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Analysis of Latvian melanoma families for *9p21* germline deletions by the multiplex ligation-dependent probe amplification approach

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Abstract

CDKN2A at chromosome band *9p21* is the most important melanoma susceptibility gene identified to date. Germline mutations of *CDKN2A* have been detected in melanoma families worldwide but the overall proportion of families with identified mutations remains moderate. Here we applied a novel method, called multiplex ligation-dependent probe amplification (MLPA), for detection of germline deletions at *9p21* in four melanoma-prone families from Latvia with no previously detected *CDKN2A* point mutations. No germline deletions were identified, excluding *9p21* deletions as a causal event in the patients analysed. However, we describe the application of MLPA and show the advantages of the method in gene dosage analysis.

Key words: *9p21*, *CDKN2A*, gene dosage, germline mutations, melanoma families.

Introduction

Many melanoma prone families show linkage to markers at chromosome region *9p21* (Newton Bishop et al. 1999) where the *CDKN2* locus is situated. The *CDKN2* locus is complex and consists of the *CDKN2A* and *CDKN2B* genes.

CDKN2A has been shown to be a melanoma susceptibility gene (Kamb et al. 1994). *CDKN2A* encodes the tumour suppressor protein p16 that acts through the retinoblastoma cell cycle control pathway (reviewed in Ortega et al. 2002). Germline mutations of *CDKN2A* have been identified in melanoma families around the world. However, the proportion of pedigrees in which *CDKN2A* is mutated remains moderate. Only approximately 25 % of families with two or more cases of melanoma have detectable mutations within the *CDKN2A* coding region (Goldstein, Tucker 1997). In the *CDKN2A* promoter region germline mutations occur rarely (Liu et al. 1999; Harland et al. 2000; Pollock et al. 2001). Recently, the lack of detectable mutations has been partly explained by the observation of noncoding mutations deep in the introns (Harland et al. 2001; Majore et al. 2004; Harland

et al. 2005a). However, there are still a significant proportion of *9p21*-linked families in which the susceptibility to melanoma remains unexplained.

The *CDKN2A* locus is complex and besides the above mentioned protein p16 gives rise to the second completely unrelated protein p14ARF through alternative splicing and translation of the products in different reading frames. The p16 and p14ARF mRNAs are transcribed from different first exons, 1α and 1β respectively, and utilise the same second and third exons. Like p16, p14ARF is also a tumour suppressor protein (Ortega et al. 2002). Mutations specifically altering *p14ARF* such as a *CDKN2A* exon 1β deletion (Randerson-Moor et al. 2001), a 16 bp insertion in exon1β (Rizos et al. 2001) and exon 1β splice site mutations (Hewit et al. 2002; Harland et al. 2005b) indicate a significant role of this transcript in melanoma predisposition.

No evidence has been found that *CDKN2B* (p15) is a melanoma susceptibility gene (Flores et al. 1997; Liu et al. 1997; Platz et al. 1997; Laud et al. 2006).

In other cancer family syndromes, germline deletions of susceptibility genes have been recognised as pathogenic mutations in a significant proportion of families. Germline deletions are common in *hMSH2* and *hMLH1* genes in hereditary non-polyposis colorectal cancer (Gille at al. 2002) and in the *BRCA1* gene in hereditary breast cancer (Petrij-Bosch et al. 1997). Germline deletions involving the *CDKN2A* locus have also been reported previously (Bauhuau et al. 1998; Mistry et al. 2005).

Here we applied a new technique, called multiplex ligation-dependent probe amplification (MLPA; Schouten et al. 2002), to the analysis of four patients from Latvian melanoma-prone families for heterozygous and homozygous germline deletions at *9p21*, and describe the value of the new technique for determination of the relative copy numbers of DNA sequences.

Materials and methods

Patients

The criterion for including patients in this study was the family history of melanoma. The information about families was obtained by personal interview of patients. Melanoma patients from four families (one patient from each family) with two or more cases of melanoma were analysed. Three patients were recruited through the Latvian Oncological Center and one patient was ascertained by referral from a clinician treating melanoma. All patients were previously analysed for germline mutations in the *CDKN2A* gene (exons 1β, 1α, 2, and 3) and did not have any detectable mutations (Pjanova, unpublished data). Informed consent approved by the Central Medical Ethical Committee of Latvia was obtained from all patients who underwent DNA analysis.

MLPA

Genomic DNA was extracted from peripheral blood lymphocytes by the standard phenol-chloroform extraction method.

MLPA was carried out according to the supplied protocol using the *9p21* MLPA probe kit from MRC-Holland, Amsterdam, the Netherlands. The technique and preparation of the probes used in this kit are described elsewhere (Schouten et al. 2002) and are outlined in Fig. 1. The *9p21* MLPA kit contains 12 probes for *CDKN2* locus sites, 11 probes for other *9p* gene sites, and 16 control probes specific for DNA sequences outside the *9p21* region.

The genes included in this analysis were *TEK*, *ELAV2*, *CDKN2B*, *CDKN2A*, *MTAP*, *KIAA*, *INFW1*, *IFNB1*, *MLLT3*, and *FLJ00026*.

Briefly, 25 ng μl^{-1} genomic DNA (in volume 2.5 µl) was denatured at 98 °C for 5 min, cooled to 25 °C, and 1.5 µl of the supplied SALSA probe mix and MLPA buffer were added. The mixture was re-heated to 95 °C and then the hybridisation was carried out at 60 °C

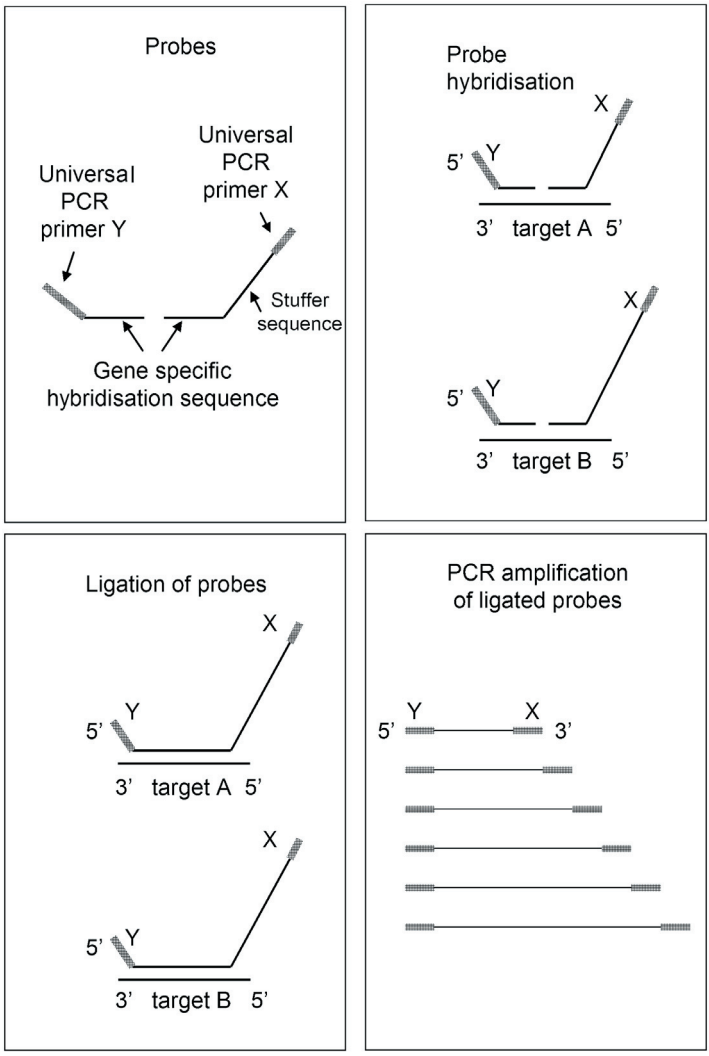

Fig. 1. Principle of multiplex ligation-dependent probe amplification (MLPA). For each specific target, a set of two probes is designed that hybridise immediately adjacent to each other. Both probes consist of a short target specific sequence and a universal forward or reverse PCR primer-binding site. In addition, one of the probes contains so-called stuffer sequence, which differs in length from probe to probe. After the hybridisation to the target sequence, the two parts of each probe are ligated by thermostable ligase. All probe ligation products are amplified simultaneously by PCR using a single primer pair. The multiple fragments can be distinguished based on their different length.

for 16 h. The hybridised probes were ligated with the ligase-65 mix (ligase-65 enzyme and ligase-65 buffers) at 54 °C for 10 to 15 min. The ligase-65 enzyme was inactivated by incubation at 98 °C for 5 min and the ligation products were than amplified by PCR according to the manufacturer's protocol using one primer labelled with 6-FAM. The amplification was performed on a GeneAmp 9700 Thermal Cycler (Applied Biosystems, Oxford, UK) with a hot-start PCR program beginning with the addition of the polymerase mix (SALSA primers, SALSA enzyme dilution buffer, SALSA polymerase) to the PCR reaction (ligation products premixed with SALSA PCR buffer) at 60 °C. PCR was carried out for 33 cycles at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s, and incubation at 72 °C for 20 min.

PCR products (1 µl) were than mixed with 8.5 µl of de-ionised formamide (HiDi formamide, Applied Biosystems) and 0.5 µl fluorescent size standard (GeneScan-ROX 500, Applied Biosystems) and analysed on the ABI3100 Automated Capillary DNA sequencer with a 36 cm capillary array and ABI POP-4 polymer (Applied Biosystems). Analysis was automated using the ABI PRISM GeneScan and Genotyper software. Specific peaks corresponding to each product were identified according to their migration relative to the size standards and exported to a Microsoft Excel spreadsheet. To obtain gene dosage quotients (DQ), peak heights were taken as the quantitative measure of DNA content and peak fractions were calculated by dividing the peak area of a certain probe by the sum of peak areas of all 16 control probes in a certain sample. Subsequently, this relative peak area of each probe was compared to the average relative area of this probe in control samples. The means and standard deviations of the DQ provide quality control of the assay. Results from samples with mean standard deviation (SD) more than 0.2 were considered as false results and the analysis was repeated in accordance with the manufacturers recommendations.

Theoretically, gene dosage quotients close to 1.0 indicate two copies present (i.e. wild-type), 0.5, one copy absent (i.e. hemizygous), and 0.00, both copies absent (i.e. homozygous deletion). Quotients were scored according to observations made from large numbers of previous samples (data not shown): wild type if the quotient exceeded 0.75, hemizygous deletion if the quotient was between 0.43 and 0.68, and homozygous deletion if the quotient was between 0.00 and 0.19.

Results

We analysed DNA from four melanoma patients with a family history of melanoma for hemizygous and homozygous germline deletions at chromosome arm *9p*. Three patients (M66, M162, M199) were from two-case melanoma families and one patient (M247) was from a five-case melanoma family (data not shown). The MLPA assay was robust and reliable in most cases analysed, as seen by peak heights in control DNA samples across the multiplex (Fig. 2) and standard deviations in dosage quotients (Table 1). One DNA sample M162 did not provide acceptable data, producing high standard deviation variations in DQ estimates. However, after re-measuring of DNA concentration and increasing the DNA concentration in the experiment a high quality result was obtained (Table 2). No deletions were identified for any *9p21* gene probes in samples analysed (Table 2), suggesting that germline deletions at *9p21* are not responsible for clustering of melanoma in these families.

Discussion

Although some reports (Bauhuau et al. 1998; Randerson-Moor et al. 2001) have detected *CDKN2A* germline deletions in melanoma-prone families, such deletions explain susceptibility only in a small proportion of families. In the first comprehensive study carried out to date only 3 % of melanoma families (three families from 93) in which germline mutations have not been found have been shown to carry *CDKN2A* germline deletions. One family was reported to have a hemizygous deletion involving *CDKN2A* exons 1α, 2, and 3, and two families had hemizygous deletions of *CDKN2A* exon 1β (Mistry et al. 2005). In the present study we did not find any *9p21* (*CDKN2A*) germline deletions in Latvian melanoma-prone families, which might be explained by the rarity of such a deletions. It seems likely that there are as yet unidentified melanoma susceptibility genes, one of which may be at *1p22* (Gillanders et al. 2003). Taking together previous reports

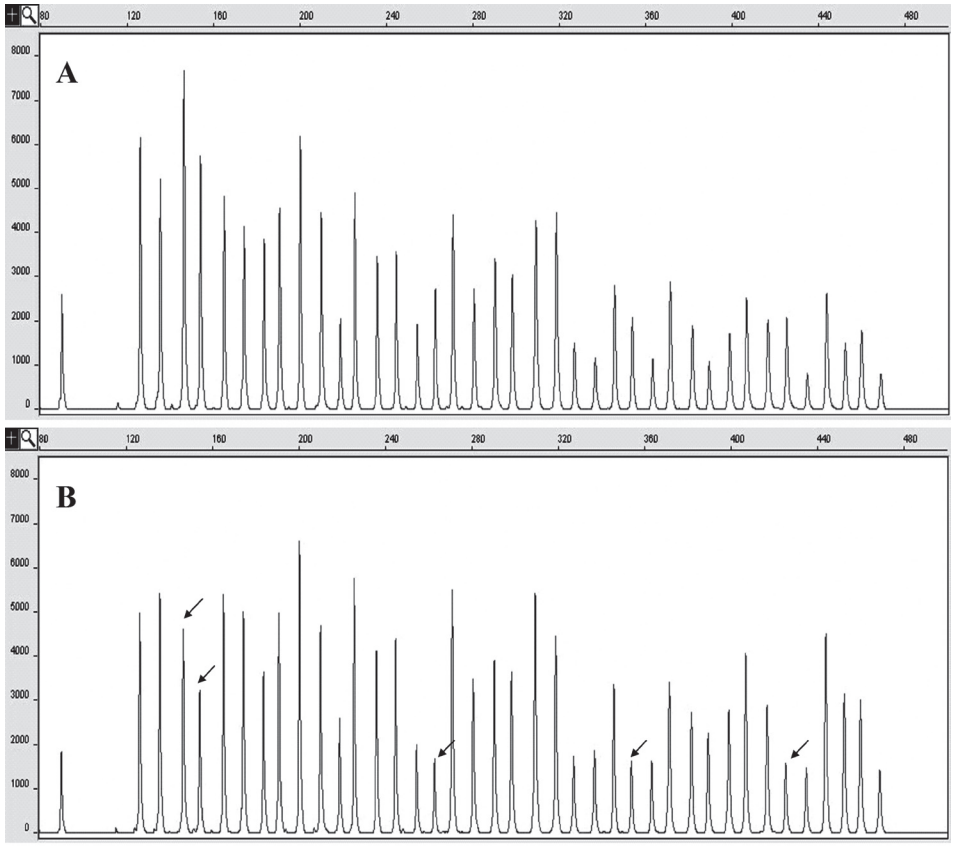

Fig. 2. GeneScan traces of MLPA products carried out on an automated capillary sequencer (ABI3100, Applied Biosystems). A, wild type control DNA. B, hemizygous deletion at *CDKN2A* exon 1β previously reported by Randerson-Moor et al. (2001) and used as a control in this study. Arrows indicate the fragments deleted in sample B..

Table 1. Gene dosage analysis by MLPA: typical dosage results showing a hemizygous deletion at *CDKN2A* 1β (bold). DNA from a melanoma pedigree previously reported by Randerson-Moor et al. (2001) and used as a hemizygous control in this study. [a], DQ (dosage quotient), the mean of DQs of each fragment against 16 control fragments. Values close to 1 indicate two copies of the fragment present and close to 0.5 loss of one copy of the fragment. [b], SD (standard deviation) used for quality control. Only results with SD ≤ 0.2 were taken into account. bp, base pairs

Fragment length (bp)	Probe	DQ[a]	SD[b]	Dosage
130	*C5q31*	0.75	0.10	Control
139	*C1p22*	0.94	0.13	Control
148	***CDKN2A* intron**	**0.53**	**0.07**	**Deletion**
157	***CDKN2A* 1β promoter**	**0.49**	**0.07**	**Deletion**
166	*MLLT3* exon 8	0.97	0.14	Normal
175	*C7p22*	0.94	0.13	Control
184	*MLLT3* exon 2	0.82	0.11	Normal
193	*C11p13*	0.96	0.13	Control
202	*CDKN2B* promoter	0.93	0.13	Normal
211	*CDKN2B* exon 1	0.95	0.13	Normal
220	*C14q24*	0.99	0.14	Control
229	*C5q35*	0.94	0.13	Control
238	*CDKN2A* exon 1	0.98	0.14	Normal
247	*C11q13*	1.06	0.15	Control
256	*CDKN2A* exon 2	0.83	0.12	Normal
265	***CDKN2A* 1β CpG island**	**0.46**	**0.06**	**Deletion**
274	*CDKN2A* exon 3	0.92	0.13	Normal
283	*C17p13*	0.99	0.14	Control
292	*MTAP* exon 7	0.97	0.14	Normal
301	*C8q24*	0.95	0.13	Control
310	*MTAP* exon 6	0.96	0.13	Normal
319	*FLJ00026*	0.82	0.12	Normal
328	*MTAP* exon 1	0.82	0.11	Normal
337	*C5q35*	1.00	0.14	Control
346	*C7q11*	0.92	0.13	Control
355	***CDKN2A* intron**	**0.68**	**0.10**	**Deletion**
364	*KIAA*	1.07	0.15	Normal
373	*C22q11*	0.98	0.14	Control
382	*IFNW1*	1.02	0.14	Normal
391	*CDKN2A* 1α promoter	1.29	0.18	Normal
400	*IFNB1*	1.12	0.16	Normal
409	*C2p14*	1.23	0.17	Control
418	*ELAVL2*	1.00	0.14	Normal
427	***CDKN2A* 1β exon 1**	**0.49**	**0.07**	**Deletion**
436	*TEK*	1.19	0.17	Normal
445	*C22q13*	1.09	0.15	Control
454	*CDKN2B* intron	1.44	0.20	Normal
463	*C10p14*	1.14	0.16	Control
472	*C8p23*	1.11	0.16	Control

Table 2. MPLA gene dosage quotients of patients from Latvian melanoma-prone families. [a], the probes are ordered from top to bottom across *9p21*, probes for *CDKN2* locus are highlighted in bold. DQ, dosage quotients. Values close to 1 indicate both copies of the fragment present. SD, standard deviation

	Probe[a]	Patients							
		M66		M162		M199		M247	
		DQ	SD	DQ	SD	DQ	SD	DQ	SD
Centromeric	*TEK*	1.10	0.10	0.85	0.10	1.49	0.26	1.36	0.16
	ELAVL2	1.13	0.10	0.91	0.11	1.27	0.22	1.07	0.13
	***CDKN2B* promoter**	**0.99**	**0.09**	**1.14**	**0.14**	**1.09**	**0.19**	**0.91**	**0.11**
	***CDKN2B* exon 1**	**0.96**	**0.09**	**1.17**	**0.14**	**1.01**	**0.18**	**0.87**	**0.10**
	***CDKN2B* intron**	**1.22**	**0.11**	**0.91**	**0.11**	**1.45**	**0.25**	**1.04**	**0.12**
	***CDKN2A* 1β CpG island**	**0.93**	**0.09**	**1.03**	**0.12**	**0.96**	**0.17**	**1.04**	**0.12**
	***CDKN2A* 1β promoter**	**1.03**	**0.10**	**1.15**	**0.14**	**1.00**	**0.17**	**0.96**	**0.11**
	***CDKN2A* exon 1β**	**1.22**	**0.11**	**0.84**	**0.10**	**1.03**	**0.18**	**1.22**	**0.14**
	***CDKN2A* intron**	**1.05**	**0.09**	**1.12**	**0.12**	**1.02**	**0.20**	**1.08**	**0.12**
	***CDKN2A* intron**	**1.02**	**0.10**	**1.15**	**0.13**	**0.97**	**0.18**	**0.95**	**0.13**
	***CDKN2A* 1α promoter**	**1.18**	**0.11**	**0.81**	**0.10**	**1.28**	**0.22**	**1.08**	**0.13**
	***CDKN2A* exon 1**	**1.09**	**0.10**	**1.08**	**0.13**	**1.06**	**0.18**	**0.98**	**0.11**
	***CDKN2A* exon 2**	**0.93**	**0.09**	**1.03**	**0.12**	**0.96**	**0.17**	**1.04**	**0.12**
	***CDKN2A* exon 3**	**0.92**	**0.08**	**0.96**	**0.11**	**0.92**	**0.16**	**0.92**	**0.11**
Telomeric	*MTAP* exon 7	0.97	0.09	1.10	0.13	1.02	0.18	1.04	0.12
	MTAP exon 6	1.07	0.10	0.99	0.12	0.99	0.17	0.87	0.10
	MTAP exon 1	0.94	0.09	0.93	0.11	0.84	0.15	1.11	0.13
	KIAA	0.96	0.09	0.99	0.12	1.16	0.20	1.00	0.12
	IFNW1	1.08	0.10	0.94	0.11	1.29	0.22	1.13	0.13
	IFNB1	1.09	0.10	0.91	0.11	1.21	0.21	1.05	0.12
	MLLT3 exon 2	1.01	0.09	1.13	0.13	0.89	0.16	0.90	0.11
	MLLT3 exon 8	1.09	0.10	1.14	0.14	1.08	0.19	0.81	0.09
	FLJ00026	0.98	0.09	1.07	0.13	0.93	0.16	1.06	0.12
	Mean SD		0.10		0.12		0.19		0.12

and results of this study, it is possible to exclude *CDKN2A* germline deletions as a major genetic determinant in melanoma susceptibility and to consider such a deletions as a rare event in melanoma-prone families.

The present study shows that MLPA is reliable, simple, and sensitive technique for relative quantification of nucleic acids, which is easy to perform and works as a multiplex assay. MLPA is a multi-step process, however the hands-on time is minimal, and detailed information is obtained. At present, different techniques are used for the detection of the copy number of genes including standard chromosome analysis, fluorescent *in situ* hybridisation (FISH), Southern blots, and loss of heterozygosity (LOH) assays. These methods are time consuming, difficult to use as a multiplex or require large amount of sample DNA (Southern blots). The PCR-based mutation detection methods are not able to

detect deletions and duplications when a normal allele is also present. The MLPA technique permits relative quantification of 40 different target sequences in a single reaction and only a thermal cycler and gene analyser or electrophoresis equipment are needed (Schouten et al. 2002). Ready-made commercial kits are available for a number of genes, as well as the tools to prepare custom kits. Moreover, the assay is suitable for high-throughput screening of DNA.

DNA concentration is important for high quality data. This would also be expected for any quantitative assay, as probe signal strengths depend on the relative amount of the target sequences present in the sample.

In summary, the present study shows that the MLPA assay is suitable for detecting deletions at *9p21*. It is sequence specific and sensitive. However, germline deletions at *9p21* are not responsible for melanoma susceptibility in Latvian melanoma-prone families, in which germline point mutations in *CDKN2A* have not been found. The susceptibility gene hunting must be continued.

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Delēciju analīze *9p21* lokusā ar MLPA metodi iedzimtās melanomas pacientiem Latvijā

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Kopsavilkums

Gēns *CDKN2A*, kas atrodas devītās hromosomas īsajā plecā *9p21*, ir galvenais šobrīd identificētais melanomas jutības gēns. *CDKN2A* mutācijas novēro melanomas pacientu ģimenēs visā pasaulē, tomēr ģimeņu skaits ar identificētām *CDKN2A* mutācijām joprojām ir neliels. Dotajā darbā meklēja iespējamās *9p21* reģiona delēcijas četrās Latvijas ģimenēs ar atkārtotiem melanomas gadījumiem, kurās mutācijas *CDKN2A* gēnā līdz šim nav atrastas. Analīzes veica, izmantojot jaunu MLPA (*multiplex ligation-dependent probe amplification*) metodi. Nevienā gadījumā nenovēroja delēcijas *9p21* reģionā. Tas norāda, ka analizētajos pacientos *9p21* delēcijas nav saistītas ar melanomas attīstību. Darbā arī aprakstīta MLPA metode un tās pielietošanas iespējas, kā arī parādītas un apspriestas metodes priekšrocības gēnu delēciju noteikšanā.

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The distribution and occurrence frequency of Gomphidae (Odonata: Gomphidae) in river Gauja

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Abstract

The article contains data on four gomphid dragonfly species known in Latvia – *Gomphus flavipes*, *Gomphus vulgatissimus*, *Onychogomphus forcipatus*, *Ophiogomphus cecilia* and the latest data on their distribution, occurrence frequency and density of individuals. Gomphidae were obtained from macrozoobenthos samples in 1998. In total 280 quantitative und 65 qualitative samples were collected in the River Gauja from the town Taurene upstream to below Carnikava. For complete analyses, the observations of adult individuals used – bibliography, unpublished (personal) observations and data from 1933 to 2005. Three species of Gomphidae – *G. vulgatissimus*, *O. forcipatus* and *O. cecilia* were recorded. Data with regard to observations of larvae/exuviae/imago stages showed that all gomphid species are encountered in throughout Latvia. *G. flavipes* is infrequent for Latvia and this species has been recorded only in the Gauja. The occurrence frequency of gomphid was 13.2 % of obtained samples. *G. vulgatissimus* was found in 10 % of samples, *O. forcipatus* in 5 %, *O. cecilia* in 0.7 %. Ecological analysis of bottom substrate showed that *O. forcipatus* prefers a hard substrate situated in the rhitral stretches or in the rapids. The density of *G. vulgatissimus* reached 0.919 ind. m^{-2}, and *O. forcipatus* 0.514 ind. m^{-2}.

Key words: distribution, Gomphidae, occurrence frequency, Odonata, River Gauja.

Introduction

There are four gomphid dragonfly species known in Latvia: *Gomphus flavipes* (Charpentier, 1825), *Gomphus vulgatissimus* (Linnaeus, 1758), *Onychogomphus forcipatus* (Linnaeus, 1758) and *Ophiogomphus cecilia* (Geoffroy, in Fourcroy, 1785). All, except *G. flavipes*, are distributed throughout Latvia (Spuris 1956), although the frequency of their occurrence varies. *G. vulgatissimus* and *O. forcipatus* are considered as widespread and common (Spuris 1956; Spuris 1993). *O. cecilia* is also widespread but has been considered to be rare (Spuris 1993). Recent investigations indicate that it is either more common than previously thought or has increased in numbers (Kalniņš, Inberga-Petrovska 2005). *G. flavipes* is also a fairly rare species but is found in various regions of Latvia (Spuris 1956; Spuris 1993).

In to the literature (Spuris 1956; Askew 1988; Spuris 1993), habitats occupied by dragonfly larvae are usually described in general, with insufficient information on the microhabitats occupied by the larvae, their frequency and density of individuals. Fragmentary data are found in some records of zoobenthos investigations (Spuris 1953; Spuris 1966; Balode et al. 1981; Cimdin et al. 1989; Parele 2001) and regarding fish feeding (Mitans 1971). However, these records also present only general information about the

frequency of dragonfly larvae and their specific habitats, or they do not identify individual species.

For many species, including dragonflies, when a trend of decreasing numbers has been observed, it is important to have more information about these species, including their habitats and occurrence frequency. This knowledge may help to explain the change and avert decline for species especially regarding the rare species *O. cecilia* (Sahlen et al. 2004) and *G. flavipes* (Schmidt 1977). *O. cecilia* is included in the Red Data Book of Latvia in the 3rd category, as a rare species with no threat of extinction. This species is encountered in small numbers or in limited areas and specific sites that may probably disappear. Therefore they require protection (Spuris 1998). To ensure protection, this species is included in the Regulations of Cabinet of Ministers No. 396 (14.11.2000.) "List of specially protected species and limited available specially protected species". *O. cecilia* is also included in Appendix II of the Bern-Convention 1979 and Appendix II, IV of the Habitat and Species Directive (EU Directive 1992).

Materials and methods

Study area

Gauja is one of the largest rivers in Latvia. It's length is 452 km with a fall of 234.5 m (0.5 m km^{-1}) and a basin of 8.9 thousand km^2. Yearly water discharge 2.2 km^3 (average flow rate - 69.7 m^3 s^{-1}). Due to variation of water level, stream rate and features of flow, the River Gauja could be characterized as a very heterogeneous watercourse. It deposits 560 thousand metric tons of sediments per year, which is more than any another river in Latvia. About 30 % of its basin is covered by forests, 5 % – by bogs. The volume of flow rate of the river Gauja in spring periods reaches 870 m^3 s^{-1} compared to only 6 m^3 s^{-1} in winter. Its average flow rate is 0.2 ÷ 0.4 m s^{-1} but reaches 0.6 ÷ 0.8 m s^{-1} in some places. In the upper course the River Gauja flows through several lakes and millponds. In the region of the Augšgauja lowland the riverbed is 10 to 20 m wide with variable depth from 0.4 to 2 m. The riverbed is sandy, gravely; occasionally muddy, pebbly or with small boulder rapids. Below Rēveļi it flows through the lowlands of Melnupe and Lejasciems. In this region the riverbed reaches 20 to 30 m in width and its depth is 0.4 ÷ 1.5 m in rhitral and 2.5 m in the potamal stretches. Until Lejasciems the riverbed is mainly sandy or gravely, rarely pebbly or with boulder rapids. Below outflow of the Tirziņa the Sikšņu rapids begin, which are over 4 km in length. From the Sikšņu rapids until Vireši the river falls 14 m in a length of 11 km (1.3 m km^{-1}). There are dolomite outcrops in the riverbed and on the banks. In the Trikātas rising the riverbed is sandy and rough, with sandbanks and deep pools, the banks are steep and easily eroded and occasionally have collapsed. There are many oxbows. The width of the river is 30 to 80 m. The biggest rapids are the Strenču rapids, where the river depth is mainly 1.8 ÷ 2.2 m but it does reach 3 m. Below the Abula outflow (above Valmiera) until Murjāņi the River Gauja flows through its old valley. The riverbed is 60 to 120 m wide with abrupt changes in depth (from 0.3 ÷ 1 m to 5 ÷ 7 m). The riverbed is mainly sandy, occasionally gravely and pebbly, but in some places there are boulder rapids (Valmieras, Kazu, Raiskuma, Rakšu, Ķūķu rapids). In the old valley the river collects much creek and spring water. Below Murjāņi it flows through the Rīga sandy lowland. In this stretch the riverbed of Gauja is sandy or gravely. It's width is 70 to 300 m, in the Gauja outflow area even more, and its depth is 2 m (Avotiņa 1995).

Sampling procedures and data analysis

Gomphidae larvae were collected during implementation of the project „Establishment of long-term pollution in water of Gauja" in 1998 (Kalniņš 2000). In total 280 quantitative samples of macrozoobenthos were collected at 32 sampling sites in the river Gauja from the town of Taurene upstream to Carnikava. The sampling sites were mainly above and below large (> 500 citizens) populated areas. Standardized methods were used for collecting and processing the Gomphoidae material (Standart… 1992). At each sampling site two to four quantitative macrozoobenthos samples were collected (by both river banks and in the middle part of river). At most of sites samples were collected in three seasons – spring, summer and autumn. An Ekmans-Berdge type grab (0.025 m^2) was used for collecting quantitative samples. The obtained densities were calculated for 1 m^2. One to two qualitative samples were collected at sampling sites using a hidrobiological hand-net. In total 65 qualitative samples were obtained. Each sample was sorted by rinsing using a sieve (pore size 1 × 1 mm). The benthos organisms were sorted in the laboratory. Data on water temperature (T °C), soil type and composition of soil, flow rate and the depth as well as vegetation of biotope were recorded.

For analysis, the following data were also included:

(i) observation data on adult individuals from literature, unpublished (personal) observations and data from 1933 to 2005; all data were entered into a data base of dragonfly distribution in Latvia (maintained by the author);

(ii) data collected by the author for *O. cecilia* adults on $29^{th} \div 30^{th}$ of July 2003 in the River Gauja valley between the towns Cēsis and Sigulda (45 km stretch), based on accounts of all adult individuals counted from a boat. The counts were made during clear and sunny weather, which is optimal for adult dragonfly activity, from 11 AM till 16 PM. Observations of each individual were recorded using GPS and entered on maps. To describe the potential feeding ground of dragonflies (especially *O. cecilia*) outside the watercourse, additional open habitats by the river were inspected;

(iii) information obtained by the author during the projects "Protection and management of the Northern Gauja valley" (LIFE project) and „Cross boundary river habitats as corridors for protected species migration – monitoring and management strategy" regarding the distribution and occurrence of habitats and dragonflies in the River Gauja.

In the above mentioned projects, rich Odonata material was collected. In total, in a ~100 ÷ 150 km long section of the River Gauja, both left and right banks (including boating) were inspected. In most cases, adult individuals were recorded, but also larvae and exuviae as well.

Distribution of Odonata was mapped using a grid of 5 × 5 km squares in the Baltic grid system on a Transverse Mercator projection (TM-1993) of Latvia. The current map is based on satellite maps available for Latvia (scale 1:50 000), published in 1999 - 2000. This map is graduated in 1 × 1 km (= 1 km^2) squares and the border of 5 × 5 km squares coincide with every fifth km line. In total, the terrestrial territory of Latvia includes 2785 squares (some squares are not complete). The River Gauja crosses 70 different squares; some of them contains only ~ 1-km-long river stretches. Samples were obtained in 32 sites (= 30 5 × 5 km squares; Fig. 1). In preparing Odonata occurrence maps, data from the author's data base were used. In total, data on Latvia's Odonata are available for 481 map squares.

Density of individuals was calculated from the total number of collected samples and from the number of samples containing gomphid larvae. Estimates of the density of gomphid larvae in the River Gauja were made in general and in optimum/sub-optimum habitats for each species.

Results and discussion

Distribution

Three species of gomphid larvae were found in the River Gauja – *G. vulgatissimus*, *O. forcipatus* and *O. cecilia* while *G. flavipes* has been recorded in the literature (Spuris 1956), it was not found in this study, confirming that it is a rare species in Latvia and that this information is not based on incomplete knowledge. Other Gomphidae were present in 21 squares (sampling sites). *G. vulgatissimus* was recorded in 20 squares, *O. forcipatus* - in eight squares (Fig. 2), but *O. cecilia* larvaes in five squares. Therefore, it can be concluded that *G. vulgatissimus* is a widely distributed species in the Gauja. *O. forcipatus* was established frequently in upper reaches, and in the sections of overfalls. However, it is presumed that the species is encountered in other sections as well as in the middle part of Gauja and in the lower reaches. *O. cecilia* and *G. flavipes* are more locally distributed species. The position of the latter being derived from the data in the literature (Spuris 1956; Spuris 1993). *O. cecilia* larvae were distributed in all river lengths. A total of 48 adult individuals *O. cecilia* were recorded (between three and nine individuals in any one 5 × 5 km square or 0.9 individuals per 1 km) during the count from Cēsis to Sigulda in 2003, showing that the species is distributed in the River Gauja more widely (Fig. 3) than indicated

Fig. 1. Quantitative and qualitative macrozoobenthos sampling sites (5 × 5 km squares) in the River Gauja from Taurene upstreams to Carnikava downstreams in 1998 (grey squares – one sampling site in square; black squares – two sampling sites in square).

Fig. 2. The distribution of *G. vulgatissimus* and *O. forcipatus* in quantitative and qualitative samples of macrozoobenthos collected in the River Gauja from Taurene upstreams to below Carnikava in 1998 (white squares – sampling sites; grey squares – localities with *G. vulgatissimus* larvae; black squares – localities with *G. vulgatissimus* and *O. forcipatus* larvae).

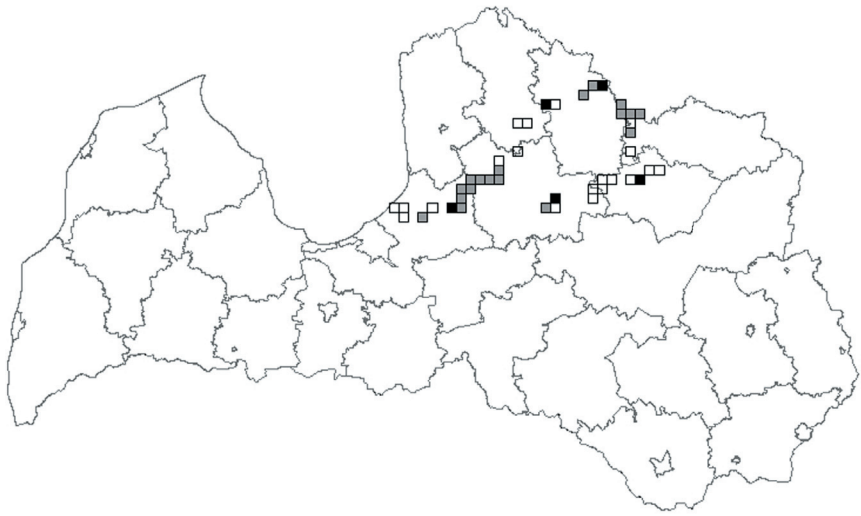

Fig. 3. The distribution of *O. cecilia* in quantitative and qualitative samples of macrozoobenthos collected in the River Gauja from Taurene upstream to below Carnikava in 1998 (white squares – sampling sites; black squares – localities with larvae; grey squares – localities with imago).

by larval counts. For example, larvae were only found in this section of the river near Sigulda. Although the adult count was performed during the period of maximum flight (Hammond 1983), it is known that a considerable proportion of adult individuals feeding

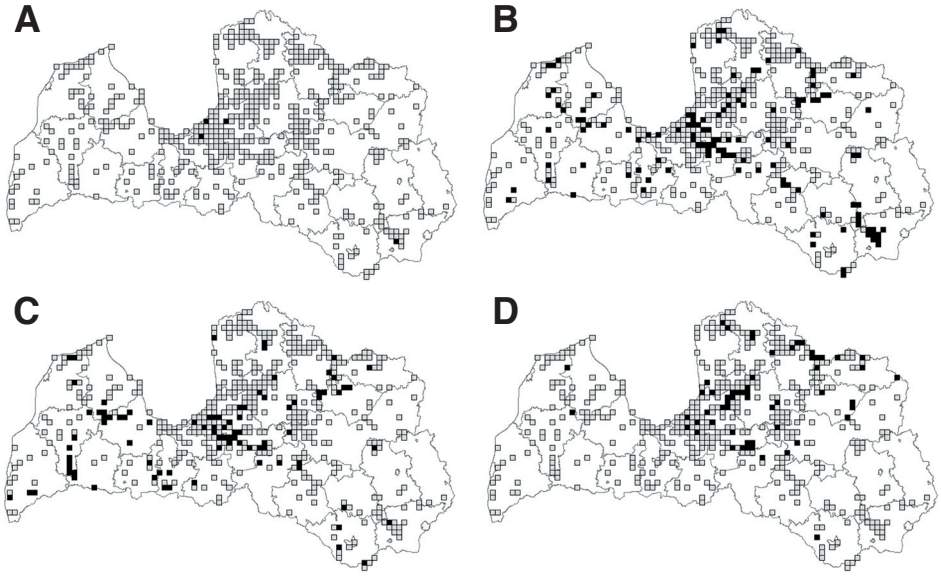

Fig. 4. The distribution of Gomphidae in Latvia (grey squares – all odonate records (n = 481); black squares – species records) on 2005. A, *G. flavipes*; B, *G. vulgatissimus*; C, *O. forcipatus*; D, *O. cecilia*. Observation data from literature, unpublished and personal observations data from 1933 to 2005; these data are entered into the data base of dragonfly distribution in Latvia (maintained by the author)..

outside of the river zone, in meadows near the river, at oxbows and other open habitats, hence the real number of individuals may be greater.

Comparing observations of gomphid larvae in the River Gauja with observations of larvae/adults throughout Latvia (author's data base on dragonfly distribution), it is clear that the above conclusion about the distribution of the species is only partly true. Species of gomphid occur throughout most of Latvia (Fig. 4). However there are differences between the distributions of the species. *G. flavipes* is the most rarely observed gomphid in Latvia found in only four squares – in the central and eastern parts of Latvia. This can probably be explained by its location in the Northern border of occurrence area of the species (Askew 1988). However it is known in more northern areas in Estonia (Ruusma 1995; Kalkman et al. 2002). Although the related species, *G. vulgatissimus*, is the most widely spread gomphid species in the country (120 squares). *G. vulgatissimus* is widespread in big rivers – Daugava and Gauja, less in the small or middle-sized rivers – Abava, Ogre and others. Since the species occurs in other regions of Latvia, there would be reason to suppose, that the species would be more widely occurred, but the less amount of the fields in other regions it is possible to explain by the less amount of inspected *O. forcipatus* is established in 88 squares, which largely coincides with current thought about the distribution of the species. This species is found throughout Latvia, but it occurrs in rapid sections of rivers, for example in the river Venta which has many rapid river sections. Potentially lower occurrence of this species is due to lack of the optimum habitats for this species (rhitral type streams covered by gravel-pebble-cobble bottom). *O. cecilia* showed large scale differences in its distribution. Although larvae were found in only five squares

Table 1. Number and occurence of gomphid larvae in quantitative samples of macrozoobenthos collected in the River Gauja from Taurene upstream to below Carnikava in 1998

Species	Total number of samples with	Total number of specimens	Ratio of all (n = 280) samples (%)	Ratio of samples (n = 37) with Gomphidae (%)
G. vulgatissimus	28	34	10.0	75.6
O. forcipatus	14	19	5.0	37.8
O. cecilia	2	2	0.7	5.4

in the River Gauja, adult individuals were observed in all rivers (23 squares) and in other sites (34 squares). Moreover, it should be noted that observations of adult individuals on the River Gauja in five squares between Gaujiena and Zīle are also referable to Estonia (cross-border river habitats).

Hence, it can be presumed that all gomphids, except *G. flavipes,* are distributed widely.

Occurrence frequency

Only quantitative data for the larvae were used for analysis of occurrence frequency. Overall, gomphid larvae were found in 37 samples from 18 sites of the Gauja. *G. vulgatissimus* larvae were present in 28 samples from all of these 18 sites, *O. forcipatus* larvae in 14 samples from eight sites and *O. cecilia* in two samples from two sites (Table 1). The presence of two larvae of *O. cecilia* in two samples indicates its rareness which was confirmed by observations of adults: *G. vulgatissimus* and *O. forcipatus* adult individuals were observed in the River Gauja in comparatively greater numbers and more often than those of *O. cecilia*.

The occurrence frequency of the different species can be explained by abiotic and biotic factors (Korkeamäki, Suhonen 2002). Gomphid larvae were typical for rhitral sections without macrophytes and a bottom is characterized by gravel. The bottom substratum was divided into components for each sample: sand, gravel, pebbles, cobbles, mud, detritus and macrophytes. In 11 % of the samples the substrate was uniform, in 49 % there were two components, in 32 % three components, and in 8 % four components. Sand occurred most

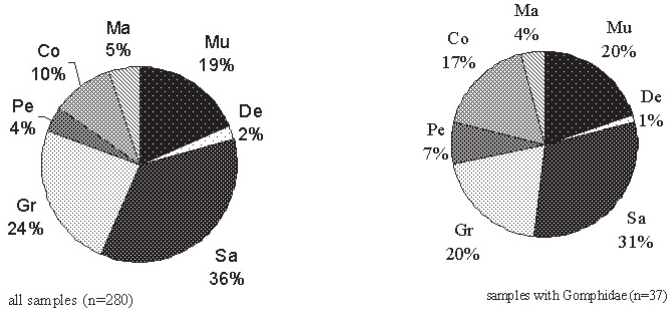

Fig. 5. Division of substratum by components of samples. Sa, sand; Gr, gravel; Pe, pebbles; Co, cobbles; Mu, mud; De, detritus; Ma, macrophytes.

Table 2. Information in literature regarding habitat and substrate preferences of gomphid larvae in Europe and Latvia

Data source	*G. flavipes*	*G. vulgatissimus*	*O. forcipatus*	*O. cecilia*
Askew 1998	Running waters, sandy banks along the lower courses of large rivers	Slow-flowing, meandering rivers and large streams with muddy beds, occacionaly with large lakes	Rivers and lakes with clear water	Running waters, sandy banks along the lower courses of large rivers
Spuris 1956	Soft clay or clay-sand bottom with little mud layer in non-vegetated places	In rapid or slow flowing river overfalls. Rarely in lakes	In fast-flowing rivers with sandy or pebbly bottom	Small streams and creeks with sandy bottom in places with sparse or no vegetation
Spuris 1993	In sandy places of large rivers	In medium, rapid and slow flowing rivers, in sandy or pebble places, very rarely in large lakes	In rapid or medium/ rapid flowing water, in medium/large rivers, in pebble and cobble bottom	In slowly and medium/rapid flowing streams and in small, poorly vegetated rivers with sandy - mud bottom
Gauja (current study)	Larvae not found	In medium or slow flowing river sections with sandy - mud, rarely gravel, bottom	In rapid or medium flowing river sections with gravel - pebble - cobble bottom	Sand or gravel bottom with mud

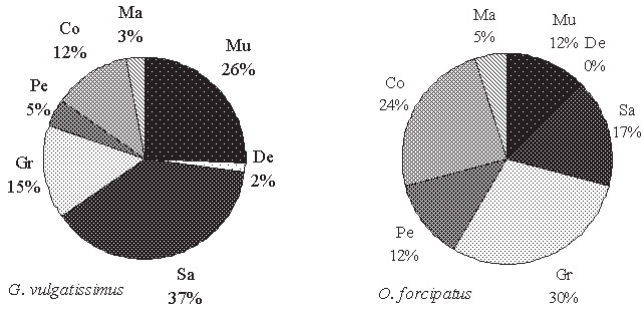

Fig. 6. Preference of *G. vulgatissmus* and *O. forcipatus* for substratum types based on number of collected individuals in the River Gauja. Sa, sand; Gr, gravel; Pe, pebbles; Co, cobbles; Mu, mud; De, detritus; Ma, macrophytes.

frequently (in 36 % of the samples), gravel was found in 24 %, mud in 19 % and cobbles in 10 %. The other components were represented in only a relatively small number of samples: 5 % contained macrophytes, 4 % pebbles and 2 % detritus. These proportions coincide with the habitat types found in the River Gauja in general (Kalniņš 2005). Comparing the

Table 3. Total density of gomphidae larvae in the River Gauja and in habitats for gomphid species in quantitative samples of macrozoobenthos collected in the River Gauja from Taurene upstream below to Carnikava in 1998

Species	Number of larvae in 1 m^2 (all samples)	Number of larvae in 1 m^2 (samples with Gomphidae)	Theoretical area (m^2) for one larva (all samples)	Theoretical area (m^2) for one larva (samples with Gomphidae)
All Gomphidae	0.19	1.48	5.1	0.7
G. vulgatissimus	0.12	0.91	8.2	1.1
O. forcipatus	0.06	0.51	14.7	1.9
O. cecilia	0.007	0.05	-	-

proportions of substrates found in the samples containing gomphid larvae with those in all samples showed a preference (Fig. 5) for hard substrate (pebble and cobble) especially regarding *O. forcipatus* larvae (42 %; Fig. 6) in regions of the river with rapids or water falls. Habitat characteristics given by different authors (Table 2) reflect the size of the rivers, and the habitats or substrata inhabited by the larvae mentioned rarely. There is general agreement between the adult distribution information found in this project and that in the literature (Spuris 1953; Askew 1988; Spuris 1993), and this is clearly very useful information with regard to locating adults. However, it does not allow any evaluation of the significance of habitats and substrata for individual species. For example, in places where *O. cecilia* larvae were found in the River Gauja and in other watercourses as well (Kalniņš, Inberga-Petrovska 2005), an obligatory component of the substrate was a thin mud layer above sand also in places where qualitative samples were collected. This indicates this microhabitat as important habitat for disguising the larvae to enable them to catch prey.

The species were not found in all suitable habitats (in river stages with moderate or slowly flow with sandy-mud, rarely gravely ground). It is possible that there are other limiting factors. Information about the density of individuals and the area of occupancy of one individual are important. On the basis of this information it can be concluded that slow flowing, sandy river regions provide more optimum habitat for *G. vulgatissimus* than rapid flowing, pebbly river regions.

Density of individuals

The density of gomphid larvae was given for the River Gauja in general and in optimum/sub-optimum habitats for each species. In addition a theoretical 'individual' area was calculated (Table 3). Clearly the greatest density of individuals and the smallest unit area per individual were established for *G. vulgatissimus*. A rather smaller density of individuals and an individual area of more than 50 % were estimated for *O. forcipatus*. However, considering that *O. forcipatus* is a more specialized species occupying rhitral stretches of the river, the density of larvae for both species were calculated using samples with *O. forcipatus* (n = 14). The relationship between densities of individuals and flow rate is opposite for these two species: the total density of *G. vulgatissimus* was 0.91 individuals per m^2 compared to only 0.42 individuals per m^2 in faster flowing water, while the total density of *O. forcipatus* was 0.51 individuals per m^2 compared to 1.35 individuals per m^2

in faster flowing water. Similarly, the area occupied by a larva of *G. vulgatissimus* in faster flowing water was 2.3 m^2 (average 1.1 m^2) compared to 0.7 m^2 (average 1.9 m^2) for *O. forcipatus*. The density of individuals of *O. cecilia* was very low with a large area occupied by a single individual but, as it was found in only two samples, a reliable value could not be calculated.

To avoid potentially inaccuracies due to larval aggregations, the dragonfly densities within a sample were examined. No indication of aggregation was found with just over 65 % of the samples in which *G. vulgatissimus* occurred containing a single larva and over 70 % for *O. forcipatus* larvae. In only two of the samples containing *G. vulgatissimus* and one of those containing *O. forcipatus* were three larvae obtained (about 6 % and 7 % respectively).

The information obtained about *O. cecilia* was limited, which confirmes the relative rarity of this species. It was only found in two samples with a large area of occupancy by an individual. However, this limited data means that little or nothing can be inferred about the factors determining the density of individuals.

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Upjuspāru Gomphidae (Odonata: Gomphidae) izplatība un sastopamības biežums Gaujā

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Kopsavilkums

Rakstā apkopota informācija par Latvijā sastopamo upjuspāru Gomphidae sugu: dzeltenkāju upjuspāres *Gomphus flavipes*, melnkāju upjuspāres *Gomphus vulgatissimus*, knaibļspāres *Onychogomphus forcipatus* un zaļās upjuspāres *Ophiogomphus cecilia* izplatību, sastopamības biežumu un indivīdu blīvumu. Pētījuma pamatā izmantots 1998. gadā Gaujā no Taurenes augštecē līdz Carnikavai lejtecē ievāktais makrozoobentosa materiāls (280 kvantitatīvie un 65 kvalitatīvie paraugi). Pilnīgākai datu analīzei izmatoti arī pieaugušo indivīdu novērojumi – literatūras dati, nepublicēti novērojumi par laika periodu no 1933. līdz 2005. gadam. Pētījumu laikā Gaujā konstatētas trīs upjuspāru sugas – *G. vulgatissimus*, *O. forcipatus* un *O. cecilia*. Apvienojot Gaujas pētījuma datus ar kāpuru/eksuviju/imago stadiju novērojumiem par visu Latvijas teritoriju, ir redzams, ka visas upjuspāru sugas ir sastopamas gandrīz visā Latvijas teritorijā. Tai skaitā, arī *G. flavipes* ir konstatēta Gaujā, bet tā ir Latvijā retāk sastopamā upjuspāru suga. Pēc sastopamības biežuma upjuspāres konstatētas 13.2 % paraugu. *G. vulgatissimus* konstatēta 10 % paraugu, *O. forcipatus* – 5 %, *O. cecilia* – 0.7 %. Analizēts paraugos pārsāvēto grunts substrātu sadalījums pa komponentiem. *O. forcipatus* vērojama izteikta saistība ar cietajām gruntīm upju straujteču vai krāču posmos. Analizēts arī kāpuru blīvums. Konstatēts, ka *G. vulgatissimus* blīvums sasniedz 0.919 indivīdus uz m^2, savukārt *O. forcipatus* – 0.514.

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Effect of cultivation conditions on morphological and biochemical characteristics of lily explants *in vitro*

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Abstract

Regeneration of bulblets from excised bulbscales has become the preferred method for vegetative propagation of lilies. For optimal *ex vitro* growth and survival during *in vitro* propagation of lilies bulblets need to have both a period of high temperature as well as cold storage. The objective of the present experiments was to determine the optimal sugar concentration in the medium and duration of pre-cold storage period for lily explants of Asiatic lily clone 'NS-94' and Trompet lily cv. 'Mezhare' and to relate morphological characteristics with peroxidase and polyphenol oxidase activities. Morphological parameters of lily microplants were affected by cultivation conditions, e.a., low temperature storage, length of pretreatment period before cold treatment, as well as by light regime and sucrose concentration in the medium. Growth of bulblets was strongly affected by cold treatment, leading to a significant decrease of relative mass. Root formation was inhibited by cold treatment. This effect was more pronounced in cv. 'Mezhare' than clone 'NS-94'. Formation of both leaves as well as bulblets was suppressed by cold storage of lily microplants. Cold treatment resulted in an increase of oxidative enzyme activity in bulblet tissues. Prolongation of duration of pre-cold period strongly increased peroxidase and polyphenol oxidase activity in bulblets of clone 'NS94', the effect was more pronounced in minimum light conditions. In contrast, for cv. 'Mezhare', higher enzyme activities were in bulblets of lily explants showing shortest duration of pre-cold period, especially in the light. A decrease in peroxidase activity appeared to be a good indicator for cold-induced slow growth of lily explants. In part this may be associated with increased antioxidative capacity in conditions of slow growth.

Key words: cold treatment, *in vitro* propagation, lily, morphological parameters, peroxidase, polyphenol oxidase.

Introduction

Regeneration of bulblets from excised bulbscales has become the preferred method for vegetative propagation of lilies. However, the necessity for specific conditions during tissue culture resembling those of natural growth of geophytes has been described. Thus, *in vitro* cultivated varieties of *Lilium speciosum* are dormant during incubation at 20 to 25 °C and need a cold treatment resembling a rest period in natural conditions prior to planting *ex vitro* (Aguettaz et al. 1990; Langens-Gerrits et al. 2003). It was suggested that development of dormancy occurs at temperatures higher than 15 °C (Delvallée et al. 1990). In contrast, lilies of the Asiatic and Trompet groups develops normally in tissue culture at warm

temperatures. We developed a protocol for micropropagation of lilies *in vitro* from small bulblets developing on scale explants (Jakobsone, Andersone 1997). However, for optimal *ex vitro* growth and survival, lily bulblets of these groups need to have both a period of high temperature as well as cold storage (Higgins, Stimart 1990; Ievinsh et al. 2003).

Peroxidase and polyphenol oxidase are enzymes catalysing oxidation of various substrates, mainly of phenolic nature, by means of hydrogen peroxide and oxygen, respectively. Attempts have been made to correlate the changes of oxidative enzymes during cultivation in tissue culture with developmental processes e.g. as indicators of explant viability or as biochemical markers of morphogenic capacity (Bouazza et al. 1993; Andersone, Ievinsh 2002). Peroxidase was shown to be involved in the dormancy of onion bulbs (Benkeblia, Selselet-Attou 1999) and in garlic microbulblets (Arguello et al. 2001). However, there is no information available on how oxidative enzymes are related to morphological changes *in vitro* during low temperature storage. Therefore, the objective of the present experiments was to determine the optimal sugar concentration in the medium and duration of the pre-cold storage period for lily explants and to relate morphological characteristics with peroxidase and polyphenol oxidase activities.

Materials and methods

Asiatic lily (*Lilium* L.) clone 'NS-94' and Trompet lily cv. 'Mezhare' cultures were produced using bulb scale explants (Jakobsone, Andersone 1997). Lily bulblets cultivated for ten months on agar-solidified Murashige and Skoog (1962; MS) medium supplemented with 3 % (w/v) sucrose were used as a source for the experiments. The cultures were grown in test tubes in a growth cabinet at 25 °C with a photoperiod of 16 h (white fluorescent lamps, 6 W m^{-2}). During this period, active growth of bulblets, leaves and roots, as well as proliferation of bulblets occurred. Lily bulblets without leaves and roots were used as initial material for experiments and were transplanted in test tubes on agar-solidified modified MS medium supplemented with 0.08 mg l^{-1} 1-naphthalene acetic acid and 2 %, 4 % or 6 % (w/v) sucrose as a source of reduced carbon. Explants were cultivated in a growth cabinet at 25 °C at the same conditions as previously for one to three weeks to test the effect of duration of the pre-cold storage period. Then explants were transferred to a cold room at 5 °C for nine weeks in dark or minimum light conditions. Control cultures were kept at the above mentioned conditions in a growth cabinet for an additional nine weeks.

Several morphological parameters were measured to characterize the physiological state of lily plantlets after cold storage, including an increase in relative fresh mass of bulblets, number of roots, number of leaves, and number of bulblets per explant. The relative increase of bulblet fresh mass was calculated based on the increase within the nine weeks of experiment divided by the initial bulblet mass.

Bulblet tissues were ground with mortar and pestle under liquid nitrogen. Soluble protein was extracted from frozen material using 25 mM HEPES buffer, pH 7.2 as described previously (Kruzmane et al. 2002). Polyphenol oxidase activity was measured spectrophotometrically using pyrocatechol as a substrate (Gauillard et al. 1993). Peroxidase activity was determined spectrophotometrically using guaiacol as a hydrogen donor.

For each treatment, 24 explants in three replicates were used. Statistical analysis and correlations were performed with KaleidaGraph® 3.6.4. (Synergy Software).

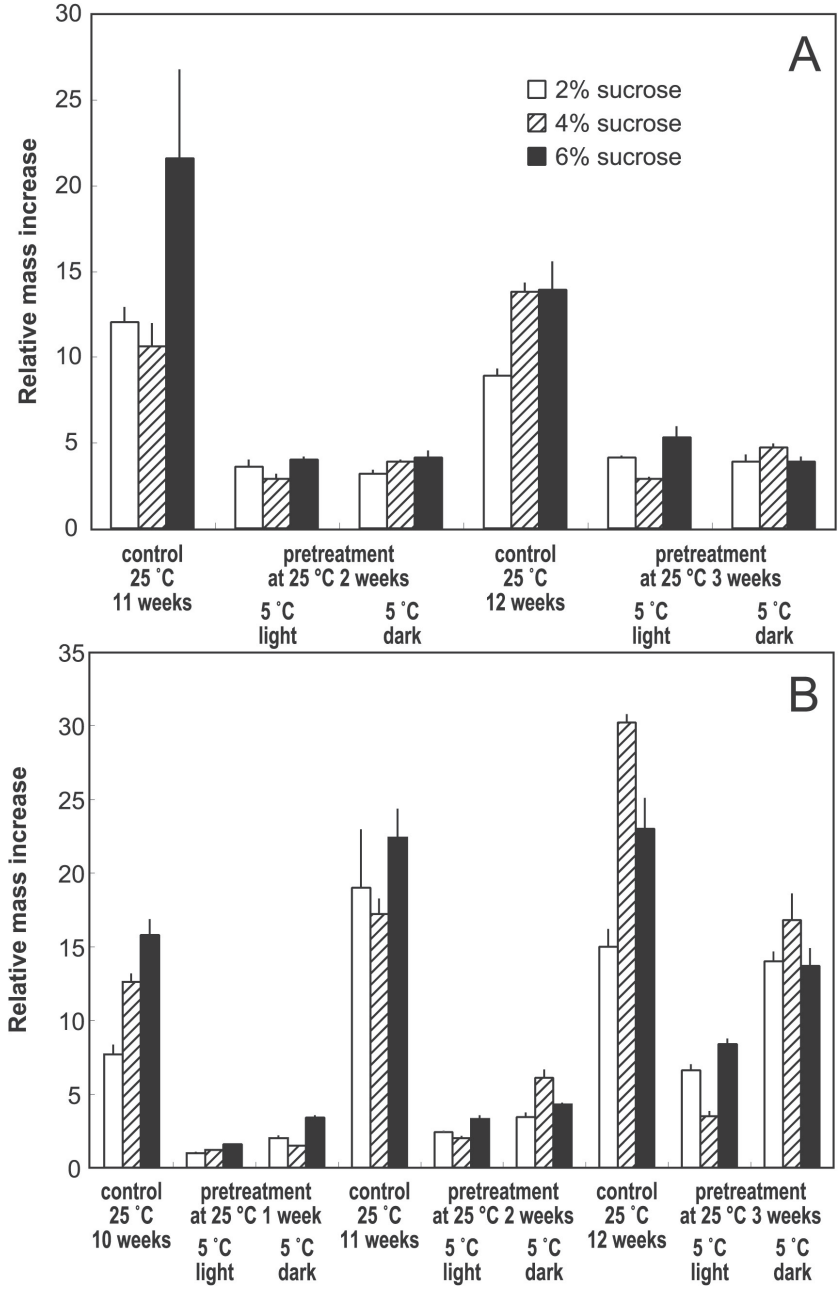

Fig. 1. Effect of pretreatment period at 25 °C and sucrose concentration in the medium on the relative mass increase of lilium explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Relative fresh mass increase of bulblets was calculated based on the increase within the period of experiment divided by the initial bulblet mass. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

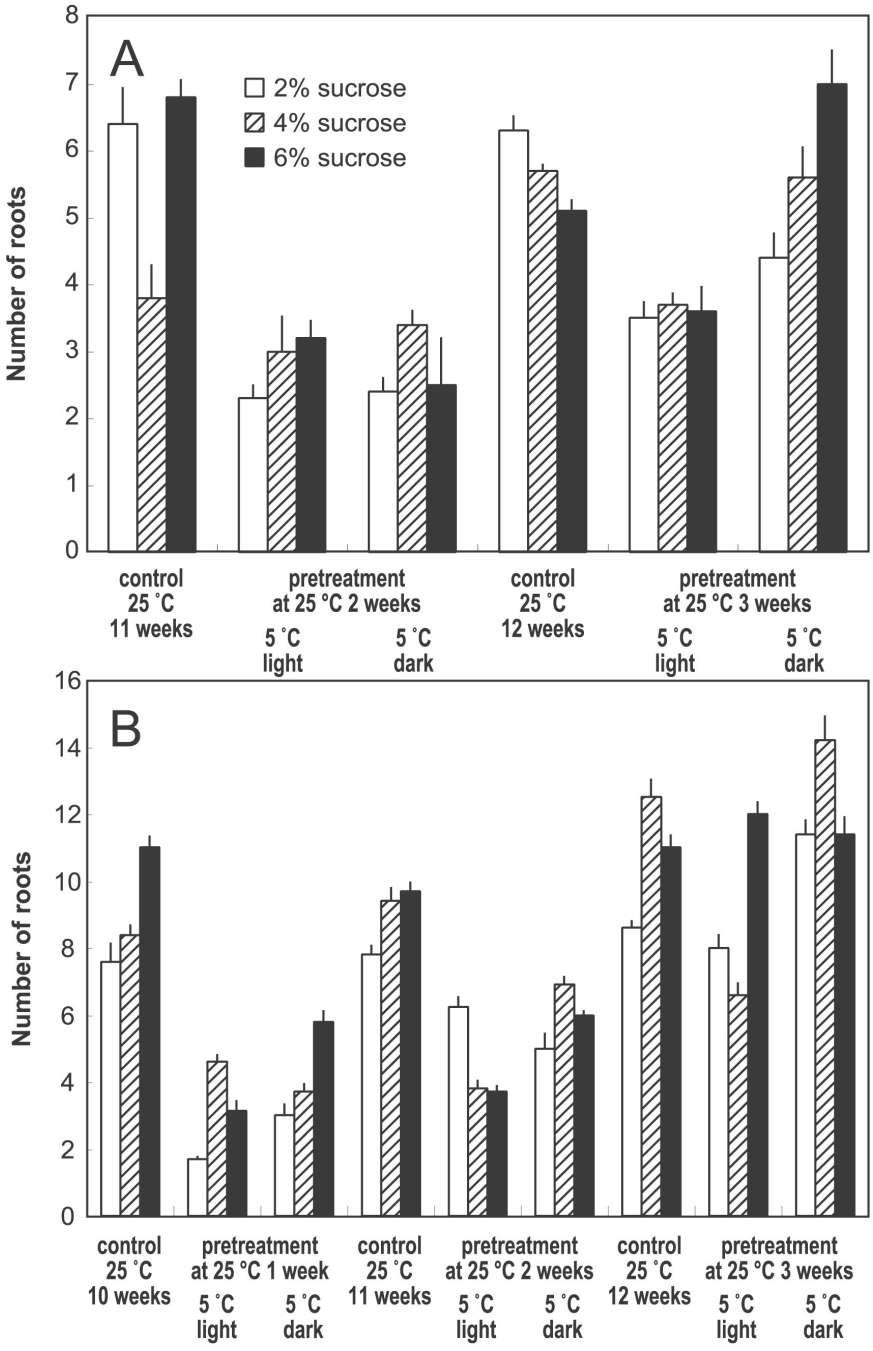

Fig. 2. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on number of roots of lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

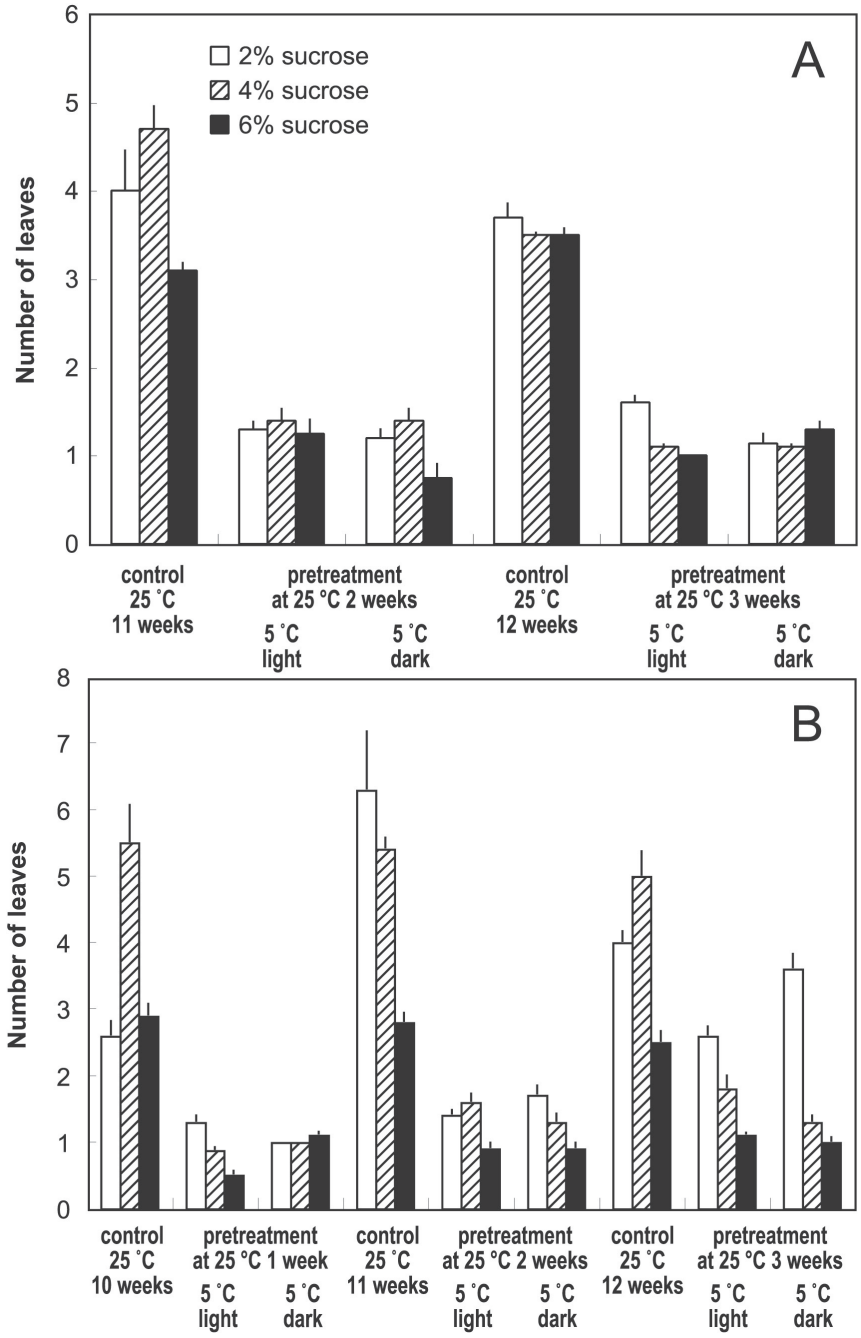

Fig. 3. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on number of leaves of lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

Results

The optimal sugar concentration in the medium and duration of the pre-cold storage period for lily explants was determined in the present experiments. High temperature-induced dormancy was practically absent for both tested lily cultivars – they developed normally at 25 °C. A moderate effect of prolonged cultivation at 25 °C was evident only as a tendency of depressed bulblet growth in clone 'NS-94' at high sucrose concentration (Fig. 1A, 4A). In cv. 'Mezhare', prolonged high temperature induced activation of bulblet growth and multiplication (Fig. 1B, 2B, 4B).

In general, the morphological parameters of lily microplants were affected by cultivation conditions, e.a., low temperature storage, length of pretreatment period before cold treatment, as well as by light regime and sucrose concentration in the medium. Growth of bulblets was strongly affected by cold treatment, leading to a significant decrease of relative mass (Fig. 1). In clone 'NS-94' the relative mass increase was not affected by length of a pretreatment period before cold storage (Fig. 1A). In contrast, prolongation of the pretreatment period significantly stimulated a mass increase of cv. 'Mezhare' bulblets, especially in the dark (Fig. 1B). Root formation was inhibited by cold treatment (Fig. 2). This effect was more pronounced in cv. 'Mezhare' than clone 'NS-94'. However in cv. 'Mezhare', the inhibitory effect of cold treatment was strongly diminished by a prolongation of pretreatment period before cold storage (Fig. 2B). Formation of both leaves (Fig. 3) as well as bulblets (Fig. 4) was suppressed by cold storage of lily microplants. For cv. 'Mezhare', prolongation of pretreatment period before cold storage had a tendency to diminish the suppressive effect of cold treatment on organ formation. This was especially pronounced for bulblet formation as well as for leaf formation at the low sucrose concentration.

Peroxidase and polyphenol oxidase activity was measured in lily bulblets at the end of the experiment. In parallel with morphological parameters, oxidative enzyme activities as well were affected by cultivation conditions. In general, cold treatment resulted in increase of oxidative enzyme activities in bulblet tissues. A longer duration of the pre-cold period strongly increased peroxidase and polyphenoloxidase activity in bulblets of cv. 'NS94'; the effect was more pronounced in minimum light conditions (Fig. 5A, 6A). In contrast, for cv. 'Mezhare', higher enzyme activity was observed in bulblets of lily explants with the shortest duration of pre-cold period, especially in the light (Fig. 5B, 6B).

The effect of sucrose concentration on oxidative enzyme activity was not clearly pronounced. Bulblets of explants of both cultivars tended to have higher polyphenol oxidase activity in their bulblets with increasing sucrose concentration in the medium (Fig. 5).

There was a strong negative correlation between peroxidase activity and relative mass increase for both lily cultivars ($r^2 = -0.81$, $p < 0.001$, clone 'NS94'; $r^2 = -0.77$, $p < 0.001$, cv. 'Mezhare'). For clone 'NS94', the peroxidase activity was also highly negatively correlated with the number of leaves ($r^2 = -0.81$, $p < 0.001$) and the number of bulblets ($r2 = -0.78$, $p < 0.001$). For cv. 'Mezhare', peroxidase activity was strongly negatively correlated with the number of roots ($r^2 = -0.72$, $p < 0.001$) and the number of bulblets ($r^2 = -0.67$, $p < 0.001$). Polyphenol oxidase activity also was negatively correlated with morphological parameters, although to a lesser extent ($r^2 = -0.64$ for mass increase, $r^2 = -0.63$ for number of bulblets, $r^2 = -0.65$ for numer of leaves). The correlation was similar for both cultivars.

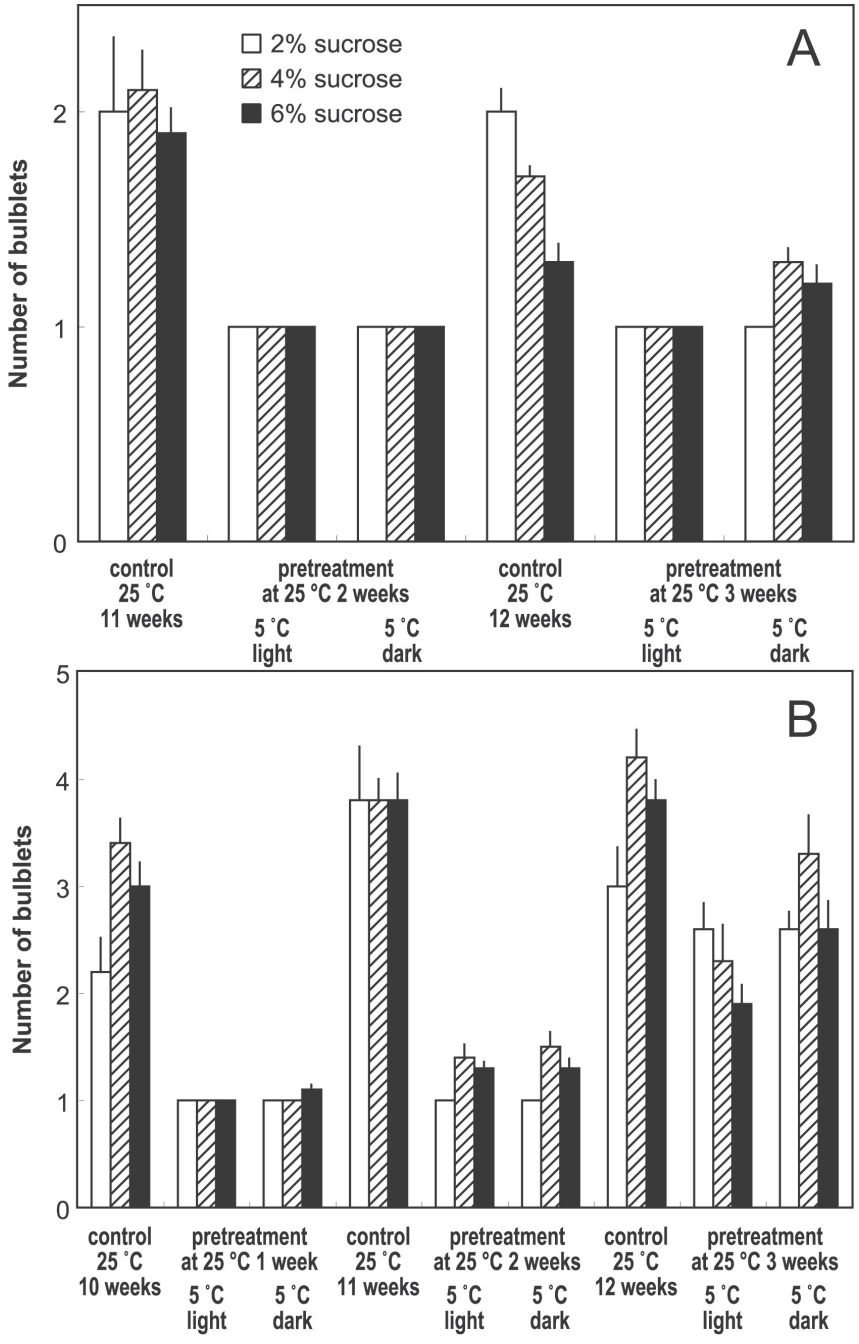

Fig. 4. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on number of bulblets of lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

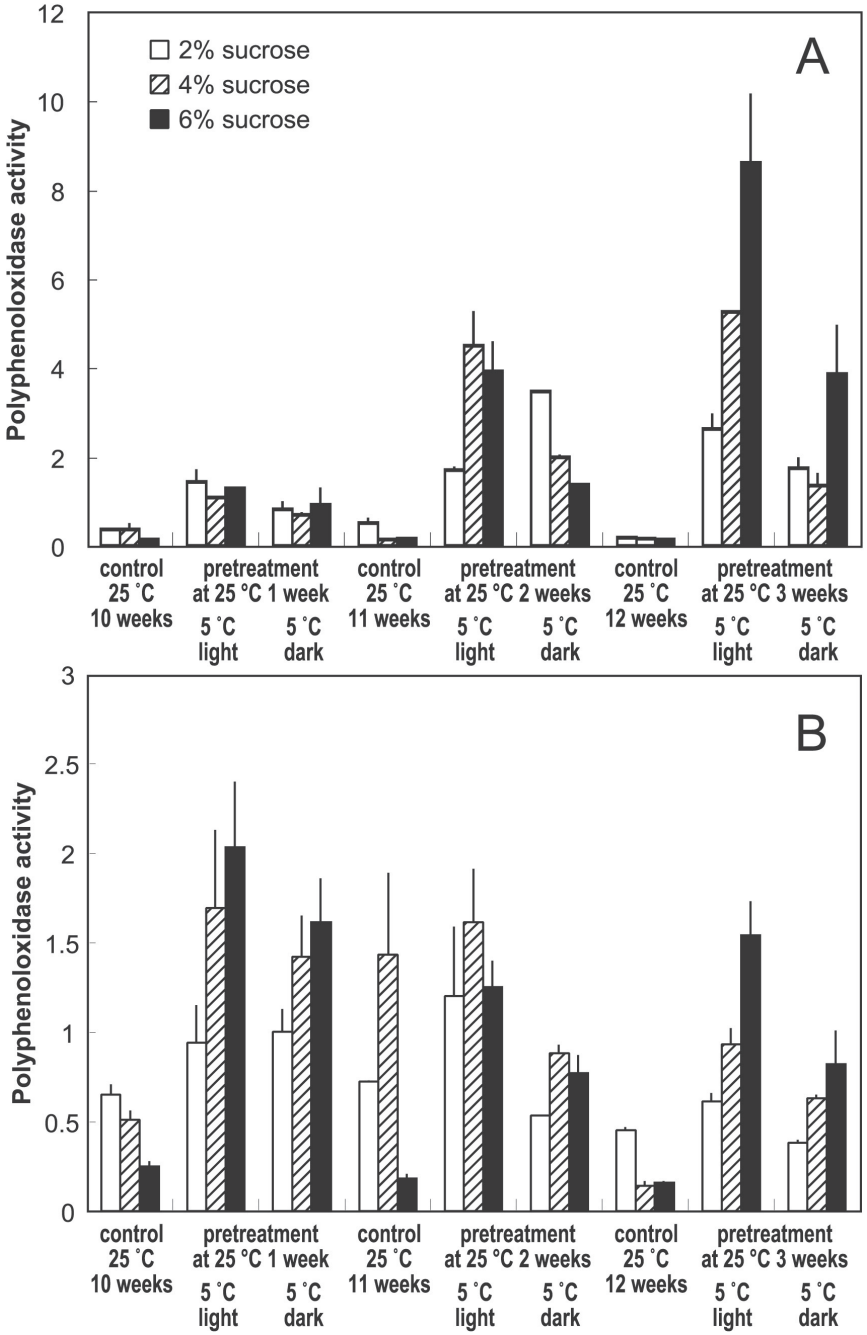

Fig. 5. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on polyphenol oxidase activity in lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

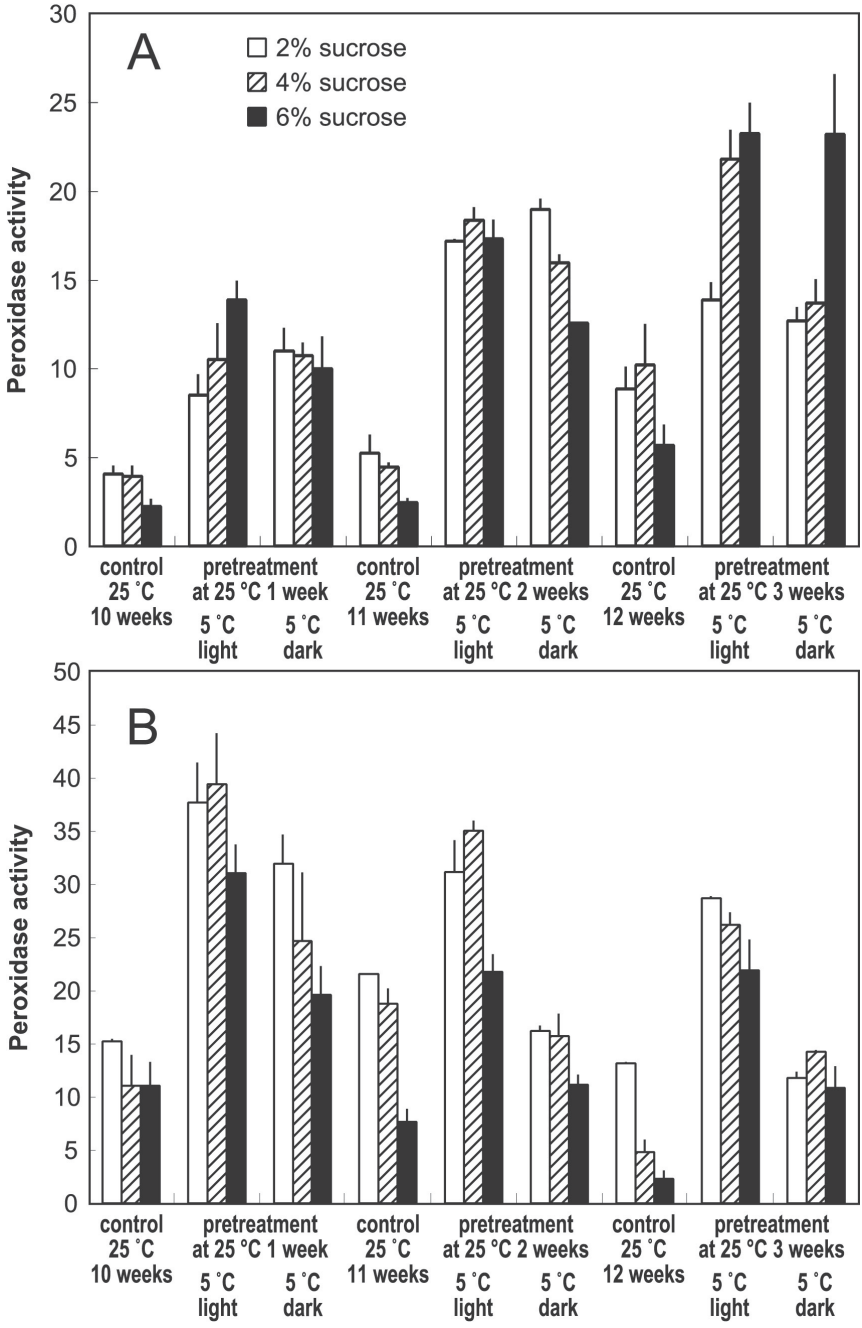

Fig. 6. Effect of pretreatment period at 25 °C, and sucrose concentration in the medium on peroxidase activity in lilium bulbscale explants after nine weeks *in vitro* at 5 °C in light or dark. A, clone 'NS-94'; B, cv. 'Mezhare'. Total duration of experiment for respective experimental variants was 10, 11 or 12 weeks. Mean values from three replicates ± SE are shown.

Discussion

During propagation of plants with a seasonal lifestyle using tissue culture, a serious problems could arise because of lack of specific environmental signals for maintaining the normal sequence of developmental events. For lilies, sensitivity to high temperature-induced dormancy is species- or even cultivar-dependent. Our results clearly showed that both a high temperature period followed by low-temperature incubation are necessary prerequisites for establishment of a normal developmental sequence of lily explants during tissue culture. While high temperature-induced dormancy was practically absent for Asiatic lily 'NS-94' and for Trompet lily 'Mezhare', the length of the high-temperature period was important for certain morphological characteristics of explants after the cold period. Both lilies remained in the juvenile stage at 25 °C as elongation of stems was not observed. The adult phase of *Lilium* begins stem growth initiated by cold treatment (Langens-Gerrits et al. 2003).

In general, 'NS-94' was relatively tolerant to an increase of preincubation period in respect to growth and development with the exception of root formation in dark at the high level of sucrose in the cultivation medium. However, a longer preincubation period in clone 'NS-94' enhanced enzyme activity in bulblets after cold storage. In cv. 'Mezhare', a prolonged preincubation period reversed the inhibitory effect of cold storage on plantlet growth and development together with a reversal of the increase in enzyme activities. Thus, it can be concluded that lilies of Asiatic and Trompet groups have different requirements for cultivation conditions during tissue culture.

In the present experiments an increase of sucrose concentration in the cultivation medium had no consistent effect on morphological parameters of lily explants and on oxidative enzyme activities in bulblets. For other species of the genus *Lilium*, a high concentration of sucrose during *in vitro* cultivation together with cold treatment has been observed to promote bulblet growth (Yamagishi 1998). Sucrose had little or no effect on shoot and bulblet growth of *in vitro* cultivated lily microplants at 25 °C in light (Bonnier, Van Tuyl 1997). However, viability as well as a regrowth *ex vitro* was increased at higher sucrose concentration. Most probably, the sensitivity of lily explants to changes in sucrose concentration in the medium is a species-specific trait.

Cold treatment-induced slow growth is a period physiologically resembling cold acclimation leading to initiation of flowering. In general, cold acclimation increases tolerance to oxidative stress due to increased ability to scavenge activated forms of oxygen (Kuroda et al. 1990; Bridger et al. 1994; Scebban et al. 1998). In our experiments, cold incubation increased both peroxidase and polyphenol oxidase activity in lily bulblets in parallel with decreased growth and development of plantlets. This effect was more pronounced in clone 'NS-94'.

An increase of peroxidase activity during cold incubation might be suggested as a result of enhanced antioxidative capacity due to increased generation of active oxygen species (Okuda et al. 1991). The most important part of chilling injury in natural conditions is associated with photoinhibition (Wise, Naylor 1987). During growth in tissue culture in darkness with high concentrations of exogenous carbohydrate supplied, this should be of less importance. Indeed, in lily 'Mezhare', cold-induced peroxidase activity was significantly higher in light-grown bulblets than in dark-grown. This was also the case for polyphenol oxidase activity in 'Mezhare'. In contrast, the enzyme activity in 'NS-94' during cold storage

were not affecetd by the light regime.

In the present experiments, a decrease in peroxidase activity appeared to be a good indicator for cold-induced slow growth of lily explants. In part this may be associated with increased antioxidative capacity during slow growth. Our data are in accordance with the fact that a transition from growth to wintering includes an increase in the antioxidative system concomitant with an increase in the cold resistance (Kuroda et al. 1990). In this respect it is interesting to note that in woody plants proteins related to cold acclimation are connected to the dormancy status of the plants (Wisniewski et al. 1996; Rowland, Arora 1997).

Acknowledgements

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Kultivēšanas apstākļu ietekme uz liliju eksplantu morfoloģiskajām un bioķīmiskajām īpašībām *in vitro*

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Kopsavilkums

Liliju veģetatīvajā pavairošanā vsibiežāk izmantotā metode ir sīksīpoliņu reģenerācija uz atdalītām sīpola zvīņām. Optimālas *ex vitro* augšanas nodrošināšanai un izdzīvošanai *in vitro* apstākļos liliju sīksīpoliņi jāeksponē gan augstā temperatūrā, gan arī aukstumā. Aprakstīto eksperimentu mērķis bija noteikt optimālās barotņu cukura koncentrācijas un pirms aukstuma perioda ilgumu Āzijas liliju klona 'NS-94' un trompešliliju šķirnes 'Mežāre' eksplantiem, kā arī saistīt morfoloģiskās īpašības ar peroksidāzes un polifenolu oksidāzes aktivitāti. Liliju mikroaugu morfoloģiskos parametrus ietekmēja tādi kultivēšanas apstākļi kā uzglabāšanā zemā temperatūrā, priekšapstrādes perioda ilgums pirms aukstuma uzglabāšanas, gaismas režīms un saharozes koncentrācija vidē. Sīpoliņu augšanu būtiski ietekmēja aukstuma uzglabāšana, izraisot būtisku relatīvās masas samazināšanos. Aukstums inhibēja arī sakņu veidošanos, un šī ietekme bija izteiktāka šķirnei 'Mežāre', nekā klonam 'NS-94'. Mikroaugu uzglabāšana aukstumā apspieda gan lapu, gan sīpoliņu veidošanos. Aukstuma apstrāde izsauca oksidatīvo fermentu aktivitātes pieaugumu sīpoliņu audos. Siltuma inkubācijas perioda paildzināšana pirms aukstuma apstrādes būtiski paaugstināja peroksidāzes un polifenolu oksidāzes aktivitāti klona 'NS-94' sīpoliņos, un šis efekts bija vairāk izteikts minimālās gaismas apstākļos. Pretēji tam, šķirnei 'Mežāre' augstākas fermentu aktivitātes bija novērojamas to liliju eksplantu sīpoliņos, kuriem bija īsākais siltuma inkubācijas periods, it īpaši, gaismā. Pazemināta peroksidāzes aktivitāte izrādījās labs indikators aukstuma inducētajai liliju eksplantu lēnajai augšanai. Tas daļēji varētu būt saistīts ar pretoksidatīvās sistēmas aktivāciju lēnās augšanas apstākļos.

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Assessment of risk factors in the development of pancreatic cancer in Latvia

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Abstract

The aim of the study was to assess the possibility of identifying individuals at risk of pancreatic cancer (PC) on the basis of information from questionnaires filled in by patients and analyses of the DNA from peripheral blood of PC patients for mutations in *BRCA1*, *CDKN2A*, *INK4/ARF* and *STK11* genes. Questionnaires showed unknown risk factors in ethiology and pathogenesis of PC besides smoking and other known risk factors. Two carriers of frameshift mutations in the *BRCA1* gene of 68 PC patients tested were detected. Screening for two founder mutations in the *BRCA1* gene should be carried out in PC patients to identify at least a part of cancer-prone families in order to offer to mutation carriers comprehensive care, surveillance and preventive procedures. No deleterious mutations were detected in *CDKN2A*, *INK4/ARF* and *STK11* genes. We conclude that mutations in these genes do not contribute significantly to PC incidence in the population of Latvia. The role of missense mutations detected can not be estimated unambigously on the basis of our data.

Key words: genes, mutations, pancreatic cancer, risk factors.

Introduction

Pancreatic cancer (PC) is one of the most aggressive and therapeutically resistant cancers of the gastrointestinal tract and is the fifth leading cause of cancer related death in both men and women. The annual mortality rate is almost equal the annual incidence rate, and the survival rate of PC patients is usually less than one year (mortality/incidence ratio is 98 %). Very few of the patients diagnosed with PC have been found to be operable at the time of diagnosis and even in these patients the postoperative five-year survival rate has remained low (< 5 %) because of a very high rate of recurrence (Jemal et al. 2002; Parkin et al. 2005). PC has been resistant to all conventional and modern therapies available, although some promising results were shown in the ESPAC-1 (European Study of Pancreatic Cancer) trial (Neoptolemos et al. 2004).

Increased mortality from PC has been recorded during the last years in Latvia. In total 375 new cases (16 per 100 000 persons) were diagnosed in 2004, but only 18 patients underwent radical operations. In 2002 and 2003 the incidence of PC was 14 cases per

100 000 persons, which is considerably higher than the overall incidence in Europe and USA. Overdiagnosis, especially in rural hospitals in Latvia, can not be excluded as one of the reasons for the higher incidence of PC found in our population.

Pancreatic adenocarcinoma represents about 90 % of all pancreatic tumours. The incidence of PC is increasing in developed Western countries and incidence and mortality rates are between seven to nine cases per 100 000 in males and five to six cases per 100 000 in females (Parkin et al. 2005). Mortality rates are lower in developing countries, which is rather due to diagnostic capacities than ethiology. Accordingly, the main problem remains early stage diagnosis and the availability of efficient screening procedures.

Much success have been achieved during the past years in understanding the mechanisms of the pancreatic carcinogenesis. Various habitual and environmental exposure factors have been reported to be associated with increased risk of PC, such as smoking, high fat diet and familial background of cancer (Ahlgred 1996; Stolzenberg-Solomon et al. 2002; Michaud et al 2003). However, the most important risk factor of cancer is genetic predisposition associated with structural alterations in cancer-associated genes (Lal et al. 2000; Efthimiou et al. 2001; Tersmette et al. 2001).

The genetic basis for neopasms of pancreas has been the subject of a number of investigations in recent years. While no specific gene(s) associated with PC development have been detected up to now, aproximately 5 to 10 % of the PC cluster in families and can be considered as hereditary. Several hereditary cancer syndromes, such as breast/ovarian cancer syndrome, hereditary non-polyposis colorectal cancer, familial atypical mole-melanoma syndrome, ataxia telangiectasia, and Li-Fraumeni or Peutz-Jegher's syndrome, may be associated with significantly increased risk of developing pancreatic cancer (Lal et al. 2000; Efthimiou et al. 2001; Vimalachandran et al. 2004). Early age at onset of disease and multiple primary tumours in cancer patients may indicate genetic predisposition, even if there are no cancer cases known in the family (too small families or no information available in some cases). There are genes such as *BRCA1*, *BRCA2* or DNA mismatch repair genes highly penetrant and strongly associated with disease in mutation carriers. A number of other genes may each make a subtle contribution to a person's susceptibility to a disease. Genes may also affect how a person reacts to environmental factors. Therefore, the characterization of genetic alterations in cancer-associated genes of PC patients may help to understand better the pathogenesis of PC, as well as to contribute to identification of risk individuals and earlier diagnosis of cancer (de Vos tot Nederveen Cappel et al.2003).

To introduce this approach in clinical practice it is necessary to characterise genetic alterations prevalent in the population. In breast and ovarian cancer patients in Latvia, a high prevalence of a limited number of the *BRCA1* gene mutations was detected indicating a strong founder effect in this population (Csokay et al. 1999; Tikhomirova et al. 2005). This allows targeted testing of cancer patients and improvement of testing yields not only in cases of breast and ovarian cancer but even more in cases of different associated cancers, including pancreatic cancer. Targeted testing in mutation carrier families allows to identify persons at elevated risk of cancer and to apply available preventive procedures.

This population-based study was designed to assess the possibilities for identification of individuals at risk of pancreatic cancer using information available from questionnairies and the results of genetic analyses of the DNA from peripheral blood of PC patients.

Materials and methods

Patients with pancreatic adenocarcinoma were recruited in the Department of Gastroenterology of P. Stradiņš Clinical University hospital in the period between 2002 and 2005. Diagnosis was established using at least two of the following methods: ultrasound, computer tomography, magnetic resonance imaging, endoscopic ultasound, positive cytology, histology, intraoperative finding and other additional diagnostic tools (oncomarkers CA 19-9, CEA, upper gastrointestinal endoscopy, etc.).

A specially designed questionnaire was created for this study, to provide information concerning family cancer history (in first and second degree relatives), history of allergic diseases and asthma, smoking habits, age, education etc. Patients, who were mentally fit to answer the questionnaire were included in the study.

Peripheral blood samples (5 ml) were collected in vacutainers from patients who agreed to participate in the study. Samples were kept in refrigerator until the isolation of DNA, but not more than week. DNA was isolated from 88 blood samples and 68 DNA samples were used further for genetic analyses.

Analysis of questionnaires was carried out to test the role of heredity in PC, the possible association of PC to other cancers in the family history, to investigate if asthma and other allergic diseases are the risk factors for PC and to assess the role of smoking habits as risk factor of PC in the population of Latvia.

Genetic analyses of DNA samples isolated from peripheral blood of PC patients included the screening of the *BRCA1* gene for prevalent mutations, determination of the entire coding sequence and adjacent intronic sequences of the *CDKN2A* gene encoding p16, determination of the alternative exon1 of *CDKN2A* gene encoding $p14^{ARF}$, and screening for mutations in the exon 3 of *STK11* gene.

Genomic DNA was isolated by conventional phenol/chloroform extraction procedure.

DNA analysis was carried out for the most prevalent *BRCA1* gene mutations (5382insC, 4154delA) and for 300T>G and 185delAG, found less frequently.

Oligonucleotide primer sequences have been desribed for *BRCA1* (Tikhomirova et al. 2005) and for exons 1 and 2 of the *CDKN2A* (Soto-Martinez et al. 2005 and Hussussian et al. 1994, respectively).

The forward primer for exon 3 of the *CDKN2A* was 5'-GATGTGCCACACATCTT-TGAC-3', rewerse primer – 5'-TGTGGACCTTCGGTGACTG-3'. Primers for the *INK4/ARF* locus were obtained from Soufir et al. (2000).

The forward primer used for exon 3 of *STK11* was 5'-GGCCATCATCCTGACGTTG-3', the reverse primer was 5'-GCCAGTCTCCTTCAAGGAG-3'.

Fragments were amplified using a MJ Research PTC100 Programmable Thermal Controller and SSCP/HDA (single strand conformation polymorphism and heteroduplex analysis) was carried out as described earlier (Tikhomirova et al. 2005).

Variants detected were identified by direct DNA sequencing (ABI PRISM 3100).

The *BRCA1* gene was screened in 68 pancreatic cancer patients. The same DNA samples were analysed for mutations in the entire CDKN2A gene. DNA samples from 20 patients diagnosed before 56 years or with positive family cancer history were analysed for $p14^{ARF}$ and 39 patients diagnosed before 65 years or with positive family cancer history were analysed for exon 3 of *STK11* gene.

An agreement to participate in the study including genetic analysis of cancer predisposing genes was obtained from all pancreatic cancer patients, and a written consent was received for the interview and blood specimen analysis. The required research permission was obtained from the Ethics Committee of Latvian Institute of Cardiology.

Results and discussion

Analysis of questionnaires

Individuals with pancreatic adenocarcinoma from Pauls Stradiņš Clinical University Hospital (138 patients) were included in this study from November 2002 until May 2005. Blood samples for further genetic analyses were collected from 60 women and 78 men: 90 (65 %) were city inhabitants, 48 (35 %) patients were from rural areas. Characteristics of patients are presented in the Table 1.

A positive family history of PC was reported only by three patients (one first degree relative in each case). A positive family history of other cancers was reported by 36 of 138 patients, slightly more cases were reported by women, probably because they are usually best informed about diseases of relatives. More than two family members with cancer were noted by 12 (33 %) of 138 patients. The overall proportion of patients with a significant cancer history in family was not high in our patient group, and it represents mainly consecutive patients from our hospital. In total 50 cases cancers other than PC were detected in family histories. Gastric cancer was found more often than all other cancers – in 12 cases, followed by gynaecologic cancers (seven cases), lung cancer (five cases), breast, kidney, urinary bladder (each one of three cases), melanoma and carcinoma of esophagus (each in two cases). Colon cancer, hepatocarcinoma, sarcoma, leukaemia, larynx and brain cancer were found in single cases. Overall cancer localisations reported in family members coincided with data found in the literature. More than one cancer location was reported by three patients: one women had breast cancer (radical mastectomy was performed), another woman had gastric cancer (radical gastrectomy was performed) and a third patient (man) had urinary bladder cancer and palliative therapy due to metastasis in liver (possibly from pancreatic cancer) was performed.

It is impossible to estimate the contribution of hereditary factors in PC reliably taking into account only the information provided by patient. One problem was that large families are not typical in the population of Latvia, and in small families it is less likely to find a family history of cancer. In addition, because of the specific historical situation in Latvia in the past century, information about cancer cases in relatives may be lost or

Table 1. Characteristics of pancreatic cancer (PC) patients included in the study

Characteristics of PC patients	PC patients included in the study		
	Total 138 (100 %)	Female 60 (43 %)	Male 78 (57 %)
Mean age (years)	65.7	68.0 (42 ÷ 87)	63.5 (37 ÷ 83)
Pancreatic cancer in family	3 (2.1 %)	2 (1.4 %)	1 (0.7 %)
Other cancers in family	36 (26.0 %)	20 (33.3 %)	16 (20.5 %)
Allergic diseases	22 (16.0 %)	15 (25.0 %)	7 (8.9 %)
Smoking	69 (50.0 %)	6 (10.0 %)	63 (80.0 %)

not available in many families. On the whole, the patients we included in our study had unremarkable family histories of cancer. It should be noted that we cannot confirm the family history data given by patients, and this information may not be reliable in some cases. Characterisation of genetic alterations in the genomic DNA of patients may help to estimate the role of heredity in the incidence of PC in Latvia.

Smoking is a well-known risk factor for lung cancer and was shown to be an important risk factor for PC as well (Ahlgred 1996; Stolzenberg-Solomon et al. 2002). Our study confirms that 69 (50 %) of PC patients were smokers. Analysis of smoking as a risk factor for PC showed that 80 % (63 cases) of men with PC and 10 % (six cases) of women were smokers. Closer analysis of smoking duration showed that 63 % (mostly men) were heavy smokers, it means that they had smoked more than 20 pack years. A small percentage of smoking women has PC, but an almost equal sex incidence of PC indicates that there are many other known and unknown factors in the ethiology and pathogenesis of PC.

Age is one of the significant risk factors of PC. About 75 % of PC develop after 65 years of age and it is quite unusual in persons younger than 50 years, if there are no predisposing conditions such as familial genetic predisposition to cancer, hereditary pancreatitis or specific genetic abnormalities present (Löhr et al. 1999; Lowenfel et al. 1993). Overall in our study, 100 (72 %) of patients were older than 60 years, however we had a quite large subgroup of patients – 38 (28 %, 29 men and 9 women) who were younger than 61 years. Additional risk factors, such as significant cancer history in the family or hereditary pancreatitis were not found in these patients (positive, but not significant family cancer history was detected only in 28 % cases), except in two patients who had another cancer localization (breast cancer or urinary bladder cancer).

Regarding allergic diseases, the literature suggests that allergy is associated with reduced risk of PC, especially allergies related to atopy (Gandini et al. 2005). The analysis of questionnaires showed that 14 % of patients in our study had a history of allergic diseases (22 % of females and 9 % of males). Four patients had asthma, others had different types of allergies (atopic, food or drug allergy). We can conclude that a large population based on case-control studies can establish a relation between PC and allergic diseases.

BRCA1 *gene mutations*

Out of 68 pancreatic cancer patients tested only one woman reported a breast cancer in her family. Hence, according to common criteria, it did not seem that patients with breast/ovarian cancer syndrom in their families could be detected among patients included in this study. However, a strong founder effect detected earlier in the population of Latvia suggested that the same prevalent mutations in the *BRCA1* gene strongly associated with susceptibility to breast and ovarian cancer may have a cosiderable effect in the ethiology of other tumours. This indicates that targeted screening in mutation carrier families might facilitate the identification of risk individuals predisposed not only to breast or ovarian cancer, but to other oncological diseases as well. Well-timed preventive and diagnostic procedures may be especially useful in these individuals.

SSCP analysis was carried out for exon 5 and the 3'-end of exon 11, and heteroduplex analysis was carried out for exons 2 and 20. Positive controls were included in each experiment. No mutations were detected in exons 2 and 5, however one carrier of a deleterious mutation in exon 20 (5382insC) and one mutation in exon 11 (4154delA) was detected.

A patient carrying the mutation 5382insC was diagnosed at 55 years of age and he did not report cancer cases in his family members. The other mutation (4154delA) carrier was diagnosed at 70 and no cancer cases in the family were reported. This corresponds to our previous data (Tikhomirova et al. 2005) as well as to data from International Hereditary Cancer Center, Pomeranian Medical University (Poland) concerning a less pronounced pathogenic effect of the framshift mutation 4154delA as compared to the 5382insC mutation (Gorski et al. 2005), associated with earlier age at diagnosis. The absece of cancer history in family indicated in questionnaries may be associated with a limited number of family members or absence of information in proband.

No other genetic variants were detected in DNA fragments of the *BRCA1* gene analysed.

Two BRCA1 mutation carriers among 68 PC patients tested amounted to 3 %, which is a rather high frequency of deleterious mutation prevalence. It was suggested that BRCA2 gene mutations may be associated with risk of PC in some populations (Lal et al. 2000), however, the association of *BRCA1* gene mutations with PC has been established as well (Lynch et al. 2005). The analysis of family histories of breast and ovarian cancer patients showed that PC is a rather frequent associated cancer localization in breast/ovarian cancer syndrome (Thompson, Easton 2002) and the managment of *BRCA1* gene mutation carriers should include comprehensive care, surveillance and preventive procedures.

CDKN2A *gene mutations*

It has been shown that mutations in the *CDKN2A* gene predispose individuals carrying these mutations to hereditary melanoma and mutations in *CDKN2A* can be detected very frequently in pancreatic tumours (Schneider, Schmid 2003). We assessed the role of mutations in this gene in inherited predisposition to PC by characterization of the prevalence of mutations in the *CDKN2A* gene in the genomic DNA of 68 patients diagnosed with PC. *CDKN2A* gene mutations are known as an important risk factor in the development of familial atypical mole melanoma (Hall, Peters 1996). Only two patients included in this genetic testing had melanoma in their families (each reported one first degree relative with melanoma).

The entire coding sequence of the *CDKN2A* gene and adjacent intronic sequences were analysed by SSCP/HDA, followed by direct sequencing of the variants detected. Results of the analyses are presented in Table 2.

No genetic alterations were detected in exon 1. Missense mutation A148T in exon 2 (alanine to threonine in codon 148) was detected only in two patients. It was suggested recently that this genetic variant may be associated with slightly increased risk of breast cancer (Debniak et al. 2005a). Our results do not suggest an increased risk of pancreatic cancer associated with this mutation. Low prevalence of this common variant do not indicate that it as an important factor affecting susceptibility to pancreatic cancer in the population of Latvia. More data will help to evaluate the role of this missense mutation. At present, it may be considered as one of the possible genetic factors which may modify the risk of cancer in the association with another genetic variants or epigenetic mechanisms and might therefore affect cancer susceptibility to some degree. Significantly increased frequency of the A148T variant among patients with melanoma (7 %) in comparison with the general population (3 %) was observed in Poland (Debniak et al. 2005b). However, the role of the A148T missense mutation in exon 2 of *CDKN2A* gene should be assessed

Table 2. Genetic variants detected in the genomic DNA of 68 pancreatic cancer (PC) patients. M, mother; F, father; B, brother; S, sister; gM, grandmother; ca, cancer

Patients Nr	Age	Cancer in family history	*CDKN2A* (68 patients)	*STK11* (39 patients)	*BRCA1* (68 patients)
1	66	unknown cancer in 2nd degree relative (gM)		IVS3 +12 G>T	
3	71	-	500 C>G		
6	64	melanoma (B)	500 C>G		
9	55	-			5382insC
10	56	-		IVS3 +12 G>T	
14	67	-	500 C>G		
17	43	-	500 C>G		
20	68	-	500 C>G		
23	69	-	500 C>G		
31	45	two gastric ca (M, B)	500 C>G		
32	63	gastric ca (F), cervical ca (M)	500 C>G		
34	37	-	500 C>G and A148T	IVS3 +12 G>T	
35	80	lung (S), two gastric ca (M, F)		IVS3 +12 G>T	
39	51	-	500 C>G	IVS3 +12 G>T	
41	70	-			4154delA
43	50	-	500 C>G	IVS3 +12 G>T	
45	67	brest ca (S)	500 C>G	IVS3 +12 G>T	
47	62	-		IVS3 +12 G>T	
53	71	-	500 C>G		
58	76	larynx ca (B), unknown ca (S)		IVS3 +12 G>T	
63	65	bronchial ca (M)	500 C>G	IVS3 +12 G>T	
64	76	-	500 C>G		
67	69	esophag ca (M), leikosis (S)	500 C>G		
69	57	gynaecological ca (M)	500 C>G and A148T		

more carefully taking into account very early onset of disease (37 years) in one of patients and not late (57 years) diagnosis in another patient, both having in addition a 500C>G variant in the noncoding region of exon 3 and gynaecological cancer in the mother in one of them.

A frequent missense mutation 500C>G in 3'-non-coding region (29 nucleotides behind the stop codon) of the exon 3 of the *CDKN2A* gene was detected in 17 patients, in some patients it was found in the homozygotic state G/G, indicating that it is a rather frequent alteration in the population. The prevalence of the 500C>G variant was characterised in the Polish population. It was not found overrepresented in Polish cancer patients compared to control subjects (Debniak et al. 2005b) and therefore was considered as a nonsignificanct risk factor.

The alternative exon 1 of CDKN2A *gene encoding p14^{ARF}*

DNA samples from 20 patients with pancreatic cancer diagnosed before the age of 56 years or reporting cancer history in their families were tested for genetic alterations in p14^{ARF}. No genetic alterations were detected in the DNA samples analysed.

Exon 3 of the STK11 *gene*

Pathogenic mutations in the serine/threonine kinase *STK11* (alias *LKB1*) causes Peutz-Jeghers syndrome (PJS) in most affected individuals. PJS is an autosomal dominantly inherited disease characterized by hamartomatous gastrointestinal polyps and mucocutaneous pigmentation, with an increased risk for various neoplasms, including gastrointestinal cancer. Recently, the PJS gene encoding serine/threonine kinase *STK11* was mapped to chromosome 19p13.3, and germline mutations were identified in PJS patients (Lim et al. 2004).

It was suggested previously (Lim et al. 2004) that genetic alterations in exon 3 of *STK11* gene may be associated with increased risk of pancreatic cancer. However, the data from different populations are controversial (Grutzmann et al. 2004). To characterise genetic alterations in the genome of pancreatic cancer patients in Latvia, along with other genes we analysed exon 3 of *STK11* gene. DNA samples from 39 patients with pancreatic cancer diagnosed before the age of 65 years or reporting cancer history in their families were tested.

No mutations in coding sequence were detected, however a frequently represented missense mutation in adjacent intron 3 (+12G>T) was detected in 10 of 38 DNA samples analysed, several of them in homozygotic state +12T/T, indicating that this variant probably may be prevalent in the population as well.

It should be noted that one patient (Nr 34) diagnosed at 37 years of age carried A148T missense mutation in exon 2 of the *CDKN2A* gene with possible pathogenic effect, along with two variants of unknown significance, namely, 500C>G in the noncoding region of exon 3 and +12G>T variant in intron 3 of the *STK11* gene. Its possible role in correct splicing can not be excluded, regardless of the high frequency of this alteration. We can suggest that two other genetic variants detected may enhance the pathogenic effect of the A148T mutation in this patient.

We can conclude that the role of genetic factors in PC incidence can not be assessed only on the basis of questionnaires concerning family history of cancer because of the small size of typical families in Latvia and the absence of information in many cases. Questions about family size and availability of information should be included in all cases as well as age at diagnosis of cancer in first degree relatives.

Taking into account the high prevalence of founder mutations in the *BRCA1* gene in the population of Latvia, screening for the two mutations would be useful for identification of at least a part of cancer-prone families.

No known or new deleterious mutations were detected in *CDKN2A*, *ARF* or *STK11* genes. The role of missense mutations detected can not be assessed in an unambigous manner and more data must be accumulated concerning prevalence of these variants in the population of Latvia and association with cancer cases. These genes do not contribute significantly to incidence of PC in our population. Nevertheless it should be noted that PC patients included in this study had no significant cancer histories in their families, therefore we hardly could expect to find a deleterious mutations in these genes. However,

the significance of missense variants detected needs to be studied more carefully, by segregation studies in families and probably by the characterization of the prevalence in control samples.

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Riska faktori vēža slimniekiem

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Kopsavilkums

Mūsu pētījuma mērķis bija novērtēt riska personu identificēšanas iespējas, balstoties uz informāciju, kuru snieguši aizkuņģa dziedzera vēža slimnieki, aizpildot anketas, un gēnu (*BRCA1*, *GDKN2A*, *INK4*/*ARF* un *STK11*) analīzēm, izmantojot slimnieku perifērās asins DNS. Slimnieku aizpildīto anketu analīze norāda, ka, neskaitot smēķēšanu un citus zināmos riska faktorus, slimības etioloģija ir saistīta ar citiem, nezināmiem riska faktoriem. Analizējot 68 aizkuņģa dziedzera vēža slimnieku DNS, noteikti divi *BRCA1* gēna patogēnu mutāciju nesēji – tas nozīmē, ka veicot skrīningu tikai pēc divām visbiežāk izplatītajām *BRCA1* gēna mutācijām, iespējams identificēt vismaz daļu no riska ģimenēm un piedāvāt mutāciju nesējiem atbilstošus profilakses un aprūpes pasākumus. *CDKN2A*, *INK4*/*ARF* un *STK11* gēnu pilnas analīzes rezultātā mutācijas ar skaidri paredzamu patogēnu efektu netika atrastas, no kā mēs varam secināt, ka šo gēnu mutācijām nav būtiskas nozīmes saslimšanā ar aizkuņģa dziedzera vēzi Latvijas populācijā. Balstoties uz līdz šim iegūtajiem datiem noteikto ģenētisko variantu („missense" mutāciju) nozīmi slimības izpausmē nav iespējams raksturot viennozīmīgi.

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Biological basis of biological diversity: physiological adaptations of plants to heterogeneous habitats along a sea coast

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Abstract

To understand the mechanisms of adaptations to heterogeneous habitats along a sea coast, information is needed on the causal character of phenomena in the sequence: environmental factors » adaptive plant features » endogenous control mechanism. Abiotic conditions on the sea coast form a unique environment: sand burial, high temperature, soil salinity, soil flooding. The responses of coastal plants to these conditions are analyzed in order to define possible mechanisms of adaptation. Special attention is given to the cellular mechanisms of protection of photosynthetic machinery and antioxidative protection of cellular constituents. Hormonal and cellular control mechanisms of putative adaptive responses are analyzed. Several characteristics that may possess adaptive significance are discussed e.a. phenotypic plasticity and clonality, seed dormancy, mycorrhizal symbiosis.

Key words: adaptation, heterogeneous environmental conditions, hormonal and cellular regulation, mechanisms of cellular protection, plant conservation biology.

Introduction

In order to meet the targets of the Global Strategy for Plant Conservation, understanding the biology of rare plant species is of extreme importance. More than one third of the rare and endangered vascular plant species of Latvia listed in the Red Data Book occur in coastal habitats close to the Baltic sea. Understanding of the biology of these plants is required for successful development of models with protocols for plant conservation in Latvia. In addition coastal habitats are among most vulnerable zones throughout the world, therefore the information on coastal plant biology is of general interest.

Already in the 1980s there was a debate in the scientific literature for the necessity of establishing basic physiological mechanisms for survival of plants in the heterogeneous coastal environments (Ernst 1985; Lee, Ignaciuk 1985; Rozema et al. 1985). An excellent insight into the ecophysiology of early stages of coastal dune plants was given by Maun (1994). Despite the growing body of literature on the subject within the last decades our knowledge in this field is still incomplete (fragmented in ecological terms).

Some relationships in the coastal zone are more than obvious e.g. vegetation zonation in dune and salt marsh habitats, relative abundance of some species in contrast to the relative rarity of other species in the coastal zone etc. Both coastal sand dune and salt

marsh habitats show a clear sequence of plant species along a transect from the beach to inland. The characteristic spatial distribution of plant species or zonation in sea-affected habitats was first considered as initial stages of succession (Odum 1969; Doing 1985). Knowing that similarities in coastal plant communities in different parts of world in part are due to specific environmental factors, emphasis has been given mainly to plant community analysis. Using this approach, habitat types in fore-dunes are described in terms of floristics, geomorphology, ecology and climate without analysis of the biological features of adaptive value.

Another approach tends to analyze zonation from the point of putative effective environmental factors. Recent studies suggest that salt marsh zonation can not be explained only on the basis of soil salinity and tidal regime (Silvestri et al. 2005). Root oxygen availability is considered to be of extreme importance. In addition to oxygen availability, nutrient disbalance or even toxicity of several ions should be considered.

There is a continuous debate in the scientific literature on whether plant community zonation in sand dunes is predominantly determined by salt spray or by sand burial (Maun, Perumal 1999; Wilson, Sykes 1999; Dech, Maun 2005). Again, possible adaptive life form characteristics and metabolic adaptations mostly have been disregarded. To move further it would be reasonable to quote here Osmond et al. (1987) "The survival of plants in any ecosystem depends on their physiological reactions to various stresses of the environment".

It becomes clear from the above that the nature of the relationships in a coastal vegetation can not be understood simply by means of statistical data analysis based on temporal and spatial distribution of individuals. We need to know the causal character of phenomena in the sequence: environmental factors » adaptive plant features » endogenous control mechanism. While relatively good information is available on the environmental factors, we lack here mostly knowledge on what lays between the perception of a certain signal and the resulting putative adaptive feature. The need for this type of knowledge clearly shifts plant conservation biology from ecological studies towards physiological investigations.

From a physiological point of view, the rarity of a certain plant species (both in terms of populations and individuals) might be the result of two alternative mechanisms. First, species well adapted to particular environmental conditions may become rare because of a decrease of availability of potential habitats. Second, species on the distributional border or with a patchy distribution might have suppressed adaptive potential, decreased fitness and consequently a decreased number of individuals in a particular habitat.

The aim of the present paper is to discuss the recent primary scientific literature relevant to the subject and to refer the reader to most important reviews in this respect for further reading. For better understanding, the most important relevant publications from the past decades are cited. Thus the present paper is not a comprehensive review on the enormously complex problem but rather an insight into the field of coastal plant conservation from the point of view of plant biology, with emphasis on recent literature.

Coastal habitats: heterogeneous, not stressful

Coastal habitats, both sand dunes and salt marshes, are often characterized as being "stressful". While the term has been used mostly to point out that environmental conditions

there are outside the optimal range for majority of the plants, it does not mean that the plants growing in a coastal zone continuously face stress conditions. Common misconceptions in use of the term "stress" have been analyzed by Otte (2001). Therefore, instead of using the term "stressful", coastal habitats can be characterized as highly heterogeneous. According to Stuefer (1996) environmental "heterogeneity" may refer to various fundamentally distinct aspects of environmental variability including scale, contrast, predictability, temporality, spatiality etc. In respect to coastal habitats it is obvious that spatial and temporal variability of both resources and abitoic factors should be considered first. Thus in temperate sand dunes a patchy distribution of nutrients and water is spatially and temporally variable (Alpert, Mooney 1996).

In ecosystems, nutrient levels not only affect primary production, but also composition of species, dominance structure and, subsequently, vegetation succession (Di Tomasso, Aarssen 1989). On the other hand, the effects of nutrient availability on single plant species strongly depend on the presence of other species (Kiehl et al. 1997).

It is well known that coastal sand dunes are low in available phosphorus. However, the effect of mineral nutrients on growth of dune plants has been less studied than that for salt marsh species. The conditions differ in a driftline communities because of annual peaks of decomposition of algal litter and consequently the amount of available mineral nutrient. The response of annual driftline species *Salsola kali* to added phosphate was shown to be more pronounced than the response to nitrate (Lee, Ignaciuk 1985). However it was argued based on the experimental data that in conditions of sand dunes with severe abiotic conditions and limited water supply, plants are unable to respond to the increased availability of mineral nutrients (Houle 1997).

Other soil-related factors besides nutrient levels are also important for coastal plants. It is commonly accepted that vegetation composition on sand dunes is related to soil development. Thus grey dunes which have the largest spatial variability in soil pH, also support the highest diversity of plant species (Iserman 2005). In coastal wetlands, the main negative impact on plant physiology is related to flooding-initiated changes in soil chemistry e.a. soil reduction (Ponnamperuma 1972). Therefore, one of the possible problems facing plants on flooded soils is related to soil reduction-dependent changes in availability of mineral nutrients.

Plasticity and clonality

Plants in general can be characterized by a relatively high level of phenotypic plasticity. Coastal plants, in particular, have a higher level of adaptation in comparison with plants from less heterogeneous environments. Clonality is a manifestation of morphological and reproductive plasticity. Biochemical or regulative plasticity is seen as induced metabolic responses to changes in environmental conditions.

Clonal growth is manifested by the ability of plants to vegetatively produce potentially independent genetically identical daughter plant units (ramets). There are different ways how clonal growth is exhibited. Evolution of clonal plant life histories has been reviewed recently by Fischer and van Kleunen (2002). The most important question about the putative adaptivity of evolution of clonal traits (seen as an optimization of fitness in different environments) was discussed. It has been considered that all four clonal life history traits, namely (i) clonal plants can reproduce both generatively and vegetatively,

(ii) vegetative reproduction can be realized with spacers of different length, (iii) vegetative offsprings can be placed in the most suitable microhabitats, (iv) ramets of clonal plants can maintain prolonged physical and physiological integrity, affect plant fitness. It appears that phenotypic plasticity should have a special role in evolution of clonal life histories.

Clonal plants are extremely abundant in coastal plant communities. It is generally believed that this is mostly due to the fact that clonal growth/clonal integration provides adaptive means for resource capturing in the highly heterogeneous environment (Price, Marshall 1999). However, experimental evidence in support to the concept is still sparse or absent. It has been even argued that clonal integration is not necessarily advantageous in all habitat types (Kun, Oborny 2003). Potentials and limitations of concepts regarding the response of clonal plants to environmental heterogeneity have been reviewed by Stuefer (1996) therefore this aspect of clonality and plasticity in coastal habitats will not be analyzed in detail here. Rather several examples and discussion on possible control mechanisms will be given below.

It is generally believed that the degree of clonal integration, which is highly variable among different species, is related to the degree of environmental patchiness and therefore to average resource availability in a heterogeneous environment. However only limited experimental evidence so far is available to support this hypothesis (D'Hertefeldt, Jonsdottir 1999). The sand sedge *Carex arenaria* from sand dunes in temperate regions forms extremely long clonal fragments (up to 12 m) with older ramets containing only rhizome and associated roots. Extensive clonal integration in *Carex arenaria* with water and nutrients being taken up by older ramets and translocated acropetally while photosynthetically fixed carbon being translocated basipetally was found (D'Hertefeldt, Jonsdottir 1999).

Resource sharing between ramets in clonal plants can be modified by hormones, similarly to their action between separate organs of an individual non-clonal plant. Resource sensing by individual ramets can be expected, as resources are generally transferred from ramets situated in microsites with relatively abundant resources to those in microsites with relative shortage of resources. However sharing of carbon can be bidirectional in contrast to sharing of nitrogen that is mostly unidirectional towards younger ramets. Consequently, the existence of a tightly coordinated control system can be expected instead of gradient-driven transport mechanisms. Limited experimental evidence so far suggests the existence of hormonal control of resource sharing in clonal plants. Thus, application of exogenous indole-3-acetic acid modulates the degree of resource sharing induced by low light intensity or low nitrogen level (Alpert et al. 2002). However there is no evidence for shifting of resource flow by hormonal treatment in a situation when resources are abundant. In addition, no physiological mechanisms have been proposed for regulation of storage of resources in particular ramets of rhizomatous clonal plants.

Regarding the persistence of integration, clonal plants can be divided in to two large groups. Genet splitter species do not form persistent physiological integration. When established, a daughter ramet splits up from the genet. Clonal fragmentation in other clonal species (genet integrators) has been proposed, but there is insufficient evidence about how often this may happen in natural conditions and of its physiological importance. It has been proposed that clonal fragmentation may result from dieback of individual ramets due to abiotic or biotic stress factors or from physiological disconnection caused by internal or external factors (Kun, Oborny 2003). The two types of clonal plants clearly represent

alternative ways of dealing with environmental heterogeneity.

In general, morphological plasticity of stoloniferous clonal plant species has been shown to be considerably higher than that of rhizomatous plant species (Dong et al. 1996).

In addition to physiological integration, a less studied aspect of clonal plant networks is related to intra-genet transport of non-resource agents. Very recently experimental evidence has been shown that induced systemic resistance against herbivores exists in a stoloniferous herb *Trifolium repens* (Gomez, Stuefer 2006). It appears that a plant systemic signal generated in response to generalist herbivore damage is transported through phloem following source-sink gradients. A similar systemic response within a genet has been proposed for pathogen resistance of clonal plants (Stuefer et al. 2004). There is, however, no information available so far on how clonality affects mycorrhizal symbiosis. It remains to be demonstrated also that adaptation of clonal plants to abiotic constraints can involve systemic control within a clonal network. If demonstrated, such a mechanism will give a completely new picture on abiotic adaptations in highly heterogeneous coastal habitats.

Seed germination and dormancy

Several patterns of germination have been characterized for dune plants (Maun 1994), which reflect different mechanisms of dormancy by means of delayed germination until the optimal conditions in the environment are met. Dormant but viable seeds in soil form a seed bank as a propagule reserve for establishment of species especially after heavy environmental disturbance (Luzuriaga et al. 2005). Seed banks are important for the spatial and temporal distribution of annual coastal plant species which mostly lack vegetative propagation characteristic for many perennial coastal plants.

Halophyte seed germination has been reviewed recently in great detail (Khan, Gul 2005). Adaptations to high salinity at the germination level are represented not by maintenance of germination ability at high salinity levels but rather by the recovery potential of germination after conditions of saline imbibition. Consequently, saline-enforced dormancy of seeds represent a selective advantage in conditions of fluctuating soil salinity, such as coastal salt marshes, thus allowing seedling recruitment from a viable seed bank. It has been shown that precipitation in a salt marsh considerably reduces soil salinity leading to increased seedling establishment (more than 50 %) of a number of salt-tolerant species (Shumway, Bertness 1992). On the other hand, the ability of seeds or fruits to float in sea water for prolonged periods of time may be important for dispersal of annual plant species on temperate beaches with major sand accretion (Lee, Ignaciuk 1985). In a study using a large number of coastal plant species it was revealed that species with higher germination at relatively low NaCl have a high recovery germination after high NaCl (Hanslin, Eggen 2005). Some exceptions of the rule have been described: the marsh/shingle boundary species *Limonium bulidifolium* was observed to germinate better in any salinity than in fresh water (Woodell 1985).

It is widely accepted that the species composition of a seed bank always differs from existing vegetation (Ungar 2001; Jutila 2003). Seed bank studies of salt tolerant plant species have shown that the size and species composition of a persistent seed bank in temperate coastal salt marshes are controlled mostly by the combined action of the degree of salt stress and tidal action (Ungar 2001). However, some highly salt-tolerant plant

species do not produce a persistent seed bank in the conditions of a salt marsh. It appears that certain species-specific physiological characteristics of seed germination should be taken into account.

It has been shown recently that changes in salinity and water regime during seed dormancy affect seed response at the time of seed germination (Espinar et al. 2005). Thus, the annual fluctuations in salinity and the duration of flooding affect the persistence of seed banks as well as seedling recruitment from a bank. Salinity-enforced dormancy is thought to be dependent on the endogenous balance of hormones affected by high NaCl, since it is possible to counteract the inhibition produced by salinity by fusicoccin, ethephon, and nitrogenous compounds (Khan et al. 2003).

Another aspect of seed dormancy besides seed banks is related to seed dispersal. For plants from coastal areas, seed dispersal is mostly by wind-driven sand drifting as well as by sea water transport. Besides anatomical and morphological adaptations to water transport, potentially sea-dispersed seeds should have a considerably stable dormancy status to perform successful transportation without germination. It is most reasonable to argue that the high osmotic potential of the seawater preventing water uptake is sufficient for preventing germination. However other dormancy-resembling mechanisms should not be ruled out, as seeds of many plant species readily imbibe seawater without any visible signs of germination (Khan, Gul 2005). Alternatively, seeds of both glycophytes (*Medicago sativa*) and halophytes (*Atriplex lentiformis*) can imbibe salt water with similar consequences for mobilization of stored resources and initiation of radicle protrusion (Malcolm et al. 2003). Only at the stage of hypocotyl elongation and cotyledon spreading, glycophyte seedlings become flaccid and die while halophyte seedlings are protected by means of tissue succulence.

Seeds exposed on the surface usually have very poor germination (Zheng et al. 2005) probably due to seed desiccation and light exposure. The light-dependent inhibition of germination in coastal dune plants has been suggested as a surface-avoiding mechanism in sandy habitats (Thanos et al. 1994).

Sand burial

Wind-driven sand deposition leading to burial of plants is one of the main stress factors on foredunes. Sand accretion alters physical features of the microenvironment where plants grow. Moisture and nutrients increase together with decrease in soil temperature and aeration, and light intensity (Maun 1994). It is necessary to distinguish sand burial of established plants from the effects of the burial at the stages of germination and seedling establishment. In addition, seedling establishment from germinated seeds physiologically is a more complex problem than the germination itself.

In general, the optimum depth for successful germination and seedling establishment for sand dune species is 2 to 6 cm (Maun, Lapierre 1986; Chen, Maun 1999; Zheng et al. 2005). Seed size positively correlates with rate of seedling establishment. This may be because the emergence of a seedling primarily depends on the amount of energy contained in a seed. Seeds with higher energy reserves may have a better chance for establishment in less favorable conditions. Heavier seeds in habitats with a high probability of sand burial are an advantageous trait, since they have a better chance to emerge from a greater depth. It was observed that species with relatively light seeds had a high emergence rate only at

a 0.5-cm depth while species with relatively heavy seeds emerged at a higher rate from relatively greater depths (Zheng et al. 2005).

Several life form characteristics that allow to survive sand burial are important for sand dune plants. Creeping growth along a sand surface is a characteristic for coastal plants well adapted both to heavy winds and sand burial. First, many plants with a clonal growth habit have life forms similar to climbing plants. Second, many herbaceous non-clonal plants have morphological adaptations that favour emergence from sand. Third, true climbing plants are well adapted to sand dune conditions.

Clonal plants growing on sand dunes have characteristics similar to those of true climbing plants – young branches of stolones or rhizomes act as searchers and explorers reaching for resources in the highly heterogeneous environment. For climbing plants, a juvenile phase might represent a self-supporting growth habit maintained by suppressed elongation growth due to suboptimal conditions, e.a. low light. Similarly, clonal growth by elongation can be significantly suppressed during periods when inadequate conditions prevail. Linaoid plants growing on open sand dunes with few climbing opportunities have adopted a modified growth strategy where older perennial stems form underground axes generating shorter-lived stems above the surface of the sand (Isnard et al. 2003).

A number of plants exhibit clonal features only after being buried by sand at an intensive rate. On burial *Honckenya peploides* forms numerous adventitious roots from a newly formed subvertical rhizome (Gagne, Houle 2002). At the end of the vegetation season, buried stems become rhizomes with numerous adventitious roots and buds. In the following spring overwintered buds develop morphologically distinct shoots with smaller regularly and densely positioned leaves, allowing efficient growth of the shoot through the thick layers of sand (Ievinsh, unpublished data). Formation of morphologically different shoots upon severe burial is characteristic also for other sand dune plants e.a. *Alyssum gmelinii* and *Salsola kali* (Ievinsh, unpublished data).

Several plant species well adapted to sand burial represent "obligate buried species", as a certain level of sand accretion is necessary to maintain optimal growth of these plants. An extreme example is represented by *Salsola kali*, in which shoot dry mass can be stimulated up to two times following 14 weeks of sand accretion with the rate of 12 mm week^{-1} (Lee, Ignaciuk 1985). Sand buried plants of the well-adapted dune plant *Cakile edentula* exhibit higher leaf chlorophyll concentration than unburied plants (Zhang 1996). Another coastal pioneer species *Triplasis purpurea* has been shown to increase both plant size and seed production after partial burial by sand (Cheplick, Demetri 1999). Increased fitness (both biomass and seed production) of buried plants depends not only on sand burial itself but rather from the cumulative effect of burial and improved moisture and nutrient status associated with it (Zhang, Maun 1992; Zhang 1996). In experiments where washed sand instead of natural sand was used for plant burial the growth increase of buried plants was only temporary.

Formation of new stem nodes is a prerequisite for burial-induced shoot elongation (Voesenek et al. 1998). Endogenously produced ethylene has been shown to be involved in stem elongation of buried plants by stimulating the process of stem node formation (Voesenek et al. 1998). Soil burial-stimulated internode elongation also seems to be regulated by ethylene (Suge et al. 1997). Other investigations report an increase of the length of existing internodes (Maun 1994). For grasses partial burial of seedlings stimulates growth of new leaves and tillers (Maun 1994).

However even typical sand dune species can negatively respond to sand burial. Burial was shown to significantly reduce the biomass of the embryonic dune and foredune species *Leymus mollis,* having high survival rates (87 %) during burial by 2 cm of sand per week for seven weeks (Gagne, Houle 2002). Another typical foredune species *Honckenya peploides* appeared to be completely intolerant to burial – no seedling survived high burial rates of 1.5 or 2 cm of sand per week (Gagne, Houle 2002).

Tolerant-to-burial coastal dune species exhibiting no burial-dependent increase in elongation can shift biomass accumulation from below-ground to above-ground parts during prolonged partial burial (Brown 1997). In addition, nitrogen allocation can be directed from roots to stem and leaves emphasizing the importance of resource allocation for burial tolerance.

Resistance to sand burial involves tolerance to periods of prolonged dark conditions. Another possible stress factor during sand burial is a reduction of gas diffusion. Therefore it can be proposed that some common metabolic resistance mechanisms exist between adaptation to sand burial and flooding. Even species from dune habitats exhibit different degrees of sand burial tolerance that can be related to light exclusion. In a study with 29 New Zealand dune species survival in complete darkness ranged from 19 to 140 days (Sykes, Wilson 1990). It was suggested therefore that dark survival of dune species often might be considered as a pre-adaptation.

As a physiological adaptation to survive sand burial an ability to maintain photosynthetic intensity while in the buried state as well as a capacity to restore photosynthetic activity after the burial have been demonstrated (Kent et al. 2005). Thus elasticity of photosynthetic response allows buried plants to quickly replenish carbohydrate reserves between two burial episodes. The exact mechanism of the ability to maintain photosynthesis in the buried conditions (low light intensity, low rate of oxygen diffusion) is not known. In the case of clonal plants, physiological integration might be one of the mechanisms. The formation of aerenchyma-like tissues might solve the problem by oxygen diffusion, but no experimental evidence so far has been published.

Mycorrhizal symbiosis play a role in plant adaptation to sand burial (Perumal, Maun 1999). Mycorrhizal plants of both *Agropyron psammophilum* and *Panicum virgatum* subjected to sand burial had a higher CO_2 exchange rate, leaf area and biomass in comparison with the respective non mycorrhizal plants.

As opposed to sand burial, wind-driven removal of surface sand layers should be considered in the coastal environment. This may have both positive and negative consequences for driftdline and dune plant physiology. For already established plants, sand removal leads to exposure of rhizomes and roots and eventual death of plants in the most severe situations. However clonal fragmentation as a result of sand erosion may have a positive effect on the dispersal of clonal plants. For seeds in a seed bank, removal of an uppermost sand layer may result in loss of burial-imposed dormancy and establishment of seedlings in favorable conditions.

Light, temperature, salt – protection of photosynthesis

Protection of photosynthetic machinery is a crucial in maintenance of plant fitness in conditions of suboptimal conditions. Sand dune habitats in temperate regions in summer are characterized by a high irradiance of incident and reflected light as well as by high

substrate temperatures. On sunny days surface sand temperatures on south dune slopes can rise up to 50 °C (Maun 1981). Consequently high light stress in combination with heat stress are inevitable consequences of existence in sand dunes in summer.

Acquired thermotolerance as an ability of plants to survive otherwise lethal high temperature stress can be induced by a mild high temperature (for a review see Sung et al. 2003). In general acquired thermotolerance is associated with a well studied heat shock response through induction of synthesis of heat shock proteins (Schoffl et al. 1998; Queitsch et al. 2000). While acquired thermotolerance has been studied in different model systems in controlled conditions it is not known what mechanism can count for thermal tolerance in natural conditions. In the context of the present paper it would be important to understand if adaptation of dune plants to high substrate and air temperatures involves mechanisms similar to those for acquired thermotolerance. It is reasonable to suggest that different parts of a dune plant in conditions of full sunshine will be exposed to a temperature gradient with a different physiological consequences. In addition the effect of root zone heat stress or thermotolerance on protection of photosynthetic machinery should be considered.

Effect of moderate heat stress on photosynthesis has been recently analyzed and new mechanisms for damage and protection have been discussed (for a review see Sharkey 2005). It was argued that mainly thylakoid reactions are affected by heat stress because high temperature tolerance can be improved by altering thylakoid lipid composition.

As major damage during high temperature stress is associated with active oxygen species-dependent damage of cellular biomolecules (see Suzuki, Mittler 2006 for a recent review) one might assume that an enzymatic antioxidative system is an important constituent during both basal as well as acquired thermotolerance. Increased peroxisomal ascorbate peroxidase gene expression is a part of the heat shock response (Shi et al. 2001). Expression of this gene from barley in *Arabidopsis thaliana* resulted in enhanced thermotolerance at 35 °C suggesting that scavenging of H_2O_2 in peroxisome under heat stress is important for a general thermotolerance.

Experimental evidence for cellular regulation against heat stress-dependent oxidative damage has been shown for *Arabidopsis thaliana* (Larkindale, Knight 2002). Calcium as a main second messenger acting in concert with ethylene, abscisic acid, and salicylic acid protects plant tissues against oxidative damage during post-stress recovery. Thus these endogenous regulators are important for basic thermotolerance after short-time heat treatment. Other studies have shown that salicylic acid-dependent signaling can promote basal thermotolerance in *Arabidopsis thaliana*, while acquired thermotolerance does not depend on salicylic acid (Clarke et al. 2004). It appears that different endogenous signaling systems may be involved during control of different types of thermotolerance.

Besides light and heat, other environmental factors in coastal habitats can affect plant physiology through photosynthesis. Salt stress is generally supposed to have a depressive effect on photosynthesis. Salt stress inhibits photosynthesis mostly through photosystem II activity (Sharma, Hall 1991). Detailed investigations have shown that salt stress enhances photodamage of photosystem II by inhibiting the repair of photosystem II via suppression of transcription and translation of light-dependent genes (Allakhverdiev et al. 2002). Decrease of photosynthesis in salt stress conditions stimulates excess excitation energy which may cause photodamage to photosystem II in the case of limited energy dissipation. The dissipation of harmful energy is thought to depend on heat formation through the

zeaxanthin/violaxanthin system at the antenna region of photosystem II (Gilmore 1997). However until recently mostly glycophyte species have been studied in this respect. Recent data suggests that for halophyte species photosynthetic machinery is extremely well adapted to conditions of high salinity.

Photosynthesis of the sand dune stabilizing obligate halophyte species *Cakile maritima* is impaired at high salinity (400 to 500 mmol l^{-1} NaCl) mainly through a stomatal limitation mechanism with no inhibitory effect on CO_2 fixation enzymes and only a minor suppressive effect on H^+-ATPase activity (Debez et al. 2006).

One of the strategies allowing to grow in a highly saline soil is increased tolerance of photosystem II against photoinhibition caused by a combination of high salinity and high light. As an extreme example, *Suaeda salsa* is an obligate halophyte with 200 mmol l^{-1} NaCl as an optimal concentration for growth. Even 400 mmol l^{-1} NaCl combined with full sunlight did not cause any significant negative effect on photosystem II photochemistry (Lu et al. 2002). The protective effect was not due to an increased amount of protective pigments, either as shown for other species under the effect of salinity (Qiu et al. 2003) or drought and light stress (Masojidek et al. 1991). Similarly, a facultative halophyte *Artimisia anethifolia* well adapted to grow in conditions of high salinity showed no signs of decrease in photosystem II photochemistry due to high salinity even in high light conditions (Lu et al. 2003a; Lu et al. 2003b). The results suggest that photosystem II of halophytes shows high resistance both to high salinity and to photoinhibition. As in *Suaeda salsa* (Qiue et al. 2003) the protective effect was not associated with an inducible increase in xanthophyle cycle activity. However, the violaxanthin/zeaxanthin cycle was involved in the protection against high light. As a result the CO_2 assimilation rate of halophytes is raised in conditions of high light but optimal salinity (Lu et al. 2003c).

Recent data suggest that obligate halophytes are better protected against heat stress than glycophytes. Studies on high salinity effects on halophyte photochemical aspects of photosystem II showed that salt-adapted plants maintain a high CO_2 assimilation rate even at extreme temperatures above 40 °C suggesting enhanced thermotolerance of halophytes (Wen et al. 2005). The increased thermostability is independent of the degree of salinity (Lu et al. 2003c). Regarding the mechanism, it was shown that the increased stability of the oxygen-evolving complex is responsible for thermostability of photosystem II during salt adaptation (Lu et al. 2003c). It is not clear however what molecular changes in the complex are involved. A rapid adaptation of photosynthesis to high temperature by salt treatment is of extreme physiological importance for sand dune species.

Increased activity of enzymatic antioxidants has been proposed as a mechanism for increased protection of photosystem II of salt tolerant species and cultivars (Meloni et al. 2003). In addition, the xanthophyll cycle may protect photosynthetic apparatus in the obligate halophyte *Atriplex centralasiatica*, which is well adapted to increased salinity by a high tolerance of photosystem II to salinity and photoinhibition (Qiu et al. 2003).

It is generally accepted that in conditions with limited CO_2 supply the C_4 pathway is advantageous over the C_3 pathway because of more efficient CO_2 use in C_4 plants. In this respect several species with induced shift between photosynthesis pathways have been described. One such species is the aquatic plant *Hydrilla verticillata*, which changes from C_3 to C_4 in conditions of dense vegetation (Reiskind et al. 1997). Operation of an inducible CO_2 concentrating mechanism allows to minimize photorespiration in conditions of limited CO_2 supply together with high temperatures and O_2 level. Also, in conditions of

water stress C_4 plants have better adaptation capacity and better water use efficiency in comparison with C_3 plants. In part this may be related to several anatomical features and biochemical characteristics at the level of CO_2 fixation (Nelson et al. 2004). On the other hand, better tolerance against water stress exhibited in C_4 plants is related to better ability to withstand endogenous oxidative stress (Nayyar, Gupta 2006). In shifting sand dunes mostly C_4 species are present because of a higher net photosynthetic rate in conditions of high temperature and light intensity accompanied by water stress episodes and, consequently, limited CO_2 supply. Recent findings indicate that C_3 species in sand dunes may exhibit characteristics of C_4 photosynthesis (Niu et al. 2006).

A switch from C_3 to CAM photosynthesis is inducible in *Mesembryanthemum crystallinum* after salinity treatment (Adams et al. 1998). Likewise, with the other inducible features, CAM becomes inducible only at the adult stage. However, compatible solute biosynthesis can be induced by salt treatment at the early stages of development. Like C_4 plants, intermediate C_3/CAM species are more resistant to environmental stress than typical C_3 species (Miszalski et al. 1998). In particular, one such species *Sedum album* shows enhanced antioxidative protection due to a drought-induced shift from C_3 to CAM photosynthesis (Castillo 1996).

Cold and freezing stress responses and the related adaptations are not analyzed in the present paper mostly because they are not specifically expressed in the coastal zone. However, it should be noted that in many respects cellular adaptations to cold and freezing involves systems (e.a. osmoprotectants, enzymatic antioxidants etc.) which are common with other stress responses (e.g. heat, dehydration by water stress and freezing, high salt).

Salinity

It is commonly thought that all plant species growing in a saline environment are halophytes. While it is reasonable from an ecological point of view to designate plant species growing on saline soils as halophytes it does not make any sense from a physiological point of view. To accentuate physiological plant responses to salinity, designation as halophytes is unbiased only for plant species which attain optimum growth at a certain increased level of NaCl. Thus, obligate halophytes are competitive only in conditions of increased salinity. Subsequently, in addition to the above mentioned obligate or true halophytes, facultative halophytes are salt-tolerant species whose growth is not affected at a certain level of NaCl in a substrate. In contrast, glycophytes are plants susceptible to even a minor increase in NaCl concentration. It should be noted that the above system of classification does not impose any absolute NaCl levels for any of the types but instead can be used only in relative terms.

True or obligate halophytes exhibit increased growth rates and higher tissue biomass at moderate salinity levels in comparison with non-saline soils (Short, Colmer 1999). Optimal salinity levels for obligate halophytes are in the range of 50 to 300 mmol l^{-1} NaCl (Lee, Ignaciuk 1985). Dependency from high salt concentrations can be manifested also by stimulation of photosynthetic electron transport in chloroplasts by high chloride levels for obligate halophytic species (Critchley et al. 1982). However in contrast to salt marshes where sea water inundation causes increased soil salinity, sand dune plants receive NaCl mainly in the form of salt spray (Rozema et al. 1985). It is reasonable therefore to distinguish tolerance to soil salinity from tolerance to salt spray (Greipsson, Davy 1996).

Also, different mechanisms of salt tolerance might be responsible for the two types of salinity tolerance. Consequently, plants tolerant to high soil salinity may be susceptible to increased salt spray and vice versa.

In general, salinity tolerance mechanisms are described as cellular, organizational, and whole plant adaptations. For an extensive review of different salt tolerance mechanisms readers are directed to Shannon (1997). Physiologically these mechanisms are oriented towards restriction of ion accumulation in shoots. Mechanistically, this can be achieved by facilitation of ion transport systems (i) transferring Na^+ taken up in roots back to the external medium, (ii) sequestering Na^+ into the vacuole, (iii) transferring Na^+ to older leaves, (iv) excreting salt into salt glands or onto leaf surfaces. Other additional means include cellular osmotic protection due to adjusting of internal osmotic balance by accumulation of compatible solutes and protection against consequences of endogenous oxidative stress.

Salt exclusion at the root level at first seems to be the best solution to the problem of salinity. However a drawback with effective Na^+ exclusion from plant cells is related to the fact that Na^+ influx through the plasma membrane is passive in contrast to active efflux (Maathuis, Amtmann 1999).

Recent studies with an emerging model species for plant salt tolerance studies *Thellungiella halophila*, a close relative to *Arabidopsis thaliana*, indicate that limitation of Na^+ influx in leaves is the main mechanism of salt tolerance in *T. halophila* (Wang et al. 2006). A large proportion of Na^+ taken up into the roots is immediately transferred back to the external medium. Obviously, a high selectivity for K^+ over Na^+ exists in all major cation uptake channels in root membranes of *T. halophila*. Thus, *T. halophila* appears to be a typical "excluding" species not relying on cellular salt tolerance mechanisms.

Damage from high salinity at the cellular level is associated with three different mechanisms. Firstly, ion toxicity is caused by excessive accumulation of Na^+ and Cl^- ions in the cytoplasm leading to ionic disbalance. This can be counteracted by an increased transport intensity of the ions to vacuole. Secondly, even if the excessive ions are compartmented in the vacuole, the osmotic potential needs to be balanced according to a decreased external water potential, otherwise damage to macromolecules will occur. Thirdly, a high cellular NaCl concentration causes an increased formation of active oxygen species with possible oxidative damage of cellular constituents. The formation of active oxygen species under high salinity can be attributed mostly to action of NaCl on the photosynthesis machinery. Here, the photosynthetic electron transfer system is a most obvious candidate for an activator of oxygen during salt stress.

The capacity of transporters to discriminate between K^+ and Na^+ and to translocate them is a critical feature to allow accumulation of high ion concentrations facilitating salt tolerance. In contrast to glycophytes that exclude Na^+ in the shoot, halophytes allow high levels of Na^+ to be translocated to the shoot. Na^+ sequestration into the vacuole is a critical feature of salt tolerance if Na^+ has been taken up in the shoot (Niu et al. 1995). In general, halophytes have higher basal and inducible Na^+/H^+ antiporter activity than glycophytes, supporting the role of Na^+/H^+ antioprters in salt tolerance through Na^+ exclusion from cytoplasm to vacuole (Zhao et al. 2006). At the cellular level, Na^+/H^+ antiporters catalyzing exchange of Na^+ for H^+ across the tonoplast membrane results in removal of Na^+ from cytoplasm into vacuole. The expression of a vacuolar Na^+/H^+ antiporter *SsNHX1* in *Suaeda salsa* is increased by salt stress, suggesting an important role in salt tolerance

(Ma et al. 2004). The antiporter uses an electrochemical gradient of protons generated by vacuolar H^+-translocating enzymes, e.a. H^+-pyrophosphatase. By expressing the *SsNHX1* from *Suaeda salsa* it was possible to increase salt tolerance in rice (Zhao et al. 2006) and by expressing the H^+-pyrophosphatase gene *SsVP* from *Suaeda salsa* – salt and drought tolerance in Arabidopsis (Guo et al. 2006).

A recent detailed review on high-affinity potassium and sodium transport systems in plants relevant to this paper was produced by Roriguez-Navarro and Rubio (2006). Readers interested on a whole array of Na^+ tolerance mechanisms related to Na^+ transport are directed to an excellent review by Tester and Davenport (2003).

Osmotic adjustment is one of the major cellular protection mechanisms against the adverse effects of salinity, drought, and high and low temperature stress. All these stress factors are extremely important in the coastal zone. Consequently, the capacity to adjust osmotic potential in tissues to a certain degree reflects a plant's ability to withstand abiotic stress of the coastal zone in general. Different species accumulate various chemical substances as osmoprotectants. In general they belong to polyols and sugars (e.g. mannitol, trehalose), amino acids (e.g. proline), and to ammonium compounds (glycine beataine) (Nuccio et al. 1999).

An increased amount of osmoprotectants in plant tissues under high salinity is an inducible response and it is thought that the more salt-tolerant species have a higher degree of salinity-induced osmotic adjustment. By tracing changes of compatible osmolytes in leaf tissues of several halophytes throughout the growing season in natural conditions it was shown that the highest osmolyte concentrations coincide with the potentially most stressful period (Murakeözy et al. 2003). Thus the amount of cellular osmoprotectants, even of salinity-adapted plants, is a tightly controlled and inducible feature.

Another line of evidence on compatible osmolytes as cellular stress protectants comes from studies with genetically engineered plants forced to produce higher amounts of these substances. The amounts of glycine betaine accumulated in genetically engineered plants with increased tolerance to a variety of abiotic stressors suggested that the protective effect can not be attributed to osmotic adjustment (Sakamoto, Murata 2000). Rather, direct protection of membranes and macromolecules can be suggested. Glycine betaine are thought to protect an oxygen-evolving complex of photosystem II and native conformation of Rubisco protein in chloroplasts against adverse consequences of wide array of abiotic stress factors including salt, high and low temperature, and drought.

Additional water supply to maintain osmobalance is mediated by a group of channel forming proteins designated as aquaporins (Hill et al. 2004). A relationship has been established between salt tolerance of plants and the activity of aquaproins. However, the role of aquaporins in salinity tolerance of halophytic plants is not clear.

Ion sequestration into salt glands, salt hairs or onto the leaf surface is one of the possible salt avoidance mechanisms. Not all halophyte species are capable of secreting salt, and for the salt secreting species a particular intensity of the secretion is both species-specific as well as strongly dependent on environmental conditions. The efficient salt secretors e.g. *Spartina anglica*, can translocate as much as 60 % of the absorbed Na^+ onto the surface, in contrast to the halophyte *Armeria maritima* which can secrete only 4 % (Rozema et al. 1981). It is reasonable to assume that at increasing salinities plants can secrete relatively larger amounts of absorbed salt. Indeed, *Spartina anglica* in the above mentioned study was from the most saline habitat (Rozema et al. 1981). Also, increasing salinity stimulated

Na^+ and Cl^- secretion by salt glands of *Glaux maritima* (Rozema, Riphagen 1977). At maximum salinity a five-fold increase in the amount of secreted ions was noted with only a two-fold raise of the osmotic potential of the plant sap.

Further, dependence of the density of salt glands and salt hairs on external NaCl concentration can be expected. It may seem paradoxical, but an increase in medium salt concentration resulted in a decrease of salt hair density in the salt marsh species *Atriplex triangularis* (Karimi, Ungar 1989). In addition, the hair density was reduced also by low light and lack of aeration. In the genus *Zoysia* (Poaceae), salt gland density was not affected by external salinity (Marcum et al. 1998). However, salinity tolerance of a particular cultivar or accession was negatively correlated with shoot Na^+ level and positively correlated with leaf salt gland density and Na^+ secretion rate.

Many of the adaptive features leading to increased salt tolerance are inducible, e.i. they appear only when high salinity is present or the intensity increases in response to salt treatment. These include leaf or stem succulence (Rozema et al. 1985). Salt-inducible shoot succulence leading to massive inorganic ion accumulation, especially of Na^+ and Cl^-, accounts for exceptional salt tolerance of several coastal halophyte species (Naidoo, Rughunanan 1990).

One of the aspects of salinity-imposed cellular damage is associated with lipid peroxidation, indicating a harmful effect of endogenous oxidative stress-associated processes (Hernandez et al. 1995). Therefore it is logical to propose that plants with higher constitutively expressed or salinity-induced antioxidative enzymatic capacity will have higher tolerance to saline conditions. Indeed, a number of recent publications show that improved resistance to oxidative stress may improve growth at increased salinity (Jungklang et al. 2004; Sharma et al. 2005). Moreover, a causal relationship has been established between high or increased activities of antioxidative enzymes and the degree of protection from salt-associated oxidative damage (Mittova et al. 2004; Wahid et al. 2006). However, mostly salt-tolerant species or salt-adapted plants have been investigated in this respect. No studies so far on comparing antioxidative defense systems between obligate and facultative halophytes in conditions of high salt have been described. Only very recently, experiments were performed with an obligate halophyte *Suaeda salsa* in which growth is enhanced by NaCl up to 400 mmol l^{-1} (Zhang et al. 2005) as well as with another obligate halophyte *Crithmum maritimum* with an optimum NaCl concentration at 50 mmol l^{-1} (Amor et al. 2005). It appears that for *Suaeda salsa* the activity of chloroplastic superoxide dismutase increases after NaCl treatment in a concentration-dependent manner contributing to resistance of salinity-dependent oxidative stress (Zhang et al. 2005). Thylakoid-bound superoxide dismutase is thought to be responsible for scavenging of photogenerated superoxide radicals in close vicinity to photosystem II. Similarly, the intensity of lipid peroxidation at 50 mmol l^{-1} NaCl for *Crithmum maritimum* was lower than in control conditions, indicating better protection against endogenous oxidative stress by means of increased activities of superoxide dismutase, catalase, and peroxidase (Amor et al. 2005). An optimal NaCl concentration increased the activity of chloroplast antioxidative system enzymes, e.a. ascorbate peroxidase and glutathione reductase, for halophyte *Suaeda salsa* with no harmful effect on the intensity of lipid peroxidation or photosynthesis (Pang et al. 2005).

A detailed study with a true mangrove *Bruguiera parviflora* revealed that four of five antioxidative enzyme activities, namely, superoxide dismutase, ascorbate peroxidase,

peroxidase, and glutathione reductase, significantly increased after treatment with 400 mmol l^{-1} NaCl (Parida et al. 2004). Only the catalase activity decreased after the treatment. It was concluded that NaCl-dependent overproduction of H_2O_2 functions as a signal causing upregulation of enyzmatic antioxidants resulting in unchanged level of lipid peroxidation. Catalase as a target for salinity-induced oxidative stress has been shown also for potato plants (Fidalgo et al. 2004).

Other exogenous factors can affect plant tolerance to salinity. Thus, salt tolerance of coastal foredune leguminous plant species was shown to increase with mycorrhizal colonization (Tsang, Maun 1999). Increased tolerance was manifested as a higher content of chlorophyll and shoot dry mass in mycorrhizal plants in conditions of high salinity in comparison with non-mycorrhizal plants. Also, as mycorrhizal plants had a higher number of bacterial root nodules, the presence of mycorrhizal symbiosis improved the nitrogen status.

In respect to endogenous control mechanisms of salinity tolerance, a close relationship between salt stress tolerance of undifferentiated glycophyte tissues and ethylene production intensity has been shown (Alvarez et al. 2003).

When more realistic combined effects of salinity and following soil drying are concerned, even halophytic species with high tolerance to osmotic stress exhibit decreased tolerance with increased duration of stress conditions (Brown, Pezeshki 2006). It appears that drought magnifies the adverse effects of high NaCl on mineral nutrition, selectively decreasing nutrient uptake (Brown et al. 2006). A possible cumulative effect of different environment extremes should be considered in further studies. In addition, due to the presumably inducible nature of defense responses, acquired resistance mechanisms should be suggested.

Water – too little or too much

Strong variations between driftline, primary dunes, and salt marshes may exist in respect to water availability. A low soil water potential is a common situation in sand dunes. However, as many adaptive morphological and biochemical features are present in sand dune plants conferring relatively high tolerance to water shortage (Ripley, Pammenter 2004), water stress should not be considered as an important physiological problem. It is evident also from ecological studies that water availability is not among the main factors limiting plant distribution in coastal dunes (Monneveux, Belhassen 1996; Dech, Maun 2005). A physiologically more serious problem can occur in salt marsh habitats when flooding by sea water is followed by a prolonged drought period.

Rainfall is extremely important for maintaining an appropriate water balance both in sand dunes and in salt marshes It appears that in salt marshes rainfall in combination with salt water inundation are the most important factors affecting water-salt relationships in plants. In addition, other edaphic factors (e.a. soil redox potential, nutrient availability) depend on inundation by sea water (Pennings, Callaway 1992).

Coastal marshes are characterized by significant water level fluctuations varying from prolonged drought conditions to complete submergence. Both avoidance and tolerance strategies are therefore important as adaptive features for coastal marsh species. Investment in shoot elongation in an effort to reach the water surface can be viewed as an advantageous trait in this respect (Macek et al. 2006). The most tolerant plant species (e.g.

Eleocharis cellulosa) can withstand more than four months of complete submergence and completely recover afterwards (Macek et al. 2006). The ability to recover photosynthetic activity after prolonged submergence is of special importance, as significant depression of the photosynthetic rate during complete submergence is a general phenomenon for macrophyte species (Mauchamp et al. 2001). Depression of vegetative reproduction of clonal plants during conditions of submergence is documented (Vretare et al. 2001).

A completely different situation is evident when the rise of water level leads only to prolonged root zone flooding. Then conditions of anoxia are formed only in root tissues while there is an abundance of oxygen in shoot tissues. Consequently, adaptive strategies leading to improved aeration of submerged tissues can be expected. Here aerenchyma development in shoots and roots represent an induced feature allowing transport of atmospheric oxygen to submerged roots. A recent review summarizing regulation of aerenchyma formation has been published by Evans (2003). In addition, formation of adventitious roots with relatively high porosity facilitating internal aeration due to waterlogging has been described (McDonald et al. 2001; Li et al. 2006).

Severity of oxygen deprivation due to flooding and resultative anoxia injury may have a clearly seasonal character (Crawford 2003). In salt marshes, inundation by sea water is accompanied by a simultaneous reduction of oxygen concentration.

According to a recent review, generation of reactive oxygen species is characteristic for all physiological phases of oxygen deprivation, being most pronounced during reoxygenation (Blokhina et al. 2003). During post-flooding in conditions of oxygen abundance, the formrd oxygen radicals and acetaldehyde undergo oxidative chain reactions in general leading to destruction of membrane lipids (Crawford 2003). Thus, while tolerance to anoxia depends mostly on the degree of metabolic adaptation to anaerobic conditions, post-anoxia tolerance will depend mainly on enzymatic antioxidative capacity of the tissues expressed constitutively or induced by anoxia.

Regulation of adaptation to soil flooding at the level of sensing and signal transduction has been reviewed by Visser et al. (2004) and recently by Voesenek et al. (2006). New mechanisms of adaptation against consequences of flooding and submergence have been proposed (Pierik et al. 2005; Mommer et al. 2006). A recent review (Bailey-Serres, Chang 2005) summarizes also findings on low oxygen sensing in plants leading to physiological adaptation to oxygen deprivation. In the context of the present paper it should be noted that both reactive oxygen species as second messengers and ethylene as an endogenous hormone appear to be involved in the control of adaptation to hypoxia. Most importantly, aerenhcyma formation as an induced response to root zone flooding has been shown to be regulated by ethylene (Viser et al. 1997). Thus, a good theoretical basis has been formed to understand a complex physiological phenomenon of plant responses and adaptations to increased water level and oxygen shortage. This knowledge should be used in further experimental work aiming at understanding of particular adaptation mechanisms for different plant species from coastal habitats. Most importantly, the data analyzed so far in the context of flooding and submergence are mostly from fresh water species. There is a serious gap in a theoretical knowledge on complex adaptive mechanisms to flooding by a salt water. Only limited experimental evidence so far indicate that increased salinity leads to suppression of morphological adaptations against hypoxia (Rolletschek, Hartzendorf 2000). Possible interactions between waterlogging and salinity from the point of agriculture has been reviewed recently (Barrett-Lennard 2003).

Mycorrhizal symbiosis and other biotic interactions

The extent of mycorrhizal colonization of coastal plants to a great extent depends on a habitat type. In shifting dunes there is a limited abundance of mycorrhiza, which increase with dune stabilization (Siguenza et al. 1996). One of the reasons for poor mycorrhizal colonization of coastal plants is possibly related to high soil salinity. It has been suggested that in general mycorrhizal fungi have a lower salinity tolerance than halophytic plants (Johnson-Green et al. 2001). Therefore, in salt marshes mycorrhizal infection is negatively related with the frequency of inundation with sea water, increasing towards the upper reaches of salt marshes. Comparing the intensity of mycorrhizal infection between inland and coastal populations of one species, *Distichlis spicata*, coastal plants had only 9 % intensity of the mycorrhizal colonization in contrast to 28 % infection of inland plants (Allen, Cunningham 1983). These plants excrete salt through leaf salt glands and the intensity of mycorrhizal colonization seems to be independent on soil salinity.

Ascomycete species have been shown to be more resistant to moderate NaCl stress (50 to 200 mmol l^{-1}) than basidiomycete species (Bois et al. 2006). Higher ascomycete resistance to salt stress in part may be due to accumulation of proline or mannitol as osmoprotectants.

Increasing salt concentrations promote mycorrhizal hyphal growth while decreasing arbuscule and vesicle numbers (Tsang, Maun 1999). Regarding the particular effect of soil salinity on spore germination and subsequent hyphal growth, it appears that the NaCl effect is species specific (Juniper, Abbott 2004). In general, spores germinated in the presence of high NaCl (up to 300 mmol l^{-1}) exhibit increased hyphal growth after transfer to non-saline conditions.

Statistical evaluation of the degree of mycorrhizal colonization is often difficult due to the several factors (Hildebrandt et al. 2001). Firstly, the degree of colonization is not constant during a life cycle and has a pronounced seasonality (Siguenza et al. 1996). Secondly, a sharp gradient of environmental conditions in coastal areas can lead to a patchy distribution of spores and hyphae in the soil (Carvalho et al. 2004).

However, the above does not imply that mycorrhizal colonization is less important for coastal plant biology. On the other hand, mycorrhizal hyphae bind sand grains facilitating dune stabilization (Sutton, Sheppard 1976).

Possible physiological mechanisms of arbuscular mycorrhizal symbiosis-dependent protection of host plants against detrimental stress effects have been reviewed recently (Ruiz-Lozano 2003). A particular emphasis was given to mycorrhiza-enhanced cellular osmotic adjustment. While the researchers all believe that mycorrhizas in general have a positive effect on host plant physiology, no conclusive evidence has been shown so far. However, while expected positive results in controlled experiments dealing with a limited number of stress factors have been shown, the effect of mycorrhizal symbiosis on plant fitness tested in field experiments mostly have been unpredictable.

Recently it was shown that arbuscular mycorrhizal symbiosis has a stress-like effect on the salt marsh species *Aster tripolium* in the early stage of development (Neto et al. 2006). However, improvement of plant growth in flooded conditions during subsequent phases of development was shown. Regarding a possible mechanism of positive effect of symbiosis, the beneficial effect of mycorrhizal colonization on flooding-induced instability of proteins in the photosystem II complex increasing maximum photochemical efficiency

was proposed.

In conditions of a heavy metal-contaminated salt marsh, mycorrhizal colonization was negatively correlated with the contents of Pb and Zn in plants while biomass was positively correlated with the degree of colonization (Carrasco et al. 2006). The beneficial effect of mycorrhizal colonization was related to the reduction of plant uptake of heavy metals, especially lead. However, the effect was reduced at high salinity.

In wetlands in general flooding is one of the main factors affecting mycorrhizal colonization in conditions where available inorganic phosphorus levels do not fluctuate significantly. The level of arbuscular colonization is positively correlated with the duration of the unflooded period (Ray, Inouye 2006). Therefore, the length of the unflooded period can be used to predict the level of mycorrhizal colonization. In studies where both flooding and salinity effects on mycorrhizal colonization of *Aster tripolium* plants were analyzed, it was shown that continuous flooding reduced both the degree of colonization and fungal growth (Carvalho et al. 2003). However tidal flooding decreased colonization only if combined with an intermediate salinity level. It appears that salinity has a more deleterious effect on mycorrhizal symbiosis than flooding, especially at the stage of initiation of the symbiosis.

From an agronomical point of view, several investigations report a positive effect of inoculation with arbuscular mycorrhiza on plant productivity in conditions of irrigation with seawater (Yano-Melo et al. 2002; Giri et al. 2003; Rabie 2005). The mechanism involved is believed to be associated with mycorrhiza-enhanced protection of photosynthesis, water use efficiency, and increase in cellular osmoprotectants (Ruiz-Lozano, Azcon 2000). Similar mechanisms of protection are likely to be present in native coastal plants infected with mycorrhizal fungi.

Biotic interactions, excepting mycorrhizal symbiosis, are a less studied aspect of biology of coastal plants. As in other habitats, plant-plant interactions in coastal habitats are expected. In ecological studies, the term "facilitation" has been widely used to describe positive interactions between plants when neighboring plants buffer one another from environment extremes. While the term has been used together with the another term, "competition", to explain secondary succession in salt marsh habitats (Bertness, Shumway 1993) it is quite clear that in addition to competition for resources, from the point of view of chemical ecology, both facilitation and competition are related also to the promotive or inhibitory effect of certain plant-derived chemicals.

Due to the lack of experimental evidence, plant-plant interactions among sand dune and salt marsh plants will not be further analyzed within the present paper. It should be noted though that in the light of ecological data on putative facilitation and competition (sensu Bertness, Shumway 1993) between coastal plants, experimental studies are clearly needed to understand the important aspect of coastal plant biology.

It seems to be somehow logical to assume that in highly heterogenous coastal environments biotic interactions play a minor role in plant biology than in more "predictable" habitats. Similar to that discussed above for mycorrhizal fungi, other microorganisms and arthropod herbivores are supposed to be less adapted to environment extremes. Seasonal changes in a microbial community in a salt marsh studied over a year revealed a higher microbial abundance in a late summer (Keith-Roach et al. 2002). The few available studies on the distribution of both pathogens and insect herbivores in coastal habitats indicate that both pathogens and phytophagous insects may have non-identical distribution patterns

along an environmental gradient. It was hypothesized that mainly changes of host plant quality along the gradient account for different performance of the pests. The performance of a polyphagous stem borer *Agapanthia villosoviridescens* (Coleoptera) on *Aster tripolium* was negatively correlated with an increase of soil salinity (Hemminga, van Soelen 1988). In contrast, the monophagous leaf miner *Bucculatrix maritima* on the same species was not affected by environmental conditions (Hemminga, van Soelen 1992).

Herbivory in coastal habitats has been studied mostly in respect to plant succession both in sand dunes and salt marshes. Several experimental studies have evaluated the impact of herbivory on flowering and survival of salt marsh plants during different phases of succession (Dormann, Bakker 2000; Dormann et al. 2000). It was shown that species more tolerant to salinity were most significantly affected by herbivory. It has been even argued that well adapted plants contain higher concentrations of stress proteins, becoming more attractive to herbivores (White 1984). However it is well known that general stress proteins of adaptive nature may have antinutritive features as well. Therefore the above hypothesis does not seem to be suitable as an explanation of the phenomenon.

Some physiologically more useful information can be gained from studies that compare the effects of herbivory under different regimes of suboptimal abiotic factors. It was shown that in salt marsh plants, survival is affected much more by herbivores than by differences in abiotic conditions between marsh zones (Rand 2002). In general, the data on herbivore effects in salt marsh conditions suggest that herbivores have a major impact on plant survival and seed production. During long-term studies in coastal marshes of Louisiana it was established that under herbivore pressure flooding- and salinity-dependent reduction of species richness and biomass was further stimulated (Gough, Grace 1998).

Studies with a model species *Arabidopsis thaliana* in natural conditions of sand dunes showed that weevils have a strong effect on seed production in contrast to no significant effect of leaf-eating herbivores (Mosleh Arany et al. 2005). In tropical dunes burial by sand is thought to mask the negative effects of herbivores on leaf production (Bach 1998).

Physiological experiments in controlled conditions have shown that effects from biotic stress factors (both pathogens and herbivores) are strongly affected by suboptimal environmental factors. Both a cumulative negative impact as well as some level of induced resistance have been described, depending on the timing and intensity of stress factors (Inbar et al. 2001; De Bruin et al. 2002).

Conclusions and perspectives

On sand dunes and in coastal salt marshes both high level of morphological plasticity visible as environmental constraint-induced development of morphological adaptations as well as a high level of biochemical or regulative plasticity expressed as induced antioxidative protection and protection of physiologically critical macromolecules are of special importance for plants.

In the field of plant conservation biology where every particular rare or endangered species represents immensely high value, primary scientific data is of extreme importance. Consequently, new model species need to be established that represent different adaptation strategies of coastal plants. Several physiological questions of fundamental importance should be considered for further studies. These include measurement of general metabolic costs of different morphological and biochemical adaptations to coastal environment,

hormonal and cellular mechanisms of regulation of induced responses etc. Physiological models need to be found for the study of biologically relevant characteristics beyond resource sharing for clonal plants of different life forms.

The potential of broader understanding of plant species biology in addition to reproduction data is far from being completely understood. The present review was intended at least to generate some general interest in this respect in hope for applied outcomes in the form of practical measures in plant conservation.

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Bioloģiskās daudzveidības bioloģiskais pamats: augu fizioloģiskās adaptācijas eksistencei heterogēnos jūras piekrastes biotopos

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Kopsavilkums

Lai izprastu augu adaptācijas mehānismus eksistencei heterogēnos jūras piekrastes biotopos, nepieciešams atklāt likumsakarības sekojošā secībā: vides faktori » adaptīvas augu īpašības » endogēnie kontroles mehānismi. Apbēršana ar smiltīm, augsta temperatūra, augsnes sāļums, augsnes appludināšana ir abiotiskie faktori, kuri veido unikālos apstākļus jūras krastā. Piekrastes augu atbildes reakcijas uz šiem faktoriem analizētas, lai aprakstītu iespējamos adaptācijas mehānismus. Īpaša uzmanība pievērsta šūnu aizsardzības mehānismiem fotosintēzes aparāta pasargāšanai un šūnas komponentu pretoksidatīvajai aizsardzībai. Analizēti iespējamo adaptīvo reakciju hormonālās un iekššūnas kontroles mehānismi. Papildus analizētas vairākas īpašības ar iespējamu adaptīvu nozīmi, piemēram, fenotipiskais plastiskums un klonalitāte, sēklu miera periods, mikorīzu simbioze.

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Initial responses of explants from rare and endangered coastal plant species during initiation of tissue culture

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Abstract

The strategy of minimum interference was used in the present study to establish *in vitro* cultures of 29 rare and endangered plant species from the coastal zone of Baltic sea in the territory of Latvia by using preferentially seeds as initial plant material. In respect to germination behaviour all species fell into three groups. Seeds of eight species showed uniform germination without any visible signs of dormancy. For 13 species sporadic germination was observed. The remaining species were designated as in an undefined or incompletely understood state. Species-specific morphogenic responses were found during development of plants on hormone-free agarized medium. Annual plants flowered, gave seeds and completed the life cycle within 6 months of tissue culture. Clonal species exhibited pronounced clonal development according to a particular growth form. Axillary bud formation was not stimulated on a hormone-free medium. Root formation was a characteristic for the majority of species. Initiation of tissue culture with shoot explants was performed for seven species. The obtained results form the basis for development of suitable methods for conservation of rare and endangered plant species in tissue culture.

Key words: Baltic Sea, coastal zone, conservation of genetic resources, rare and endangered plant species, tissue culture.

Introduction

In addition to micropropagation plant tissue culture provides a means for conservation of genetic resources. This is especially important for rare and endangered native plant species as the method allows establishing cultures from a minimum amount of starting plant material with possible further multiplication (Benson 2000). In combination with another *in vitro* technique, so-called slow growth, it is possible to establish long-term collections of germplasm with minimal resources (Watt et al. 2000).

Two different strategies are used in respect to tissue culture of rare and endangered plant species. The first startegy can be designated as the minimum interference approach aimed at preserving the genetic identity of source plants. Preferentially using seeds as material for culture establishment, this can be done without damaging of the source plants. This strategy can be effectively used for preservation of particular genotypes, especially using the slow growth procedure. The second strategy is aimed at increasing genetic variation through callus culture with extensive use of synthetic growth regulators

(Seliskar, Gallagher 2000; Wang et al. 2003; Wang et al. 2004; Wang et al. 2005). Sometimes this strategy is also used for mass multiplication needs through a callus culture (Dhar, Joshi 2005).

Coastal habitats are characterized by heterogeneous environmental conditions and have a unique flora that includes many rare and endangered species (Ievinsh 2006). In Latvia more than one third of Red Data Book plant species are located in the coastal zone of the Baltic Sea.

Development of *in vitro* techniques for collecting and preserving threatened plant species has been successfuly started at the National Botanic Garden of Latvia recently (Kļaviņa et al. 2004). In the present study, the strategy of minimum interference was used to establish *in vitro* cultures of several rare and endangered plant species from the coastal zone of the Baltic Sea in the territory of Latvia. Primary growth responses in tissue culture of 29 plant species are reported here.

Materials and methods

During summer of 2005 seeds from 29 rare and threatened plant species from the coastal zone of the Baltic Sea in the territory of Latvia were collected (Table 1). Tissue culture was initiated immediately after collection or within three days. For every species 10 to 50 seeds were used for culture establishment. Seeds were surface sterilized with commercial bleach ACE for 10 to 20 min followed by three washes with sterile deionized water. Sterilized seeds were germinated in 19 × 110 mm test tubes on agar-solidified (6 g l^{-1}) half-diluted Murashige and Skoog medium (pH 5.8) under a 16-h photoperiod provided by a fluorescent light with a photon flux density 10 to 15 μmol m^{-2} min^{-1} at 22 to 25 °C. If the seeds failed to germinate within a prologed period of time different treatments were used to break dormancy including cold stratification at 5 °C, darkness, and thermoperiod.

Several species with no available seeds were tested for a possibility to establish tissue culture using shoot meristems. Explant tissues were surface sterilized with half-diluted

Table 1. Rare and endangered species used in the present experiments for initiation of tissue culture. RDB, Red Data Book of Latvia (2003); 1, endangered species; 2, vulnerable species decreasing in number; 3, rare species

Species	Status RDB	Location	Habitat type	Date of collection	Germination or seed status
Alopecurus arundinaceus Poir.	3	57°50' N, 24°20' E	Coastal meadow	Jul 12	sporadic
Alyssum gmelinii Jord.	3	56°18' N, 20°59' E	Dunes	Jul 27	sporadic
		57°34' N, 21°42' E		Sep 5	sporadic
Angelica palustris (Besser) Hoffm.	1	56°59' N, 23°53' E	Coastal meadow	Aug 18	sporadic
Atriplex calotheca (Rafn) Fr.	3	57°20' N, 23°08' E	Shore	Sep 22	dormant
Blysmus rufus (Huds.) Link	2	57°20' N, 23°08' E	Coastal meadow	Jun 28	dormant
		57°20' N, 23°08' E		Sep 22	dormant
Carex ligerica J. Gay	2	57°50' N, 24°20' E	Coastal meadow	Jul 12	sporadic

(continued)

Species	Status RDB	Location	Habitat type	Date of collection	Germination or seed status
Carex reichenbachii Bonnet	3	57°19' N, 23°08' E	Dune forest	Jun 6	dormant
		57°36' N, 21°59' E		Sep 5	dormant
Centaurium littorale (Turner) Gilmour	2	57°37' N, 22°02' E	Coastal meadow	Sep 5	sporadic
Cephalanthera rubra (L.) Rich	1	57°36' N, 21°57' E	Dune forest	Sep 5	dormant
Eryngium maritimum L.	1	57°14' N, 21°25' E	Dunes	Sep 6	dormant
Euphorbia palustris L.	2	56°58' N, 23°33' E	Coastal lake	Aug 18	explants
Glaux maritima L.	1	57°20' N, 23°08' E	Coastal meadow	Jun 28	explants
		57°20' N, 23°08' E		Sep 22	sporadic
Gypsophila paniculata L.	2	56°18' N, 20°59' E	Dunes	Jul 27	sporadic
Hydrocotyle vulgaris L.	2	57°19' N, 23°08' E	Salt marsh	Jun 28	explants
		57°15' N, 23°08' E	Coastal lake	Jun 28	explants
		57°19' N, 23°08' E	Salt marsh	Sep 22	dormant
Juncus balticus Willd.	3	57°00' N, 23°56' E	Dune slacks	Aug 18	uniform
		57°26' N, 21°39' E	Coastal lake	Sep 5	uniform
Juncus gerardii Loisel.	2	57°19' N, 23°08' E	Salt marsh	Jun 28	explants
		57°19' N, 23°08' E	Salt marsh	Sep 22	uniform
Lathyrus maritimus (L.) Bigelow	2	56°18' N, 20°59' E	Dunes	Jul 27	uniform
		57°15' N, 21°25' E		Sep 6	uniform
Linaria loeselii Schweigg.	3	57°36' N, 21°57' E	Dunes	Sep 5	sporadic
Phleum arenarium L.	1	57°34' N, 21°43' E	Coastal meadow	Sep 5	uniform
Plantago maritima L.	1	56°30' N, 21°02' E	Coastal lake marsh	Aug 24	uniform
		56°30' N, 21°02' E		Sep 18	uniform
Puccinellia capillaris (Lilj.) Jaksen	1	57°00' N, 23°56' E	Shore	Aug 18	sporadic
Schoenus ferrugineus L.	3	57°16' N, 23°09' E	Coastal meadow	Jun 28	dormant
		57°16' N, 23°09' E		Sep 22	dormant
Silene borysthenica (Geuner) Walters	2	56°18' N, 20°59' E	Dunes	Jul 27	uniform
		57°36' N, 21°57' E		Sep 5	uniform
Spergularia salina J. et C. Presl		157°52' N, 24°21' E	Shore	Jul 12	sporadic
Tofieldia caliculata (L.) Wahlenb.	1	57°34' N, 21°43' E	Dune forest	Sep 5	uniform
Tragopogon heterospermus Scweigg.	3	56°18' N, 20°59' E	Dunes	Jul 27	sporadic
		57°34' N, 21°42' E		Sep 5	sporadic
Trifolium fragiferum L.	1	56°29' N, 21°02' E	Coastal lake	Jul 27	sporadic
		57°00' N, 23°56' E	River gulf	Aug 18	sporadic
Triglochin maritimum L.	2	57°20' N, 23°08' E	Coastal lake marsh	Jun 28	sporadic
		57°50' N, 24°20' E	Coastal meadow	Jul 12	sporadic
		57°20' N, 23°08' E	Coastal meadow	Sep 22	sporadic
Tripolium vulgare Nees	1	56°30' N, 21°02' E	Coastal lake marsh	Sep 18	uniform

Table 2. Species of rare and endangered coastal plants with uniform seed germination without dormancy. Seeds were surface sterilized with ACE and germinated in test tubes on agar-solidified half-diluted Murashige and Skoog medium under a 16-h photoperiod, 22 to 25 °C. *, immature seeds

Species	Duration of seed storage (days)	Start of germination (days)	Germination (%)	Seedling establishment from germinated seeds (%)
Juncus balticus	1	7 - 24	95	96
Juncus gerardii	1	2 - 40	35	100
Lathyrus maritimus	1	12 - 104	83*	80
Phleum arenarium	3	4 - 56	97	91
Plantago maritima	6	2 - 17	94	94
Silene borysthenica	3	4 - 11	78	79
Tofieldia calyculata	3	15 - 95	97	28
Tripolium vulgare	2	3 - 76	86	90

Table 3. Species of rare and endangered coastal plants with sporadic seed germination in conditions of tissue culture. Seeds were surface sterilized with ACE and germinated in test tubes on agar-solidified half-diluted Murashige and Skoog medium under a 16-h photoperiod, 22 to 25 °C. *, mature seeds; ** immature seeds

Species	Duration of seed storage (days)	Start of germination (days)	Germination (%)	Seedling establishment from germinated seeds (%)
Alopecurus arundinaceus	80	3 - 73	30	67
Alyssum gmelinii	1	15 - 47	63	20
Angelica palustris	1	11 - 70	50	55
Carex ligerica	1	27 - 42	23	67
Centaurium littorale	3	12 - 76	15	33
Glaux maritima	1	2 - 24	29	50
Gypsophila paniculata	1	12 - 34	63	40
Linaria loeselii	3	116	7	100
Puccinellia capillaris	1	-	-	-
	42	9 - 75	30	100
Spergularia salina	1	131 - 133	73	100
Tragopogon heterospermus	1	-	0*	-
	2	12 - 35	100**	100
Trifolium fragiferum	1	15 - 81	82	78
Triglochin maritimum	1	8 - 26	67	50

ACE and placed in 19 × 110 mm test tubes in the same conditions as described above for seeds. In the case with *Euphorbia palustris* and *Cephalanthera rubra* up to 0.5 mg l^{-1} of 6-benzylaminopurine was added to the medium.

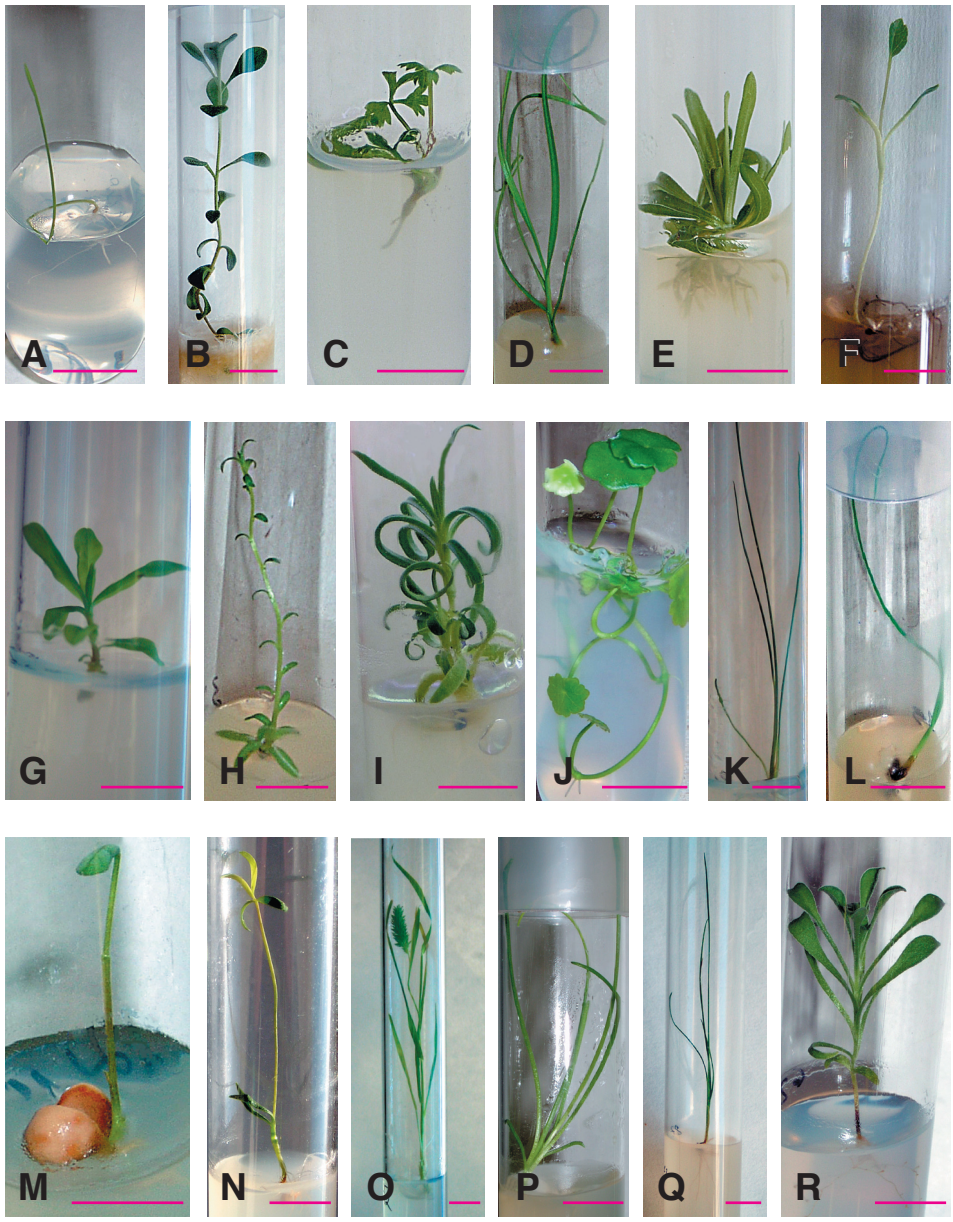

Fig. 1. Primary morphogenic responses of rare and endangered coastal plant species of the Baltic Sea in tissue culture. A, *Alopecurus arundinaceus*; B, *Alyssium gmelinii*; C, *Angelica palustris*; D, *Carex ligerica*; E, *Centaurium littorale*; F, *Eryngium maritimum*; G, *Euphorbia palustris*; H, *Glaux maritima*; I, *Gypsophila paniculata*; J, *Hydrocotyle vulgaris*; K, *Juncus balticus*; L, *Juncus gerardii*; M, *Lathyrus maritimus*; N, *Linaria loeselii*; O, *Phleum arenarium*; P, *Plantago maritima*; Q, *Puccinellia capillaris*; R, *Silene borysthenica*. Bar represents 1 cm. All species shown were established in tissue culture from seeds except *Euphorbia palustris* and *Hydrocotyle vulgaris* for which shoot explants were used.

(continued)

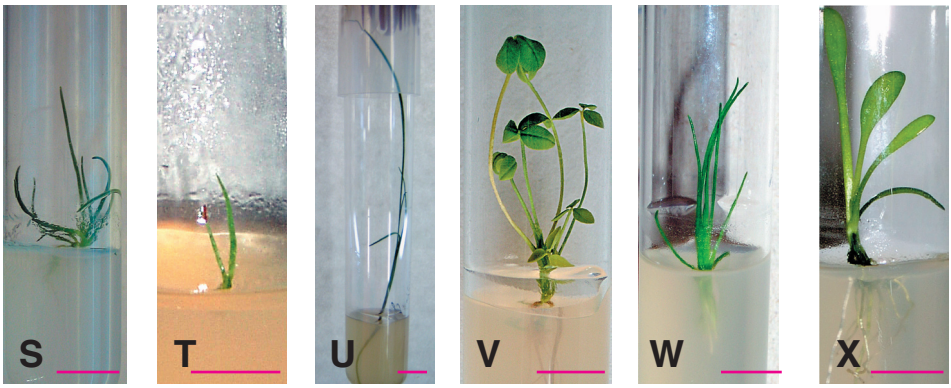

Fig. 1. *(continued)* Primary morphogenic responses of rare and endangered coastal plant species of the Baltic Sea in tissue culture. S, *Spergularia salina*; T, *Tofieldia calyculata*; U, *Tragopogon heterospermus*; V, *Trifolium fragiferum*; W, *Triglochin maritimum*; X, *Tripolium vulgare*. Bar represents 1 cm. All species shown were established in tissue culture from seeds.

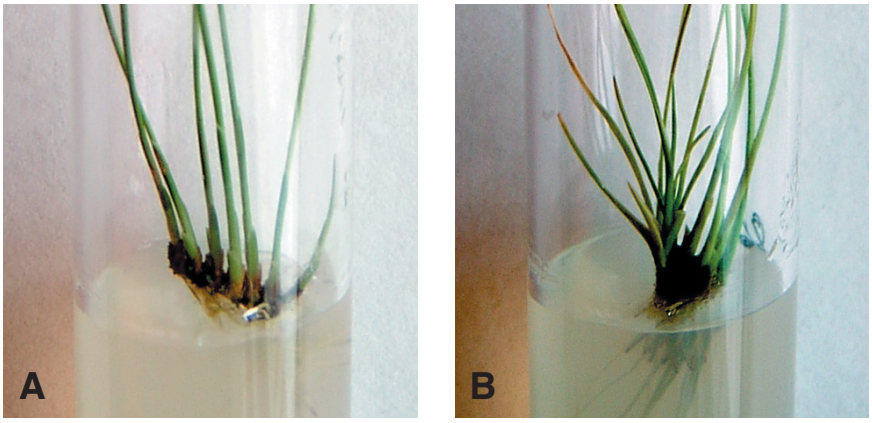

Fig. 2. Clonal growth of *Juncus balticus* (A) and *Juncus gerardii* (B) during cultivation on hormone-free Murashige and Skoog medium for 6 months.

Results

Large variability of seed germination was found for the studied species. In general, in respect to germination behaviour, all species fell into three groups: (i) species with uniform seed germination without any visible signs of dormancy (Table 1, Table 2); (ii) species with sporadic seed germination with a characteristic wide period of start of germination for every particular species (Table 1, Table 3); (iii) species with an undefined or incompletely understood state (most probably, apparent seed dormancy or uncompleted seed maturation; Table 1, Table 4).

In total, seeds of eight species showed uniform germination (Table 2). The majority of seeds from the first group species started to germinate within a week in conditions

Table 4. Species of rare and endangered coastal plants with an undefined or incompletely understood state of seeds. Seeds were surface sterilized with ACE and germinated in test tubes on agar-solidified half-diluted Murashige and Skoog medium under a 16-h photoperiod, 22 to 25 °C

Species	Treatment or status	Start of germination (days)	Germination (%)	Seedling establishment from germinated seeds (%)
Atriplex calotheca	thermoperiod 24 / 5 °C	4 - 100	20	90
Blysmus rufus	thermoperiod 24 / 5 °C		0	-
Carex reichenbachii	2 months at 5 °C	130	0	-
Cephalanthera rubra	darkness, 15, 24 °C	60 - 90	2	necrotic after 2 months
	15, 24 °C		2	
Eryngium maritimum	2 months at 5 °C	110 - 116	20	100
	afterripening + 2 months at 5 °C	88 - 100	50	100
Hydrocotyle vulgaris	seeds at different stages of ripeness	-	-	-
Schoenus ferrugineus	seeds at different stages of ripeness	-	-	-

Table 5. Species of rare and endangered coastal plants established in tissue culture by shoot explants. Explants were cultivated on agar-solidified half-diluted Murashige and Skoog medium medium for 6 months. *, indicate infection.

Species	Sterile explants (%)	Developing explants (%)	Rooting (%)	Coefficient of propagation
Alyssium gmelinii	20	0	-	-
Euphorbia palustris	80	60	0	1.2 - 1.5*
Glaux maritima	56	100	100	2 - 6
Hydrocotyle vulgaris	7	50	88	2 - 4
Juncus gerardii	0	27*	0	-
Silene borysthenica	0	0	-	-

of tissue culture. High germination energy of *Juncus balticus*, *Plantago maritima*, *Silene borysthenica* and *Tofieldia calyculata* was observed. Other species germinated during a longer period of time. Germination of *Lathyrus maritimus* succeeded only in the case when immature seeds were used.

For 13 species sporadic seed germination was observed (Table 3). Seeds for some species started to germinate within a week or two weeks with a low germination energy leading to low number of germinated seeds (*Alopecurus arundinaceus*, *Glaux maritima*). Some species of this group failed to germinate in a few months (*Linaria loeselii*, *Spergularia salina*). Germination of *Tragopogon heterospermus* were observed only in the case of immature seeds. After-ripening of seeds by dry storage at warm temperature (25 °C) was a prerequisite for germination of *Puccinellia capillaris*. Germination of *Spergularia salina* was

only delayed and the seeds successively germinated eight days after seed transplantation on a fresh medium consisting only of water with agar. Transplantation was performed 119 days after initiation of sterile culture.

The other species were designated as being in an undefined or incompletely understood state. Most probably they had dormant or incompletely developed seeds (Table 4). Thus, seeds of *Eryngium maritimum* germinated only after two months of cold stratification and after ripening for two weeks (warm stratification at 24 °C) followed by cold stratification for 2 months. After ripening the seeds were removed from the seed coat. The procedure significantly enhanced the percentage of germination. Dust-like seeds of *Cephalanthera rubra* represent only undeveloped embryo consisting only of few cell layers. However seed germination and protocorm formation with primary leaf scales was observed. Later the tissues became necrotic within two months of cultivation. Seeds of the annual species *Atriplex calotheca* were apparently partially dormant and only a very low percent of germination was achieved after cold stratification. However, in the experiments with seeds collected in previous years, *Atriplex calotheca* germinated sporadically, developed well and finished growth within six months of tissue culture (Kļaviņa et al., unpublished data).

Seeds of *Blysmus rufus*, *Hydrocotyle vulgaris*, *Schoenus ferrugineus* and *Carex reichenbachii* exhibited hard seed coats and were dormant. Low temperature stratification for two months did not result in any signs of germination for these species.

Species-specific morphogenic responses were found during development of plants on hormone-free agarized medium in sterile conditions (Fig. 1). The annual plants *Phleum arenarium*, *Spergularia salina*, and *Atriplex calotheca* flowered, gave seeds and completed the life cycle within 6 months of tissue culture. Some of the seeds of *Spergularia salina* germinated already in the cultivation tube. In general axillary bud formation was not stimulated on a hormone-free medium leading to formation of single shoots. However root formation was characteristic for the majority of the species except *Tofieldia calyculata*. Root formation was considerably depressed for *Tragopogon heterospermus* due to root wounding during transplantation after four months of cultivation leading to callus formation. Many seedlings from sporadically germinated seeds of *Alyssum gmelinii* and *Gypsophila paniculata* were vitrified. Several seedlings of *Glaux maritima* exhibited a characteristic albino phenotype.

The clonal species *Juncus balticus* and *Juncus gerardii* exhibited pronounced clonal development in conditions of tissue culture on a medium without growth regulators within six moths of cultivation (Fig. 2). The phenotype of these plants was similar to that characteristic for plants in natural conditions except that the rhizome internodes were extremely short.

Initiation of tissue culture with shoot explants was performed with seven species (Table 5). Culture establishment from apexes of *Alyssum gmelinii* and *Silene borysthenic*a collected at the end of July failed because active growth had been terminated and the infection rate was relatively high due to hairy leaves. Rosy bacteria infection was found in culture of etiolated buds of *Juncus gerardi* but the presence of the bacteria appeared not to affect growth. Shoot explants of *Euphorbia palustris* did not show any signs of growth on a hormone-free medium and no roots developed. Therefore 6-benzylaminopurine at low concentration (up to 0.5 mg l^{-1}) was added to the cultivation medium which initiated explant development. However no root formation was visible.

Shoot explants of *Glaux maritima* and *Hydrocotyle vulgaris* showed a relatively high

rate of multiplication on a hormone-free medium indicating that propagation of these species can be easily achieved with nodal explants. The clonal plant *Hydrocotyle vulgaris* exhibited extensive development of rhizomes with leaves, inflorescences and roots formed at nodes.

Discussion

To maintain a wide genetic basis it is preferred to establish tissue cultures of rare and endangered plants from seeds (Benson et al. 2000). Therefore, in our experiments seeds in different stages of development and maturity, if available, were collected as the preferred plant material for culture establishment. By using appropriate germination techniques it was possible to establish tissue cultures of 21 out of 29 species from seeds.

Several germination patterns were established for coastal plant species possibly reflecting different environmentally imposed dormancy strategies. Seeds with different genotypes may have a stronger or weaker dormancy potential and the intensity may differ for various populations. Several authors have showed that temporal variation in seed germination depends on hydration intensity, temperature regime, light conditions, as well as on ontogenic experience during dormancy release (Garvin, Meyer 2003; Walck, Hidayati 2004; Zia, Khan 2004; Kagaya et al. 2005). Therefore, in natural conditions seedling emergence occurs sporadically only when environmental conditions necessary for a particular genotype are met. In addition, a number of seeds in each population of the particular species may be programmed to remain dormant even in suitable environmental conditions indicating the existence of multiple-level dormancy (Garvin, Meyer 2003). Thus, in the present study seed coat-dependent maturation was found for *Eryngium maritimum* together with cold stratification-released dormancy.

From the point of view of the minimum interference approach, *in vitro* methods possibly leading to somaclonal variation must be avoided (Benson et al. 2000). Among them, propagation through callus culture (Arene et al. 1993) and the use of high concentrations of growth regulators in order to achieve high rate of multiplication (Karp 1992) are the most dangerous. Therefore, in the present experiments only a minimum amount of cytokinin-like substances was used in the most critical case with *Euphorbium palustre* to initiate tissue culture.

At present, further experiments are being performed to develop suitable methods for slow growth (including cold storage) techniques and subsequent efficient micropropagation of rare and endangered coastal plant species successfuly established in tissue culture.

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Reto un apdraudēto piekrastes augu eksplantu sākotnējā reakcija audu kultūru iniciācijas procesā

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Kopsavilkums

Dotajā pētījumā konsekventi izmantoja minimālās iejaukšanās stratēģiju, lai uzsāktu *in vitro* kultūru 29 retu un apdraudētu sugu augiem no Baltijas jūras piekrastes zonas Latvijas teritorijā, pēc iespējas izmantojot sēklas kā izejas materiālu. Visas sugas varēja iedalīt trīs grupās pēc to dīgšanas veida. Astoņu sugu sēklām bija raksturīga vienmērīga dīgšana bez redzamām miera perioda pazīmēm, bet 13 sugu sēklām – sporādiska dīgšana. Pārējo sugu sēklas apzīmēja kā ar nenoteikti vai nepilnīgi izpētītu statusu. Sugu specifiskas morfoģēniskās reakcijas parādījās uz bezhormonu agarizētas barotnes. Viengadīgie augi sešu mēnešu laikā audu kultūrā uzziedēja, veidoja sēklas un pabeidza dzīves ciklu. Klonālajām sugām bija raksturīga izteikta klonālā attīstība atbilstoši konkrētajai augšanas formai. Aksilāro pumpuru attīstību nenovēroja bezhormonu barotnē. Sakņu veidošanās bija raksturīga lielākajai daļai sugu. Audu kultūru ar dzinuma eksplantu palīdzību veica septiņām sugām. Iegūtie rezultāti veido pamatu piemērotu metožu izstrādāšanai reto un apdraudēto sugu augu saglabāšanai audu kultūras.

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Species diversity, abundance and dynamics of small mammals in the Eastern Latvia

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Abstract

The paper presents the results of small mammal monitoring carried out in Eastern Latvia in the period from 1991 to 2005. Rodents and shrews were snap-trapped in early summer and autumn using the method of trap-line census. During the investigation, 12 small mammal species were found. In open type habitats *Microtus arvalis* prevailed with *Apodemus agrarius* and *Sorex araneus* subdominating. In forest habitats *Clethrionomys glareolus*, *Apodemus flavicollis* and *Sorex araneus* were more common. The average abundance and species diversity was low in the studied grassland habitats (17.1 ± 2.2 individuals per 100 trap-days; Shannon index H = 1.084; Simpson index c = 0.471). In forest habitats the average small mammal abundance was 10.5 ± 1.4 ind. per 100 trap-days and species diversity H = 1.153; c = 0.407. No cyclicity of small mammal population dynamics and no temporal synchrony among coexisting species were found, although fluctuations of small mammal density were observed in the area in different years. Nevertheless, small mammal populations exhibited spatial synchrony among the fluctuations of density in the sample areas in distances of 10 to 25 km.

Key words: abundance, dynamics, Latvia, small mammals, species diversity.

Introduction

Small mammal communities are little studied in Latvia. The information is available about small mammal distribution in Riga city (Zorenko, Leontyeva 2003) and small mammal dynamics have been studied in North-western part of Latvia in Slitere National Park in 1991 - 1998 (Brauna 1992; Brauna, unpublished data). No information regarding small mammal species diversity and abundance in Eastern Latvia has been published up to this time.

The long-term monitoring of small mammals was started in the territory of the Teiči Nature Reserve, in the study area "Apsalas", in 1991. The initial aim of the research was to clarify relationships between breeding success of the Lesser Spotted Eagle and abundance of their pray – small mammals (Bergmanis 2005). Subsequently two new study areas for small mammal monitoring were established in the region – "Lisiņa" and "Žūklis", respectively, at the monitoring study areas of the Lesser Spotted Eagle. The aim of this paper is to present data obtained about species composition, species diversity and population dynamics of small mammals resident in the area.

Table 1. Habitat description of sites of small mammal monitoring in the eastern Latvia. *, according to the forest ecosystem classification (Bušs 1997)

Habitat type	Description
Grassland habitats	
Apsalas	Earlier a cornfield where agro activity has not occurred since 1993. Now this habitat can be considered to be 100 % abandoned grassland with *Dactylis glomerata* and *Phleum pratense* dominating. Overgrowing of the field by bushes and trees has not occurred, but separate young birch trees and *Salix* sp. are found on the edge.
Lisiņa	70 % agricultural land (corn or leguminous cultivated) and 30 % new eutrophic abandoned grassland where *Tussilago farfara*, *Dactylis glomerata*, *Cirsium arvense* dominates.
Žūklis	100% eutrophic abandoned grassland (since year 2003 it has been mowed once a year) with *Dactylis glomerata*, *Urtica dioica*, *Anthriscus sylvestris* dominating.
Forest habitats	
Apsalas	*Myrtillosa turf. mel.**, pine and spruce forest on mesoeutrophic drained peat soil. Tall shrub-sapling layer: *Frangula alnus*, *Sorbus aucuparia*; groundcover: *Vaccinium myrtillus*, *Oxalis acetosella*, *Maianthemum bifolium*.
Lisiņa	*Oxalidosa turf. mel.**, spruce stands mixed with birch and black alder, forest on eutrophic rich drained peat soils. Tall shrub-sapling layer: *Frangula alnus*, *Sorbus aucuparia*, *Salix cinerea*; groundcover: *Oxalis acetosella*, *Dryopteris* sp., *Convallaria majalis*, *Stellaria nemorum*, *Urtica dioica*.
Žūklis	*Mercurialiosa mel.**, spruce and birch forest on rich eutropic drained mineral soil. Tall shrub-sapling layer: *Frangula alnus*, *Sorbus aucuparia*; groundcover: *Mercurialis perennis*, *Oxalis acetosella*, *Impatiens nolintangere*, *Athyrium filix-femina*.

Materials and methods

The monitoring of small mammals was carried out by the researchers of the Teiči Nature Reserve and financed by the state budget. The work was started in 1991 in the sample area "Apsalas" at the boundary of the Teiči Nature Reserve, in Eastern Latvia (N 56° 41', E 26° 27'). In 2002 and 2003 two new sample areas were established – "Lisiņa" (N 56° 41', E 26° 38') and "Žūklis" (N 56° 51', E 26° 25'), respectively. All of the study areas are situated in the monitoring sample areas of the Lesser Spotted Eagle (*Aquila pomarina*). The distances between the study areas are 10 to 25 km.

Trapping was conducted in one grassland habitat site and in one forest habitat site in each study area. For grassland habitats extensively exploited agricultural or semi agricultural lands were chosen, as typical feeding sites for Lesser Spotted Eagle. For forest habitats the prevailing forest type in the area was chosen for small mammal trapping. Trap

Table 2. Small mammal community parameters in the sample areas. N, number of trap-days operated; s, number of species; n, number of individuals; H, Shannon's indices; EH, Shannon's equitability; c, Simpson's indices

Parameter	Apsalas (1998 - 2005)		Lisņa (2002 - 2005)		Žūklis (2003 - 2005)	
	Grassland	Forest	Grassland	Forest	Grassland	Forest
N	10.368	7.320	2.960	2.100	2.400	1.680
s	9	8	10	6	9	5
n	1.854	651	491	275	273	206
H	1.041	1.147	1.033	1.075	1.283	1.071
EH	0.501	0.552	0.470	0.600	0.617	0.665
c	0.521	0.439	0.533	0.419	0.384	0.421

lines where set along the drainage ditches existent in the habitats.

Several persons were involved in the small mammal trapping. Monitoring was initiated by U. Bergmanis and J. Rubenis, later G. Dambenieks participated and finally, since 2000, A. Pupila and U. Bergmanis conducted the work.

Trapping was conducted biannually (late May/early June and September) using the method of trap-line census. One hundred plastic snap traps were placed in grassland habitats and 70 in forest habitats at 5 m intervals. Traps were set for three or mostly for four nights and checked once a day. Initially, from 1991 to 1998, animals of genus *Microtus* and *Sorex* were not identified to the species. Later till 2005, animals were identified to species, except *Microtus rossiaemeridionalis* which were considered as *M. arvalis*.

The relative small mammal abundance was estimated as the number of animals caught per 100 trap-days. Calculating the total abundance of small mammals in the area, all "attended" traps were entered into the calculations. *Traps "attended"* are defined here as those where presence of small mammals in the trap was evident but no animal was found in there (for example, fragment of fur or tail was found). Species diversity and dominance of small mammals was calculated using Shannon's (H) and Simpson's (c) indices. The synchronism of changes in small mammal abundance was determined by Spearman's rank correlation coefficient.

Results

Species composition and diversity

There were 12 small mammal species recorded in the area. Almost all of them, except *Mus musculus* that was trapped only once and then in the forest habitat in "Apsalas", were found in grassland habitats. Of these, three species, *Micromys minutus*, *Neomys fodiens* and *Microtus rossiaemeridionalis* were not found in forest habitats. In total voles prevailed (67.5 % of the total catch) in the small mammal community in the area with *Microtus arvalis* and *Clethrionomys glareolu*s dominating. The main parameters of small mammal community in the sample areas are shown in Table 2.

In grassland habitats 11 species of small mammals were identified, belonging to five genera: *Microtus* (72.5 %), *Apodemus* (14.2 %), *Sorex* (12.2 %), *Clethrionomys* (0.8 %) and *Micromys* (0.5 %). The most numerous species were *Microtus arvalis*, *Apodemus*

Table 3. Mean abundance of small mammal species in studied habitats in early summer (first row, respectively) and autumn (second row) trapping sessions in the period of 2003 to 2005. *, traps "attended" have been taken in account

Species	Grassland habitats			Forest habitats		
	Apsalas	Lisiņa	Žūklis	Apsalas	Lisiņa	Žūklis
Microtus arvalis	2.6 ± 2.1	1.4 ± 0.6	3.6 ± 1.6	0	0	0
	15.9 ± 7.8	8.9 ± 4.5	7.5 ± 1.8	0	0	0
Microtus agrestis	0.1 ± 0.3	0.2 ± 0.1	0.3 ± 0.3	0	0	0
	0.5 ± 0.1	1.0 ± 0.5	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0
Clethrionomys glareolus	0	0	0	0.6 ± 0.4	1.0 ± 0.4	2.7 ± 0.8
	0.6 ± 0.4	0.3 ± 0.1	0.2 ± 0.2	4.1 ± 1.0	7.6 ± 2.7	10.0 ± 4.2
Apodemus agrarius	0.2 ± 0.2	0.8 ± 0.6	0.9 ± 0.8	0	0	0
	4.9 ± 3.2	3.8 ± 1.5	3.1 ± 1.5	0	0	1.1 ± 1.1
Apodemus flavicollis	0.2 ± 0.2	0	0.1 ± 0.1	0	1.3 ± 0.5	1.4 ± 0.8
	1.9 ± 1.3	1.1 ± 0.9	0.2 ± 0.2	0.1 ± 0.1	5.5 ± 1.9	4.4 ± 2.3
Apodemus uralensis	0	0.1 ± 0.1	0	0	0.4 ± 0.4	0
	0	0.1 ± 0.1	0	0	1.6 ± 1.6	0
Sorex araneus	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	-	-	0.2 ± 0.2
	1.2 ± 0.7	0.5 ± 0.4	2.3 ± 0.5	1.6 ± 1.1	0.1 ± 0.1	1.9 ± 1.4
Sorex minutus	0	0.1 ± 0.1	0.1 ± 0.1	0	0	0
	0.2 ± 0.1	0	0.4 ± 0.2	1.6 ± 1.4	0.1 ± 0.1	0.1 ± 0.1
Micromys minutus	0	0	-	0	0	0
	0	0.2 ± 0.1	0.5 ± 0.5	0	0	0
Total *	3.7 ± 2.1	2.6 ± 1.0	5.8 ± 2.7	0.8 ± 0.5	3.3 ± 1.4	5.1 ± 2.1
	27.7 ± 9.7	19.3 ± 6.5	16.9 ± 3.1	8.1 ± 4.0	17.0 ± 4.5	19.4 ± 3.1

agrarius and *Sorex araneus*. Abundance of small mammal species in each of the sample areas is shown in Table 3. The rarest species in grasslands were *Neomys fodiens*, *Apodemus uralensis*, *Micromys minutus* and *Clethrionomys glareolus*. Species composition did not differ significantly in the "Apsalas" and "Lisiņa" grasslands, where *Microtus arvalis* composed 70.5 % to 71.4 % of the small mammal community respectively and *A. agrarius* and *S. araneus* were subdominant. In "Žūklis" *Microtus arvalis* contributed 57.2 % of the total small mammal number in the habitat, at which the most abundant were *Apodemus agrarius*, *Sorex minutus* and *Micromys minutus*.

In the period from 2003 to 2005 the mammal species diversity was similar in all the grasslands observed ($H = 1.114 \div 1.317$, $c = 0.373 \div 0.466$, $p < 0.1$).

In years when populations reached their maximum, species diversity was lower than in minimum years ($H_{max} = 0.739 \div 0.866$, $H_{min} = 1.222 \div 1.520$; $p < 0.01$), since the proportion of dominating species was much higher in peak years.

In forest habitats nine species of four genera of small mammals have been registered: *Clethrionomys* (59.1 %), *Apodemus* (24.5 %), *Sorex* (15.4 %) and *Microtus* (1.0 %).

The most common species in forest habitats were *C. glareolus* (59.1 %), *A. flavicollis* (20.4 %) and *S. araneus* (11.6 %). Subdominating species in coniferous forest were *S.*

araneus and *S. minutus*, while in mixed forests *A. flavicollis*, *A. uralensis* and *S. araneus* formed the greatest proportion of the small mammal community (SMC) after *C. glareolus* (Table 3).

Species diversity of SMC in the forest habitats was higher than in the open type habitats ($H = 1.153$; $c = 0.407$, $p < 0.1$). In all the habitats in the study period of 2003 to 2005 the species diversity was similar ($H = 1.062 \div 1.096$; $c = 0.383 \div 0.421$, $p < 0.1$).

Species diversity in forests was the highest in the autumn trapping session when prevalence of dominating species was less pronounced than in early summer ($H_{September} = 1.103 \div 1.113$, $H_{May/June} = 0 \div 0.986$). However, in contrast to grassland habitats, the proportion of dominating species in the SMC was higher in years of population minimum than in years of minimum ($H_{max} = 0.928 \div 1.143$, $H_{min} = 0.548 \div 0.693$; $p < 0.01$).

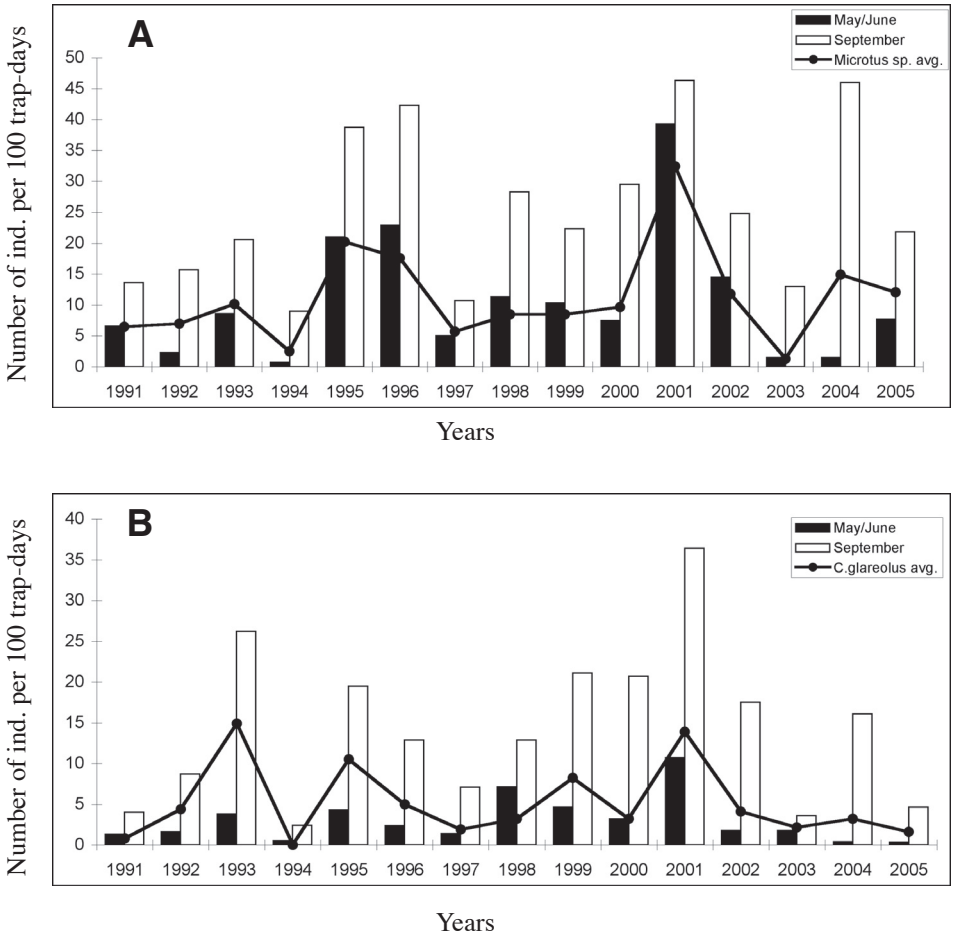

Fig. 1. Density fluctuations of the total small mammal number (columns) in relationship to the mean number of dominant species (line) in grassland (A) and forest (B) habitats in the sample area "Apsalas" in summer and autumn trapping sessions.

Abundance of small mammals

The average abundance of small mammals in the area was 13.8 ± 1.4 ind. per 100 trap-days. In the grasslands the average abundance of small mammals was 17.1 ± 2.2 ind. per 100 trap-days (9.9 ± 2.2 in early summer, 24.4 ± 2.6 in September). In the period 2003 to 2005, on average from both trapping sessions, the highest relative abundance of small mammals in the field areas was found in the old abandoned grassland "Apsalas" (15.5 ± 4.8) compared to 11.1 ± 3.7 and 11.4 ± 2.9 ($p < 0.1$) in the "Lisiņa" and "Žūklis", respectively.

The average abundance of *M. arvalis* in the grasslands was 10.1 ± 1.7 ind. per 100 trap-days. In the abandoned old grassland "Apsalas" the abundance of this species was about 1.7 times higher than in other grassland territories. The average abundance of *M. agrestis* in grasslands was 0.5 ± 0.1 ind. per 100 trap-days, *C. glareolus* 0.1 ± 0.0, *A. agrarius* 1.7 ± 0.5, *A. flavicollis* 0.4 ± 0.2, *A. uralensis* 0.2 ± 0.0, *S. araneus* 1.6 ± 0.4, *S. minutus* 0.2 ± 0.1 and *M. minutus* 0.1 ± 0.0.

The abundance of species in early summer and autumn trapping in the sample areas is shown in Table 3.

The total average abundance in forest habitats in the area was 10.5 ± 1.4 ind. per 100 trap-days, 3.6 ± 0.6 in late May/early June and 16.6 ± 2.2 in September. The highest abundance was found in mixed forest (12.3 ± 2.1) in the sample area "Žūklis", and the lowest in coniferous forest in "Apsalas" 4.5 ± 1.9 (three times lower). To a great extent, the general abundance in forest habitats was determined by the dominant species *C. glareolus*. The average degree of abundance of *C. glareolus* in forest habitats in the area was 5.6 ± 1.1 ind. per 100 trap-days, in mixed forest on mineral soils the abundance of *C. glareolus* was the highest (6.4 ind. per 100 trap-days in the period of 2003 to 2005), and comparatively 2.7 times lower in coniferous forest. The average abundance of *A. flavicollis* in forest habitats was 1.8 ± 0.5 (2.9 ± 0.2 animals per 100 trap-days in mixed forests and only 0.1 ind. in coniferous forests). The average degree of abundance of the *S. araneus* was 1.1 ± 0.3 and of *S. minutus* 0.4 ± 0.2 ind. per 100 trap-days. The rarest species in the forest habitats were *A. uralensis*, *A. agrarius* and *Microtus* species (Table 3).

Dinamycs and sinchronism of small mammals

The fluctuation of small mammal relative population density was observed in the study period investigations. Although there were pronounced peak and low population phases in several years, the dynamics were not clearly regular and the amplitudes of the fluctuation were variable (Fig. 1). For three years, from 1998 to 2000 the number of small mammals in the area was nearly stable. Subsequently from 2000 to 2004, a fluctuation cycle was observed where the number of small mammals trapped in the grassland habitats in the low phase was 7.3 ind. per 100 trap-days and 41.4 ind. per 100 trap-days in the peak year. The average amplitude of small mammals in grassland habitats, based on combined early summer and autumn data, varied from three to ten fold (n_{max}/n_{min}), with minimum and maximum densities of 3.7 and 41.4 individuals per 100 trap-days, respectively.

For the dominating species *M. arvalis*, the amplitude in different cycles varied from four to 28 fold. The highest density of this species in the study area "Apsalas" was recorded in 2001 with 32.1 ind. per 100 trap-days and the lowest density in 2003 with 1.1 individuals trapped in 100 trap-days. In years of peak density of the total number of all small mammal species, the proportion of *M. arvalis* in grassland habitats was higher (85.6 ÷ 92.0 % of SMC) than in low years of depression (16.6 ÷ 47.6 %, $p < 0.01$). *A. agrarius* showed a

regular fluctuation of density every one to three years, with an amplitude of five to 21 fold. The maximum number of *A. agrarius* observed was 5.8 ind. per 100 trap-days in 2004. *A. flavicollis* in open areas reached a maximum density in 1993 (3.7 ind. per 100 trap-days) and the next peak was observed only after 10 years in 2003 (2.6 ind. per 100 trap-days). In forests the highest density of this species was in 1993, 1998 and in 2002 when the amplitude of cycles varied from three to 19 fold. Less expressed fluctuations was found for *Sorex* species. In the grassland habitats two peaks were observed for *S. araneus*, in 1998 and 2002, and with no remarkable low phase in that period. In forest habitat the population was almost stable until 1998 when a cycle occurred from 1999 to 2003, with minimum density 0.2 and maximum 5.2 individuals per 100 trap-days.

In forest habitats the average amplitude of the total small mammal density was nine fold varying from three to 19 fold. The minimum and maximum numbers of *C. glareolus* in woodland habitats were 0.5 in 1994 and 14.9 individuals per 100 trap-days in 1993 and the proportion of species in the community did not differ during the population peak and low years, varying from 70 % to 92 % of the total abundance.

No correlation was found between fluctuation of abundance of different species in the period of investigation. Each species reached their peak phases in different years and only low phases coincided for voles and shrews. Nevertheless, the mean density of the total number of small mammals in grassland habitats reached their maximum and minimum in the same years. Also, the total number of small mammals in the grassland and forest habitats fluctuated more or less synchronously (in "Lisiņa" and "Žūklis" in the period from 2002 to 2005 there was a positive correlation, and in "Apsalas", since 1991, $r = 0.69$, $p < 0.001$).

Small mammal species in the area exhibited spatial synchrony in population density fluctuations. For a four year period a perfect positive correlation was found for *M. arvalis* ($r = 1.00$), *M. agrestis* ($r = 0.95$, $p < 0.001$), *S. araneus* (in grasslands $r = 1$, in forest habitats $r = 0.95$, $p < 0.001$), *C. glareolus* ($r = 0.8$, $p < 0.01$) and *A. flavicollis* ($r = 0.8$, $p < 0.01$) was observed in two study areas "Apsalas" and "Lisiņa".

Discussion

Twelve small mammal species were recorded in the area inter alia all three species of family *Soricidae* and nine of 13 species of the *Murida* and *Cricetidae* registered in Latvia (Timm et al. 1998). On average voles prevailed in the area, forming 67.5 % of the small mammal community. A very similar proportion of voles (69.7 %) in small mammal communities as observed in NE Lithuania (Mažeikyte 2002).

In grasslands SMC were found to be monodominant with the dominant species *M. arvalis* and *A. agrarius* and *S. araneus* subdominating. The low diversity ($H = 1.084$, $c = 0.471$) and dominating species is similar to that observed in NE Lithuania in the anthropogenic habitats (Mažeikyte 2002). That might imply that even in the study area "Apsalas", where no agricultural activity has occurred already for 13 years, the impact of ipast land use is still present. The dominating plant species *Dactylis glomerata* and *Phleum pratense* in the habitat confirm this assumption. The other grassland habitats are still influenced by human activity, which might be the reason of low species diversity observed in the area.

The small mammal community in the forest habitats was monodominant with

C. glareolus prevailing, likewise it has been observed in NE Lithuania (Balčiauskas 2005). Subdominant species in the mixed type forests were Apodemus flavicollis and Sorex species subdominate in coniferous forest. A higher density of small mammals was observed in mixed forest on rich euthropic drained mineral soil.

Small mammal populations in eastern Latvia did not show clear cyclic dynamics. Although fluctuations of population density occurred in the period of investigation, no pronounced periodicity was observed and the amplitudes of fluctuations were low and variable. Future fluctuations of small mammal density cannot be predicted in the area. It is known that northern small mammal populations exhibit multiannual fluctuations in density (Hansson, Henttonen 1985; Hanski et al. 2001). However, not all of them can be considered to be cyclic. Hansson and Henttonen (1985) found that cyclicity of arvicoline rodents decreased from the north to south in the Fennoscandinavia, where populations below 59° N fluctuated mainly seasonally.

We also did not observe interspecific temporal synchrony among the small mammal populations in the region. However, synchrony occurs in Fennoscandinavia, for example, for voles and shrews (Hansson 1984; Henttonen 1985; Korpimaki et al. 2005), and there is also a decrease in the degree of interspecific synchrony in population oscilliations from northern latitudes to the south (Henttonen, Hansson 1986).

Spatial synchrony was observed among populations in the studied sample areas at distances of 10 to 25 km. Large-scale synchrony in population fluctuations of small mammals prevail in the northern Europe (Sundell et al 2004) whereas again the degree of synchrony decreases towards the south (Steen et al 1996). For *Microtus* voles a higher degree of spatial synchrony was found in a more agricultural landscape (Huitu et al. 2003), which is consistent with the positive correlation found in eastern Latvia, where trapping was conducted in the agricultural areas.

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Sīko zīdītājdzīvnieku sugu daudzveidība, sastopamības biežums un dinamika Austrumlatvijā

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Kopsavilkums

Šajā darbā apkopoti Austrumlatvijā veiktā sīko zīdītājdzīvnieku monitoringa rezultāti laika periodā no 1991. līdz 2005. gadam. Sīko grauzēju un ciršļu uzskaite veikta divas reizes sezonā, vasaras sākumā un rudenī izmantojot standarta slazdu līnijas veida metodi ar peļu sitamajām lamatiņām. Pētījumu laikā reģistrētas divpadsmit sīko zīdītājdzīvnieku sugas. Atklātā ainavā visbiežāk sastopamā suga bija *Microtus arvalis*, nedaudz retāk – *Apodemus agrarius* un *Sorex araneus*. Meža biotopos visbiežāk sastopamās sugas bija *Clethrionomys glareolus*, *Apodemus flavicollis* un *Sorex araneus*. Zālāju biotopos novēroja zemu sīko zīdītājdzīvnieku blīvumu un daudzveidību (17,1 ± 2,2 dzīvnieku 100 slazdu-diennaktīs; Šanona indekss H = 1,084; Simpsona indekss c = 0,471). Meža biotopos vidējais sīko zīdītājdzīvnieku blīvums bija 10,5 ± 1,4 ind. 100 slazdu-diennaktīs, bet sugu daudzveidība – H = 1,153; c = 0,407. Sīko zīdītājdzīvnieku skaits pa gadiem svārstījās, tomēr netika atrasts cikliskums to skaita izmaiņās, kā arī atsevišķu sugu skaita svārstības pa gadiem nesakrita. Tomēr konstatēja, ka sīko zīdītājdzīvnieku skaita svārstības pa gadiem sakrita dažādos uzskaites parauglaukumos 10 līdz 25 km attālumā.

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Epiphytic bryophytes in old growth forests of slopes, screes and ravines in north-west Latvia

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Abstract

Little is known about the ecology of epiphytic bryophytes in broad-leaved forests of slopes, screes and ravines. Factors determining the epiphytic bryophyte distribution in such forests were investigated. In total 45 epiphytic bryophyte species were found (12 of them were signal species, including three specially protected species in Latvia – *Antitrichia curtipendula*, *Neckera crispa* and *Plagiothecium latebricola*). The total number of bryophyte and number of signal species were higher on tree species with relatively basic bark pH (*Acer platanoides*, *Ulmus glabra*, *Fraxinus excelsior*, *Populus tremula*, *Sorbus aucuparia*, *Tilia cordata*, *Salix* sp.), but lower on tree species with acidic bark (*Alnus glutinosa*, *Betula pendula*, *Picea abies*). Tree diameter, age and distance from tree to top of slope were not related to epiphytic bryophyte distribution. The highest bryophyte species richness (including signal species) was found up to a 0.5-m height on the southern exposure of trees and on the upper side of inclined trees (on south exposure of trees on north facing slopes, and on east exposure of trees – on west facing slopes).

Key words: broad-leaved forests, bryophyte, distribution, epiphyte.

Introduction

The broad-leaved forests in Latvia are highly fragmented by agricultural land-use on previous woodland cleared for tillage (Dumpe 1999). European broad-leaved forests in Latvia are mostly restricted in river valleys or slopes, lake islands and plains in their ancient distribution areas (Priedītis 1999). Many Central European plant and animal species are associated specifically with these forests (Priedītis 2000). Rich epiphytic bryophyte diversity (including many rare and threatened species) is found in natural broad-leaved forests (Priedītis 2000; Bambe, Lārmanis 2001; Ek et al. 2001).

The understorey vegetation (Priedītis 1999), bryophyte flora (Āboliņa 1968; Āboliņa 2001a; Āboliņa 2001b; Bambe, Lārmanis 2001; Bambe 2002; Āboliņa, Rēriha 2004) and lichen flora (Piterāns 2001) have been studied in broad-leaved forests of Latvia, but knowledge about epiphyte ecology is lacking (Znotiņa 2003).

Zilie kalni of Slitere National Park is a ravine system with little previous human impact. Due to its geological history and climate, Zilie kalni (Sarma 1958) supports some of the most important undisturbed Latvian forests of slopes, screes and ravines with high biodiversity, including many rare and threatened plant species (Seile, Rēriha 1983; WWF Project 1992).

Survey of natural old growth broad-leaved forests as woodland key habitat (WKH)

in Latvia began in 1997. Rare and protected bryophyte, lichen, insect and fungi species are used as indicators for identification of these forests (sensu Ek et. al 2001), but little is known about the ecology of these species in Latvia.

The most important factors affecting the distribution of epiphytic bryophyte species are forest stand age, tree age and diameter (Barkman 1958; Kuusinen 1996; Hazell et al. 1998; Kuusinen, Penttinen 1999; Aude, Poulsen 2000; Hedenås, Ericson 2000; Ojala et al. 2000), as well as tree species (Āboliņa 1968; Snäll et al. 2004), tree bark physical and chemical properties (Apinis, Diogucs 1935; Apinis, Lācis 1936; Smith 1982; Smith 1996; Weibull 2001) and microclimate (John, Dale 1995; Hazell et. al 1998; Hazell, Gustaffson 1999; Thomas et al. 2001; Bambe 2002).

The cover of epiphytic bryophytes is much higher on deciduous tree species, such as *Fraxinus excelsior*, *Acer platanoides*, *Ulmus glabra*, *Tilia cordata* (Snäll et al. 2004), *Alnus incana* and *Populus tremula* as compared to conifers (Āboliņa 1968).

Dense bark of old trees with cracks where dust and humus has accumulated is more suitable for epiphyte growth (Znotiņa 2003) but smooth, bare bark, with low air humidity is less suitable (John, Dale 1995). The bark of older trees is porous, maintaining a humidity more favourable for better bryophyte growth (Āboliņa 1968).

Tree bark is rougher on the basal part of tree stems, explaining why bryophytes favour tree bases in comparison with the smooth upper part of tree stems, with low humidity and less nutrients (John, Dale 1995; Znotiņa 2003).

Air humidity is one of the most important factors effecting the distribution and development of bryophytes (Bambe 2002). Dense epiphytic bryophyte cover is observed in deep valleys of rivers as well as at brook edges where the air humidity is high (Āboliņa 1968).

Tree bark pH is an important factor determining epiphyte distribution (Weibull 2001, Znotiņa 2003). Bryophyte species each have an optimum substrate pH range (Apinis, Diogucs 1935; Apinis, Lācis 1936). Bark pH differs among tree species (Āboliņa 1968) and coniferous trees have a lower pH than deciduous trees (Barkman 1958; Smith 1982).

The relation between height and exposure on tree stems and epiphyte community composition have been described only in a few reports (John, Dale 1995; Moe, Botnen 1997; Hazell, Gustafsson 1999; Thomas et al. 2001).

The composition of epiphytic species on tree stems varies in relation to relative humidity, light intensity and bark properties (Smith 1982). Epiphyte cover is increased on north and south exposures on basal parts of tree stems (John, Dale 1995), but all of the above factors together determine the spatial distribution of epiphytic bryophytes on trees (Thomas et al. 2001). The aim of this work was to describe the epiphytic bryophyte flora and determine the factors affecting WKH indicator species and special protected species distributions in Zilie kalni of Slitere National Park of north-west Latvia.

Materials and methods

Study area

The Study area (Fig. 1) is situated in the north-west part of Latvia, Slītere National Park (22° 10’ 50.1”E, 59° 35’ 29.0” N). The mean annual air temperature is 5.9 °C (Sarma 1958) and the mean annual precipitation is 573 mm (Seile, Rēriha 1983).

Zilie kalni is a bow-shaped north-facing slope, relative height up to 42.5 m, spanned

Fig. 1. Map of the Baltic states showing the study area (•).

by side ravines more than 20 km along (from west to south-east direction). Old growth mixed broad-leaved forest stands are the dominant forest type (WWF Project 1992).

Data sampling

The field work was conducted in July 2003 and March 2004 in Zilie kalni (Fig. 1). One north-facing slope and one west-facing side ravine were studied.

On the north-facing slope three 2-m-wide parallel transects (about 67 m long) leading downhill (from south to north direction) were randomly established. All trees with diameter at breast height ≥ 0.10 m and canopies crossing the transect were sampled. Height and distance to the top of slope were determined for each tree. The west-facing slope was selected on a side ravine, where nine broad-leaved trees (diameter at breast height ≥ 10 cm) were selected.

Tree cores were removed by an increment corer and tree rings were counted on a Lintab table equipied with a Leica microscope (MS 5) for determination of tree age.

Cover of bryophyte species was determined using the 5-point Braun-Blanquet scale (Kent, Cooker 1992) on different sides (N, S, E, W) and heights (lowest part – until 0.5 m, highest part – 0.5 ÷ 1.5 m) of tree stems (eight plots on each tree) on all trees.

Bryophyte species that could not be identified in the field were collected for identification in the laboratory. Species nomenclature follows (Hallingbäck, Holmåsen 2000; Smith 1996a; Smith 1996b).

Determination of tree bark pH

Tree bark pH was determined in laboratory after Kermit and Gauslaa (2001). Bryophytes and lichens were removed from tree bark and samples of tree bark were cut. Each sample weighed approximately 0.5 g. There was difficulty in removing bark from some trees, therefore 24 of 76 samples weighed less than 0.5 g. Each bark sample was shaken in a 20-ml 1 M KCl solution for 1 h and pH was determined with a pH-meter (GPH 014, *Greisinger Electronic*).

Table 1. Epiphytic bryophyte species occurrence on tree species and pH amplitude (in both of slopes). *, signal species

Bryophyte species	**Tree species (number of trees)**												**pH**		
	Acer platanoides (7)	*Ulmus glabra* (7)	*Fraxinus excelsior* (13)	*Populus tremula* (15)	*Salix sp.* (2)	*Sorbus aucuparia* (2)	*Tilia cordata* (1)	*Alnus glutinosa* (2)	*Betula pendula* (11)	*Picea abies* (16)	Number of trees	Number of tree species	pH min	pH max	pH average
Amblystegium serpens	2	3	4	8	1	-	-	1	1	1	21	8	4.6	6.2	5.0
*Anomodon longifolius**	-	-	2	-	-	-	-	-	-	-	2	1	5.9	6.2	6.0
*Anomodon viticulosus**	-	1	-	-	-	-	-	-	-	-	1	1	6.2	6.2	6.2
*Antitrichia curtipendula**	1	-	1	-	-	-	-	-	-	-	2	2	5.4	5.6	5.5
Brachythecium oedipodium	-	-	2	1	-	-	-	-	-	-	3	2	5.3	5.9	5.5
Brachythecium rutabulum	2	2	4	6	2	1	-	1	1	2	21	9	3.6	6.2	4.4
Brachythecium populeum	1	-	-	1	1	-	-	-	-	-	3	3	5.3	5.4	5.3
Brachythecium velutinum	1	-	-	1	-	-	-	-	-	1	3	3	3.9	5.6	4.4
Dicranum montanum	-	1	-	3	-	-	-	2	10	9	25	5	3.4	5.8	3.9
Dicranum scoparium	-	-	-	3	-	-	-	2	11	3	19	4	3.4	5.4	3.9
Eurhynchium angustirete	-	-	-	1	-	1	-	-	-	-	2	2	5.3	5.8	5.6
Eurhynchium hians	2	1	-	4	-	-	-	-	-	5	12	4	3.8	6.8	4.5
Eurhynchium striatum	3	-	6	9	-	1	-	2	6	6	33	7	3.4	6.8	4.2
Eurhynchium pulchellum	-	-	-	-	-	-	-	-	1	1	2	2	3.6	3.8	3.7
Frullania dilatata	4	3	11	12	1	2	-	-	1	-	34	7	4.5	6.8	5.3
Homalothecium sericeum	3	4	5	9	1	-	-	-	2	-	24	6	3.8	6.8	4.7
*Homalia trichomanoides**	2	4	8	6	1	-	-	-	-	-	21	5	4.9	6.2	5.4
Hypnum cupressiforme	3	3	4	12	2	2	1	2	8	13	50	10	3.1	6.8	4.1
*Isothecium alopecuroides**	2	2	2	1	1	-	1	-	-	1	10	7	3.8	6.2	4.8
*Isothecium myosuroides**	1	3	6	1	1	-	-	-	-	1	13	6	4.2	5.6	4.8
Lepidozia reptans	-	-	1	-	-	-	-	-	4	-	5	2	3.6	5.3	4.0
Leucodon sciuroides	1	3	5	3	-	-	-	-	-	-	12	4	5.2	6.2	5.7
Lophocolea heterophylla	2	2	3	3	2	1	-	2	7	4	26	9	3.4	5.9	4.0
*Metzgeria furcata**	3	5	7	12	2	2	1	1	3	-	36	9	3.6	6.8	4.7
Mnium stellare	2	3	3	7	1	-	-	1	1	-	18	7	4.9	5.9	5.2
*Neckera complanata**	5	3	7	10	2	2	-	-	-	-	29	6	4.9	6.8	5.3
*Neckera crispa**	2	-	1	1	-	-	-	-	-	-	4	3	5.3	6.8	5.7
*Neckera pennata**	-	1	2	-	-	-	-	-	-	-	3	2	5.9	6.2	5.9
Plagiomnium affine	-	-	-	1	-	-	-	-	-	-	1	1	5.4	5.4	5.4
Plagiomnium undulatum	-	2	1	-	-	-	-	-	-	1	4	3	3.9	5.9	4.3
Plagiochila porelloides	1	2	1	4	-	-	-	-	2	1	11	6	5.8	6.2	5.9

(continued)

Bryophyte species	Tree species (number of trees)												pH		
	Acer platanoides (7)	*Ulmus glabra* (7)	*Fraxinus excelsior* (13)	*Populus tremula* (15)	*Salix sp.* (2)	*Sorbus aucuparia* (2)	*Tilia cordata* (1)	*Alnus glutinosa* (2)	*Betula pendula* (11)	*Picea abies* (16)	Number of trees	Number of tree species	pH min	pH max	pH average
Plagiothecium cavifolium	-	-	-	1	-	-	-	-	-	-	1	1	5.4	5.4	5.4
Plagiothecium curvifolium	1	-	1	7	1	1	-	-	6	3	20	7	3.6	6.8	4.4
Plagiothecium denticulatum	-	-	1	-	1	1	-	1	6	5	15	6	3.4	5.9	3.8
Plagiothecium laetum	1	-	1	1	1	-	-	-	1	3	8	6	3.8	6.2	4.2
*Plagiothecium latebricola**	-	-	-	-	-	-	-	1	-	-	1	1	4.3	4.3	4.3
Platygyrium repens	-	2	2	-	-	-	1	-	1	2	8	5	3.8	5.9	4.1
Ptilidium pulcherrimum	1	-	-	-	-	-	-	1	7	-	9	3	3.6	5.8	4.0
Pylaisia polyantha	-	2	2	3	1	1	-	1	4	2	16	8	4.6	6.2	4.8
Pleurozium schreberi	-	-	-	-	-	-	-	-	1	-	1	1	4.5	4.5	4.5
Radula complanata	7	5	12	14	2	2	1	1	3	1	48	10	3.8	6.8	4.8
Sanionia uncinata	-	-	-	1	-	-	-	-	1	-	2	2	3.6	6.8	5.2
Thuidium delicatulum	-	-	-	-	-	-	-	-	1	-	1	1	3.8	3.8	3.8
Thuidium tamariscinum	-	1	3	5	-	-	-	2	3	-	14	5	3.6	5.9	4.3
*Ulota crispa**	3	2	8	12	1	1	1	1	1	2	32	10	4.0	6.8	5.0
min	5.4	5.5	5.2	4.8	4.7	5.3	5.1	3.6	3.6	3.1					
max	6.1	6.2	6.0	6.8	5.1	5.3	5.1	4.6	4.8	4.2					
average	5.7	5.7	5.5	5.2	4.8	5.3	5.1	3.9	3.9	3.6					

Data processing

Bryophyte community structure and gradients were analysed with the TWINSPAN and DECORANA programme package (PC-ORD for Windows, B. McCune and M. J. Mefford 1999. Multivariate analyses of Ecological Data Version 4.17, Oregon, USA), DCA (Detrended correspondence analysis). Relations of bryophyte species with environmental variables were determined by regression analysis (MS Excel) and Kendall's correlation (SPSS for Windows, Release 11.5.0, *SPSS inc.*). Bark pH values were converted to H^+ concentration before calculation of mean pH values.

Results

Forest stand

The studied forests were broad-leaved mixed tree forest stand with dominant *Picea abies*, *Populus tremula* and *Fraxinus excelsior*. The cover of bryophytes were estimated on 76 trees of 10 species. Uneven age structure was observed for trees, where the oldest was *Ulmus glabra* – 250 years. Dead wood was observed in various decay stages and cut tree stumps were not found, indicating minimal human impact.

Bryophyte occurrence

In total, 45 epiphytic bryophyte species were recorded – 38 Bryopsida and seven Hepaticopsida. Eight indicator species of old growth forests (Ek et al. 2001) – *Neckera complanata*, *Isothecium myosuroides* (Latvian Republic Cabinet of Ministers 2001), *Metzgeria furcata*, *Neckera pennata*, *Ulota crispa*, *Isothecium alopecuroides*, *Anomodon longifolius*, *Anomodon viticulosus*, *Homalia trichomanoides*) and three WKH specialist species specially protected in Latvia (*Antitrichia curtipendula*, *Neckera crispa* and *Plagiothecium latebricola*), were found. Further the term "signal species" will include both the WKH indicator species and the specially protected species.

The most common bryophyte species were *Hypnum cupressiforme* and *Radula complanata* (Table 1). Among the signal species, *Ulota crispa*, *Metzgeria furcata* and *Isothecium alopecuroides* had the widest ecological valence on trees.

Preference of bryophyte species for tree species

The number of epiphytic bryophyte species varied depending on substrate tree species. The richest bryophyte flora was found on *Populus tremula* and *Fraxinus excelsior* (Fig. 2). The number of signal species was highest on *Fraxinus excelsior*. The lowest number of bryophyte species was on *Picea abies*.

Dicranum montanum and *Dicranum scoparium* were found on *Betula pendula*. *Radula complanata* was exclusive to *Populus tremula*, *Fraxinus excelsior* and *Acer platanoides*.

Relation of the tree DBH, height, age, pH and tree distance to top of slope and the distribution of bryophytes

Kendall's correlation coefficients between the number of bryophyte species, number of signal species, tree bark pH, DBH, height, age and tree distance to top of slope were determined (Table 2; Fig. 3). The number of bryophyte species was significantly related to

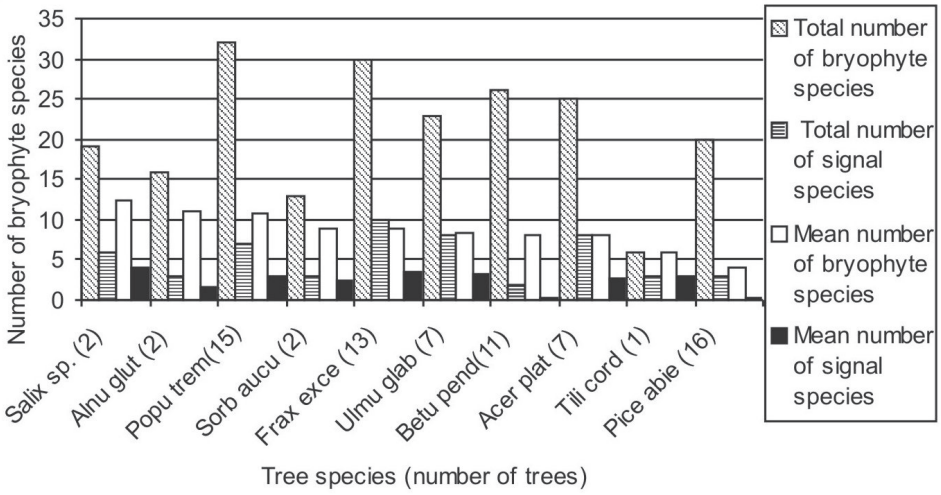

Fig. 2. Total number of bryophyte species on tree species and mean mumber per tree (in both of slopes).

Table 2. Nonparametric correlations (Kendall's correlation coefficients) among variables (on both slopes). **, correlation is significant at the 0.01 level (2-tailed); *, correlation is significant at the 0.05 level (2-tailed); DBH, tree diameter at breast height

	Number of signal species	DBH	Tree bark pH	Tree age	Tree height	Distance to top of slope
Number of species	0.536**	0.142	0.208*	0.035	0.325**	-0.016
	Number of signal species	0.013	0.469**	0.066	0.209	0.024
		DBH	-0.172	0.135	0.654**	-0.001
			Tree bark pH	0.052	-0.053	-0.009
				Tree age	0.124	0.077
					Tree height	0.052

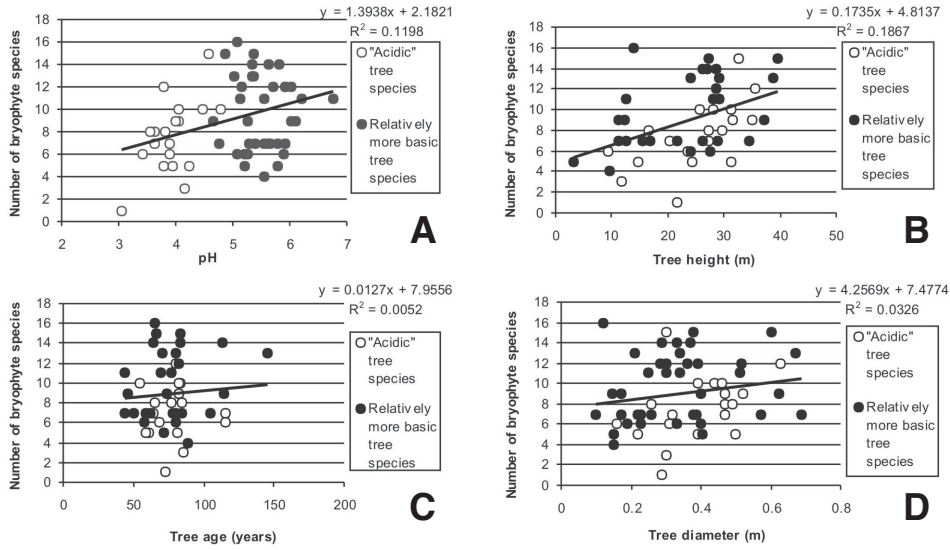

Fig. 3. Relationship between the total number of bryophyte species and tree bark pH (A), tree height (B), tree age (C), tree diameter at breast height (D).

tree height and bark pH. Number of signal species was correlated significantly only with tree bark pH (Table 2).

Tree bark pH

Tree bark pH varied between 3.1 and 6.8 for the studied trees, being lowest for *Picea abies* and *Betula pendula* and highest for *Populus tremula* and *Ulmus glabra* (Table 1). Both the number of bryophyte species and the number of bryophyte signal species was higher on trees with higher bark pH (Fig. 2; Table 1).

Based on the bryophyte preference on tree species, it was possible to divide trees into

Table 3. Number of bryophyte plots with bryophytes in various heights and exposures on trees (in both of slopes). N, north; S, south; E, east; W, west; *, signal species

Bryophyte species	Exposure				Height (m)	
	N	S	E	W	0 - 0.5	0.5 - 1.5
Amblystegium serpens	10	15	14	14	21	32
*Anomodon longifolius**	-	-	-	1	-	1
*Anomodon viticulosus**	1	2	2	2	4	3
Brachythecium oedipodium	-	1	-	-	1	-
Brachythecium populeum	-	-	2	-	1	1
Brachythecium rutabulum	3	7	3	1	14	-
Dicranum montanum	19	32	25	21	56	41
Dicranum scoparium	11	17	6	6	28	12
Eurhynchium angustirete	1	1	2	2	6	-
Eurhynchium hians	4	1	2	2	8	1
Eurhynchium striatum	16	21	22	20	64	15
Frullania dilatata	31	28	29	27	34	81
Homalothecium sericeum	18	13	11	13	17	38
*Homalia trichomanoides**	14	13	9	6	31	11
Hypnum cupressiforme	46	55	47	49	139	58
*Isothecium alopecuroides**	7	6	7	5	20	5
*Isothecium myosuroides**	10	12	9	9	25	15
Lepidozia reptans	1	5	2	5	7	6
Leucodon sciuroides	5	2	7	8	12	10
Lophocolea heterophylla	11	17	9	16	29	24
*Metzgeria furcata**	23	28	34	27	65	47
Mnium stellare	11	5	7	10	26	7
*Neckera complanata**	33	29	29	31	55	67
*Neckera crispa**	7	7	7	4	13	12
*Neckera pennata**	1	2	3	1	5	2
Plagiochila porelloides	2	2	-	2	5	1
Plagiomnium undulatum	-	1	1	1	3	-
Plagiothecium cavifolium	1	1	1	1	4	-
Plagiothecium curvifolium	9	13	14	11	40	7
Plagiothecium denticulatum	4	9	4	5	16	6
Plagiothecium laetum	1	1	2	3	7	-
*Plagiothecium latebricola**	1	1	1	-	3	-
Platygyrium repens	2	1	1	1	4	1
Ptilidium pulcherrimum	6	4	2	7	8	11
Pylaisia polyantha	2	8	5	6	13	8
Radula complanata	52	51	50	46	69	130
Thuidium delicatulum	-	-	1	1	2	-
Thuidium tamariscinum	11	11	8	12	30	12
*Ulota crispa**	12	19	15	17	9	54
Total number	386	441	393	393	894	719

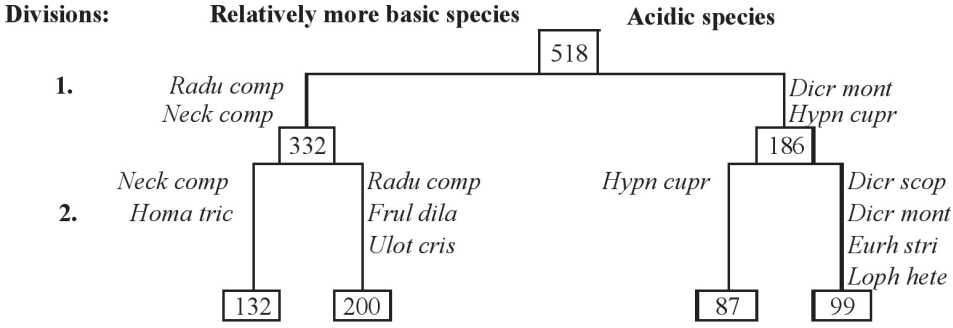

Fig. 4. TWINSPAN dichotomous division of epiphyte plots on both slopes. Indicator species are shown. *Radu comp*, *Radula complanata*; *Neck comp*, *Neckera complanata*; *Homatric*, *Homalia trichomanoides*; *Frul dila*, *Frullania dilatata*; *Dicr mont*, *Dicranum montanum*; *Hypn cupr*, *Hypnum cupressiforme*; *Dicr scop*, *Dicranum scoparium*; *Eurh stri*, *Eurhynchium striatum*; *Loph hete*, *Lophocolea heterophylla*.

those with relatively more basic bark (*Acer platanoides*, *Ulmus glabra*, *Fraxinus excelsior*, *Populus tremula*, *Sorbus aucuparia*, *Tilia cordata*, *Salix* sp. and with acidic bark (*Alnus glutinosa*, *Betula pendula* and *Picea abies*).

The first TWINSPAN division level separated plots on the basis of acidic- and relatively more basic-preferring bryophyte species (Fig. 4). Signal species, like *Neckera crispa*, *Neckera complanata* and also common species *Leucodon sciuroides* were found mostly on relatively more basic trees (Table 1). *Dicranum scoparium*, *Dicranum montanum* and *Lepidozia reptans* were generally distributed on acidic trees. *Hypnum cupressiforme* and *Brachythecium rutabulum*, *Plagiothecium curvifolium* and *Radula complanata* did not show any relation with tree species and bark pH.

Bryophyte vertical and horizontal spatial distribution on trees

The occurence and cover of bryophyte species was studied on various heights (below 0.5 m and between 0.5 and 1.5 m) and exposures (N, W, S, E) on the tree stem, together eight plots on each tree. More bryophyte species with high cover were found on the tree base (Table 3), e.a., *Brachythecium rutabulum*, *Eurhynchium angustirete* and *Plagiomnium undulatum*. The occurrence of *Radula complanata*, *Frullania dilatata*, *Homalothecium sericeum* was higher between 0.5 and 1.5 m on the tree stem.

The second TWINSPAN division level separated epiphytic communities in the different parts of the tree stem. The TWINSPAN indicator species Homalia trichomanoides and *Neckera complanata* were distributed more on the tree base, but *Radula complanata*, *Frullania dilatata* and *Ulota crispa* were found more at a 0.5 to 1.5 m height. *Hypnum cupressiforme* did not show any relation with height. *Dicranum scoparium*, *Dicranum montanum*, *Eurhynchium striatum* and *Lophocolea heterophylla* grew up to a height of 0.5 m only on acidic trees.

Differences were observed between bryophyte species occurrence on trees of the north slope of the Zilie kalni escarpment and east slope of the side ravine. Bryophyte occurrence was higher on the south exposure of trees on a north-facing slope (385 of all 1414 plots), but on the east exposure of trees on a west-facing slope (56 of all 198 plots).

The highest number of plots lacking bryophytes was observed on the north exposure on trees in the studied area (on both slopes). Plots with bryophytes (including signal species) were more common on the south exposure on trees (Table 3). The number of plots without bryophytes was similar on east and west exposures.

The species gradients extracted by DCA did not show any relation with height and direction of exposure on tree stems.

Discussion

The significance of tree bark pH

Among the studied factors, tree bark pH showed the best relationship with distribution of bryophyte species, which is consistent with other studies (Āboliņa 1968; Weibull 2001; Löbel et al. 2006).

In general, bark pH is a specific attribute for each tree species. Deciduous trees (except *Betula pendula* and *Alnus glutinosa*) have relatively higher bark pH in comparison with coniferous trees. At the same time, a specific pH amplitude exists for bryophyte species depending on substrate (Apinis, Diogucs 1935; Apinis, Lācis 1936; Barkman 1958). However, environmental factors (soil dust, acid rain) can change the value of tree bark pH, and the distribution of epiphyte species can change respectively (Barkman 1958).

TWINSPAN analysis clearly divided plots by tree species – relatively more basic (bark pH above 4.4) and acidic (below 4.4). The highest number of bryophyte species and especially the signal species, e.a., *Anomodon* sp. and *Neckera crispa* were found on reltively more basic tree species. On acidic trees, there was a lower number of species, fewer signal species (*Metzgeria furcata*, *Ulota crispa*, *Isothecium* sp.) and species with wide ecological valence (like *Hypnum cupressiforme* and *Brachythecium rutabulum*). The low epiphyte diversity on acidic trees can be explained by a possible toxic effect of tannins and resins in the bark of *Betula pendula* and coniferous trees (Barkman 1958; Gauslaa 1995).

Several authors (Hazell et al. 1998; Ojala et al. 2000) have described *Populus tremula* as a tree species which is particularly rich in epiphytes. However, in the present study, *Fraxinus excelsior* hosted a similar number of bryophyte species as *Populus tremula*.

Relationships between bark pH and tree age (Barkman 1958) and bark pH and tree diameter (McGee et al. 2002) have previously been reported, which was also found in our study (both for single tree species and for stand), but our sample size for trees was rather low.

Influence of tree age and diameter

Several authors have described an increased epiphyte richness on older trees with larger stem diameter (Trynoski, Glime 1982; Hazell et al. 1998; Aude, Poulsen 2000; Snäll et al. 2004). Some species prefer very old trees (Kuusinen, Siitonen 1998), which can be explained by relatively slow growth and colonisation rates (Crites, Dale 1998) and with changes of bark structure. With ageing of the tree, the bark structure becomes more suitable (thick and rough) for epiphyte growth (Hyvärinen et al. 1992). Also, there is more surface area for colonisation on larger trees (Lyons et al. 2000).

In our study, no significant correlation between the diversity of epiphytes and the tree age and diameter (both in general and for single tree species) was found. Possible explanations may be:

(i) in previous studies, the forest microclimate and the development history differed from the studied broad-leaved forest;

(ii) in a forest with long continuity (like the studied stand), microhabitat availability and not dispersal is the limiting factor for the establishment of epiphyte species.

A slight positive relationship was found only between tree height and the number of bryophyte species. This relation was observed for the forest stand in total, but not within tree species. The relatively lower species richness on smaller (lower) trees can be explained by shading from other trees (Lyons et al. 2000).

Influence of microclimate

Aspect of epiphytes on the tree stem is a valuable tool for detailed study of the microclimate and microhabitat niche of bryophyte species. TWINSPAN analysis divided plots into four groups based on bryophyte species: (i) growing at the base (below 0.5 m) of relatively basic trees; (ii) growing higher (0.5 to 1.5 m) on stems of relatively basic trees; (iii) growing at the base (below 0.5 m) of acidic trees; (iv) growing on acidic trees without any height preference (Fig. 4.).

The highest number of species was found at the basal part of tree stems (0.5 m). There were no species preferring the higher part of tree stems on acidic trees. *Hypnum cupressiforme*, a widely distributed species without preference to a specific substrate (Āboliņa 1968; Weibull 2001), was distributed throughout the tree stem. The low species diversity on the upper part of acidic tree stems can be explained by desiccation of bark, which is more pronounced for coniferous in comparison with deciduous trees, and with bark scaling of conifers (Barkman 1958). The base of all trees was covered with species from the surrounding soil, like *Plagiomnium affine*, *Eurhynchium striatum*, *Plagiomnium undulatum*. However, the base of basic trees provided habitat also for signal species like *Neckera complanata*, *Homalia trichmonoides*, *Antitrichia curtipendula*, *Neckera crispa*, *Anomodon viticulosus* and *Anomodon longifolius*. The epiphyte diversity decreased higher on the tree stem (between 0.50 and 1.50 m). On basic trees higher parts were typically inhabited by pioneer species *Radula complanata*, *Frullania dilatata*, *Ulota crispa*, (Barkman 1958; Trynoski, Glime 1982) adapted to desiccation (Moe, Botnen 1997).

At the tree base, high bryophyte diversity is favoured due to higher relative humidity (Barkman 1958; Āboliņa 1968; John, Dale 1995; Bambe 2002) and the physical and chemical nature of tree bark (Smith 1982). Thick and rough bark provides sheltered microhabitats for the establishment and growth of bryophytes (Barkman 1958). Also, uncovered roots of trees provide various microhabitats for bryophytes (personal observation). The high bryophyte diversity at the tree base (below 0.50 m) indicates that the humidity and the physical properties of bark (thickness, cracks) are limiting factors for the local distribution of the epiphyte species.

Several authors have described a higher bryophyte cover on the north exposure of trees and less on the south, east and west. Relatively low light and temperature and relatively high humidity on the north-facing slope which provide suitable conditions for bryophyte growth (Barkman 1958; Trynoski, Glime 1982).

In our studied forest stand, a higher number of bryophyte species, including signal species, was found on the southern exposure, but lower, on the northern part of trees. This can be explained by the relief of the studied site – on the northern slope trees were leaning northwards. On the upper part of leaning trees, there is a suitable microclimate for

bryophyte growth, because the humidity here is maintained for a relatively longer period (Barkman 1958). The influence of the inclination on the epiphytic vegetation is due to precipitation and general moisture conditions (Moe, Botnen 1997).

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Epifītiskās sūnas dabiskos nogāžu un gravu mežos ziemeļrietumu Latvijā

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Kopsavilkums

Epifītisko sūnu ekoloģija nogāžu un gravu mežos ir maz pētīta. Dotajā pētījumā noskaidroti faktori, kas ietekmē epifītisko sūnu izplatību šajos mežos. Kopumā konstatētas 45 epifītisko sūnu sugas, no kurām 12 bija signālsugas, tai skaitā, arī trīs īpaši aizsargājamas sūnu sugas Latvijā – *Antitrichia curtipendula*, *Neckera crispa* un *Plagiothecium latebricola*. Kopējais sūnu sugu un signālsugu skaits bija augstāks uz kokiem ar relatīvi bāzisku mizas pH (*Acer platanoides*, *Ulmus glabra*, *Fraxinus excelsior*, *Populus tremula*, *Sorbus aucuparia*, *Tilia cordata*, *Salix* sp.), bet mazāks sūnu sugu skaits – uz "skābajiem" kokiem (*Alnus glutinosa*, *Betula pendula*, *Picea abies*). Koka diametrs, vecums, un attālums līdz nogāzes augšdaļai maz ietekmēja epifītisko sūnu izplatību. Visvairāk sūnu sugas (ietverot signālsugas) bija izplatītas uz koka stumbra līdz 0,5 m augstumam dienvidu debespusē, kā arī stumbra augšpusē uz slīpiem kokiem (dienvidu debespusē uz kokiem ziemeļu nogāzē un austrumu debespusē – uz kokiem rietumu nogāzē).

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Probiotics as functional food: microbiological and medical aspects

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Abstract

Probiotic bacteria are sold mainly in fermented foods, and dairy products play a predominant role as carriers of probiotics. Functional dairy foods are well suited to promoting the positive health image of probiotics for several reasons: (i) fermented foods and dairy products in particular, already have a positive health image by their traditional use for centuries; (ii) people are familiar with the fact that fermented food contain living microorganisms; (iii) probiotics are used as starter to join together the positive images of fermentation and probiotic cultures. Probiotics are defined as live bacterial preparations (food or medicine) with clinically documented health effects in humans. Most probiotics exert beneficial effects by modulating the mucosal barrier function and immune activity. Probiotics have specific properties and targets in the human intestinal tract and intestinal microbiota. Understanding the mechanisms by which probiotics influence the normal intestinal microflora and counteract aberrancies in microflora can facilitate the use of probiotics for dietary management and reduction in risk of specific diseases. In reference of the immune system, many studies have pointed out that not only pro- and prebiotics, but also single micronutrients incorporated into functional foods contribute to an enhancement of immunocompetence. In this article, the effect of some functional foods and ingredients such as probiotics and selenium on health and especially immune function are reviewed.

Key words: functional dairy products, functional foods, probiotics, selenium.

Introduction

The term "functional food" was first introduced in Japan in the mid-1980s and refers to processed foods containing ingredients that aid specific bodily functions in addition to being nutritious (Swinbanks, O'Brien 1993). Generally, they are considered as those foods intended to be constituted as part of a normal diet, and that contain biologically active components, which offer the potential of enhanced health or reduced risk of disease.

Research has demonstrated that nutrition plays a crucial role in the prevention of chronic diseases, as most of them can be related to diet. Functional food enters the concept of considering food not only necessary for living but also as a source of mental and physical well-being, contributing to the prevention and reducing of risk factors for several diseases or enhancing certain physiological functions. Dairy products form the major part of functional products. To understand their success it is important to realise that milk is a natural and highly nutritive part of a balanced daily diet. Developing functionality

in dairy-based products simply means modifying and/or enriching the healthy natural characteristics of the original base. Milk and some other dairy products were recognised as important foods as early as 4000 B.C. The Roman historian Plinio recommended the use of fermented milk for treating gastrointestinal infections. The French paediatrician Tissier proposed in the early 1900s that bifidobacteria could be effective in preventing infections in infants, as they were the predominant component of the intestinal microflora in breast-fed infants. Then Metchnikoff suggested that consumption of fermented milk could reverse the putrefactive effects of the gut microflora. This concept has developed particularly over the past two decades through trend scientific evidence based on placebo-controlled clinical trials showing that particular strains have associated health benefits.

Nowadays dairy products are excellent media to generate an array of products that fit to current consumer demand for functional food. Fermented dairy products enriched with probiotic bacteria have developed into one of the most successful parts of functional foods. The food industry is especially active in studying probiotics because the gastrointestinal tract is one of the richest zones of biodiversity within the body with at least 450 known species of microorganisms commonly found there. Some of the most

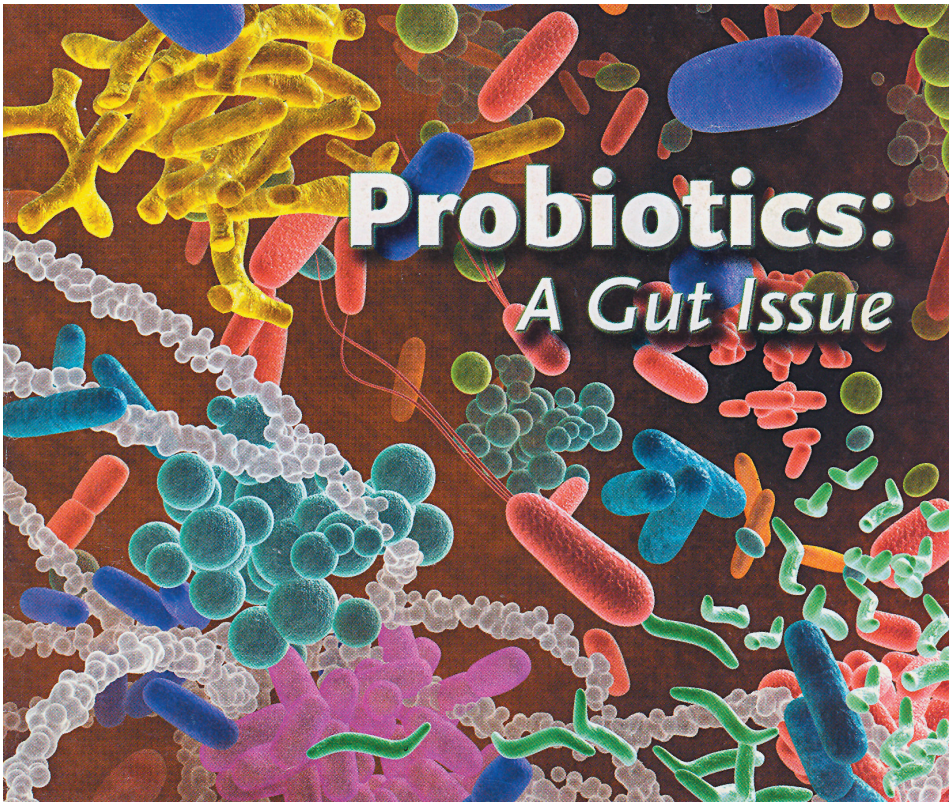

Fig. 1. Some representatives of human gut microflora: *Lactobacillus* GR-1 (dark blue); *Lactobacillus* RC-14 (light blue); *Escherichia coli* (red); *Bacteroides fragilis* (orange); *Streptococci* (green); *Staphylococci* (cyan); *Campylobacter jejuni* (blue green); *Klebsiella* (purple). Reproduced with permission from *The Scientist* Vol. 16 (2002).

Table 1. Microorganisms considered as probiotics (Holzapfel et al. 2001).

Lactobacillus	***Bifidobacterium***	**Other lactic acid bacteria**	**Non-lactic acid bacteria**
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus cereus* var. *toyoi*
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli* Nissle 1917
L. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freudenreichii*
L. crispatus	*B. breve*	*Leuconostoc mesenteroides*	*Saccharomyces cerevisiae*
L. delbrueckii subsp. *bulgaricus*	*B. infantis*	*Pediococcus acidolactici*	*Saccharomyces boulardii*
L. gallinarum	*B. lactis*	*Streptococcus thermophilus*	
L. gasseri	*B. longum*	*Sporolactobacillus inulinus*	
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			

important representatives are shown in Fig. 1. Functional dairy products have been the focus of intensive research and product developments over the last two decades regarding putrefactive intestinal bacteria; there has been much interest in the possible health benefits of probiotic microorganisms. Dairy products, accounting for 65 % of the total European functional foods market, are at the forefront of probiotic developments (Hilliam 2003).

Probiotics and prebiotics: definition and mechanism of action

Vergin first introduced the term "probiotics", when he compared in his paper "Anti- and Probiotika", the detrimental effects of antibiotics and other antimicrobial substances on the gut microbial population with factors "probiotika" favourable to the gut microflora (Vergin 1954). Then probiotics were defined as non-pathogenic microorganisms when ingested, exert a positive influence on host health or physiology (Fuller 1989). Now, the definition of Food and Agriculture Organisation of the United Nations/World Health Organisation (FAO/WHO 2001) for probiotics is "Live microorganisms, which when administered in adequate amounts, confer a health benefit on the host". This definition retains the historical elements of the use of living organisms for health purposes but does not restrict the application of the term only to oral probiotics with intestinal outcomes (Reid 2006). This is important considering that vaginal applications of probiotics have existed for more than 20 years (Reid, Bruce 2006).

Microorganisms that are probiotics (Table 1) in humans include yeast (Periti, Tonelli 2001), bacilli (Pinchuk et al. 2001), *Escherichia coli* (Midtvedt 1997), enterococci (Lund, Edlund 2001), and the more commonly used bifidobacteria and lactic acid bacteria, such as lactobacilli, lactococci and streptococci (Salminen et al. 1998; Isolauri et al. 2002). The International Dairy Federation has recently published a bulletin summarising the evidence for the effect of probiotic cultures on a range of diseases and disorders in humans. The

bulletin No 380/2003 contains a section (Ouwehand et al. 2003) reviewing the evidence for clinical effects in an extensive range of conditions including lactose maldigestion, diarrhoea, immune modulation, inflammatory bowel syndrome, constipation, necrotising enterocolitis, *Helicobacter pylori* infection, small bacteria overgrowth, colorectal cancer, breast cancer, allergy, serum cholesterol and blood pressure decreasing, coronary heart disease, urinary tract infection, upper respiratory tract and related infections. Thereby probiotics have multiple mechanisms of action (Table 2), including prevention of pathogenic bacterial growth, binding to or penetration of pathogens to mucosal surfaces, stimulation of mucosal barrier function, production of antimicrobial agents or altering immunoregulation, decreasing proinflammatory and promoting protective molecules (Sartor 2005; Novak, Katz 2006). It was demonstrated (Meier, Steuerwald 2005) that not only viable or dormant bacteria administered to the intestinal tract but also probiotic DNA is active, even if injected subcutaneously. Attention is now focusing on the intestinal survival of probiotic bacteria, their competition with the abundant resident microbiota, identification of activity and clarification of mechanisms of action. Probiotics have to survive gastrointestinal transit and arrive viable to contribute positively to the activity of the intestinal microflora, and thus, the health of the host (Table 3). A recent paper hypothesised that probiotics might even help detoxification in cases of mercury poisoning (Brudnak 2002).

Another interesting aspects concerns the antigenotoxic activities of probiotics. Our experiments have suggested that the potential genotoxic effect of furazolidone, nalidixic acid and 4-nitroquinolone-N-oxide could be strongly reduced by *in vitro* co-incubation with probiotic bacteria, belonging to three genera and probiotic yeast (Raipulis et al. 2005; Toma et al. 2005). Surprisingly, the nonprobiotic yeast *Saccharomyces carlsbergensis* also possesses antigenotoxic activity but to a minor extent (Toma et al. 2005). The antigenotoxic properties were shown only by live cells but heat treated cells did not act as an antigenotoxin. These results are of considerable interest with the increasing demand

Table 2. Some examples of target specific search for optimal probiotics (Salminen et al. 2005)

Target for probiotic action	Selection criteria
Alleviation of lactose maldigestion probiotics	High lactase producing strongly site specific symptoms
Intestinal inflammation	Site specific adhesion properties, anti-inflammatory cytokine expression, mucosal properties to alleviate permeability disorder and gut microflora abberancy
Alleviation or food allergy symptoms, reducing the risk proteolytic properties of atopic disease	Adherence to small intestine, induction of local transforming growth factor-β production
Reducing the risk of colon cancer	Target specific adhesion to distal or proximal colon, mucosal butyric acid production, competitive exclusion of inflammatory bacteria, toxin binding and promotion of nontoxigenic mucosal microflora

Table 3. The probiotic effects reported and their putative mechanisms (Sanders 2003)

Benefit	Function	Proposed mechanism
Digestive comfort	Irritable bowel syndrome, symptoms affecting the gastrointestinal tract in general (constipation, non-pathogenic diarrhea, distension, flatulence, cramp, halitosis of a digestive cause)	Change in populations or activities of the intestinal microflora
	Lactose intolerance	Delivery of microbial lactase to the small intestine
Defense	Allergy (atopical eczema, allergy to the milk, rheumatoid arthritis)	Translocation, barrier effect
	Cariogenicity	Changes in the populations, activity of the oral microflora or its ability to adhere to the teeth
	Carcinogenicity, mutagenicity, tumor	Absorption of the mutagen, stimulation of the immune system, inhibition of carcinogen production by the intestinal microflora
	Diarrhea linked to antibiotics, diarrhea caused by Rotavirus, colitis caused by *C. difficile*, nosocomial diarrhea	Competetive exclusion, translocation/ barrier effect, immune response promoted
	Helicobacter pylori	Antipathogenic activity
	Immunomodulation (immune status, vaccinal response)	Interaction with the immune cells or cell receptors leading to an increase in the phagocytic acivity of the white cells, increasing IgA levels after exposure to the antigen, increasing the proliferation of the intra-epithelial leukocytes, regulating the Th1/Th2 ratio, induction of cytokine synthesis
	Intestinal inflammation, ulcerative colitis, Crohn's disease, pouchitis	Immune response downregulated
	Excessive intestine bacterial growth	Antimicrobial activity, competitive exclusion
	Vaginosis, urinary infections	Antipathogenic activity, competitive exclusion
Others	Lowering of blood cholesterol	Deconjugation of the bile acids
	Endotoxemia combined with cirrhosis	Inhibition of the production of endotoxins by the intestinal microflora
	Hypertension	Cellular constituents or peptides derived from fermentation acting as inhibitors of ACE (angiotensin-converting enzyme)
	Renal calculi	Changes in the digestive flora influencing the breakdown of oxalate

for functional foods, especially functional dairy products, such as yogurts and fermented milks, containing *Lactobacillus* and *Bifidobacterium*.

Prebiotics are defined as nondigestible substances (dietary fiber) that exert some biological effect on humans by selective stimulation of growth or activity of beneficial microorganisms either present on therapeutically introduced to the intestine. Prebiotics undergo fermentation by probiotics in the large intestine. Prebiotics are sources of energy for probiotics. Clinical trials have shown that several different oligosaccharides can be used to stimulate bifidobacteria in the gastrointestinal tract and protect against gastrointestinal infections (Novak, Katz 2006).

Prebiotics are inulin, fructo-oligosaccharide, galactooligosaccharide and lactulose. With regard to a possible role for prebiotics in reducing the risk of diseases, the evidence is limited. The area where evidence can be considered promising is constipation (Roberfroid 2000) and gastrointestinal infections (Novak, Katz 2006). Although prebiotics improve calcium absorption (Abrams et al. 2005), their positive role in reducing the risk of osteoporosis needs to be supported by more human studies. The reduction of the risk of obesity and possibly of type 2 diabetes, both of which are known to be associated with insulin, also needs further investigation.

It has been observed that modification of intestinal microflora by inherently selectively fermented prebiotics is central in determining their nutritional properties (Van Loo 2004). Prebiotics interact positively through the large intestinal surface with various physiologic processes and are thought to improve health status by reducing risk for disease.

Probiotics, intestinal microflora and health

One of the main selection criteria for probiotics has been competitive exclusion of pathogens. Probiotics compete directly or delay the adhesion of pathogens on stereo-specific receptors on the mucosal surface of gastrointestinal tract. They also have an influence on the development of intestinal microflora in infants. The outcome of the microbiota development and competitive exclusion depends on the specificity of the microorganisms and their adhesion for the receptors and the relative concentrations of competing bacteria. The effective dosage of probiotics is thus determined by the relative affinity for receptor sites (Salminen et al. 2005). Different probiotics and even different strains have distinct modes of action and the clinical efficacy of various probiotics has been proven in distinct indications (Holst, Breves 2005).

Gut health and immunity

The gut and immune system form a complex integrated structure that has evolved to provide effective digestion and defence against ingested toxins and pathogenic bacteria. Around 60 % of functional foods, principally pro- and prebiotics, are targets of the gut and the immune system. A characteristic feature of gastrointestinal immune systems is its ability to exhibit tolerance towards innocuous dietary antigens and commensally microflora acquired during infancy and to mount a vigorous immune response to potentially pathogenic microorganisms. The execution of these disparate functions requires that the immune system surveys all the lamina antigens, to sort "harmful" from "harmless" antigens and to tightly regulate the ensign effect or responses; a failure to

regulate the mucosal immune response results in a range of clinical disorders such as allergy, inflammation and autoimmune diseases (Gill 2003). To perform these functions the gastrointestinal tract harbours the largest immune system in the whole body, over 70 % of the total immune system being located in this area. The gastrointestinal immune system consists of two main components: organized lymphoid follicles (Payer's patches and mesenteric lymph nodes, and a large number of immunocompetitive cells – the organised tissues) serve as a potential site for the induction of immune responses to new antigens, whereas the intestinal mucus serves as the effector site.

Probiotics and the immune system

The effect of probiotics on the immune system has been the subject of numerous studies over the past 20 years. There is evidence that certain strains of probiotics are able to stimulate as well as regulate several aspects of the natural and acquired immune response. It has also been demonstrated that there are significant differences between the ability of *Bifidobacterium* and *Lactobacillus* strains to influence the functioning of the immune system.

The initiation, maintenance and resolution of both innate and acquired immune responses are regulated by cell-to-cell communication via cytokines. The intake of probiotics in humans has been shown to enhance cytokine production *in vivo*, and by peripheral blood mononuclear cells *ex vivo* (de Simone et al. 1989). Probiotic intake has been reported to be effective in restoring the age-related decline in phagocyte function (Gill 2003). Strain- and dose-dependent differences in the ability of probiotics to influence immune function are well documented (Gill 1998). The intake of specific strains of probiotics has also been shown to enhance humoral immune responses to natural infections and systematic or oral immunization in human subjects (Majamaa et al. 1995; Fukushima et al., 1998). It is important to note that probiotic administration is also known to stimulate antibody responses to completely unrelated antigens as well as to themselves (Yasui et al. 1989).

Probiotics are thus suggested to confer protection against enteropathogens by:

- stimulating cytokine production;
- enhancing the phagocytic capacity of polymorphonuclear cells and macrophages;
- augmenting NKH cell activity;
- enhancing specific antibody responses to pathogens.

Minimum concentration of probiotic required for beneficial effect

The information to recommend the minimum concentrations of probiotic bacteria for effective function is still insufficient. Nevertheless, adequate numbers of viable cells, namely the "therapeutic minimum" need to be consumed regularly for transfer of the "probiotic" effect to consumers (Viljoen 2001). Consumption should be more than 100 g per day of bio-yogurt containing more than 10^6 CFU ml^{-1}. Shah (2000) amongst others has suggested a minimum viable number of 10^6 CFU ml^{-1} or gram but recommends 10^8 CFU g^{-1} to compensate for reduction through passage through the gut. Yogurt is a classic example of a functional food with probiotics. Yogurt with probiotics, called bio-yogurt, should contain living bacterial cells. According to regulation yogurt should contain 2 ×

10^6 living bacteria in 1 ml at the end of the recommended storage period. The daily dose of probiotic microorganisms should reach 1×10^9 cells. The titre of bacteria in fermented drinks reaches 10^8 to 10^9 ml^{-1} and decreases with storage. It is also possible to use tablets or capsules as additives to foodstuffs, that contain lyophilised cultures of bacteria. Probiotics are available as pharmacopoeia preparations such as Linex 1.2×10^7, Mutaflor 2.5×10^9, Lactoseven 1×10^9. Jogurt capsules 2×10^9 contain freeze-dried bacterial cells per caps, correspondingly. The question is – which is more effective way to take viable or lyophilised bacteria – in yogurt or capsules? The intake of functional dairy products also is more physiologically and more acceptable for patients or consumers as well. Within the last decade, consumers have made increasing reference to functional food, recognising the relationship between nutrition and health to the point of endowing an overreliance on pharmaceuticals and regarding prescription drugs as often being unnecessary, too expensive, unsafe and of dubious benefit once all the risks are considered (Bagchi 2006).

Safety of probiotics

The safety of probiotics can be described in short:

(i) centuries of use fermented products;

(ii) no reports of probiotic pathogens;

(iii) safe use of active cultures in thousands of subjects have demonstrated that probiotic intake is safe.

This past safe history is very important regarding use by pregnant woman and newborn, because there is some limitation for clinical trials. At the same time, some scientists have doubt about reasonability in taking a high dose of viable bacteria (Henriksson et al. 2005). A review outlining the safety of current probiotic compounds has been published (Borriello et al. 2003). Cases of infection caused by *Lactobacillus* and *Bifidobacteria* are extremely rare. Previous research into the protective mechanisms associated with probiotic bacteria focused on the bacteriology of the gut and concentrated on intestinal colonisation and probiotic-induced suppression of pathogen growth and/or invasion (Clancy 2003). Indeed, the concept of a balance existing in the intestine, involving competition between probiotic and pathogenic bacteria for specific binding sites on intestinal epithelial cells, has been well established in the literature. However, recent research has turned toward understanding the role of probiotics and their products, and in enhancing and modulating innate and adaptive immune responses in the organism by other mechanisms (Fedorak, Madsen 2004). The ability of immune and epithelial cells to discriminate between different microbial species through activation of Toll-like receptors (Kadowaki et al. 2001; Vinderola et al. 2005) indicates that probiotics may show some of their protective functions through modulation of immune activity and epithelial function in gut.

Probiotics and selenium

Selenium (Se) has been recognized as an essential nutrient in the late 1950s, when it was found that it could replace vitamin E in the diets of animals (Schwartz et al. 1957). It is hard to overestimate the importance of Se to biological systems. Its crucial role is underlined by the fact that it is the only trace element to be specified in the genetic code (Rayman 2002). It is specified as selenocysteine, now recognized as the 21st aminoacid, as it has its

own codon and specific biosynthetic and insertion mechanism (Gladyshev 2001). About 40 mammalian selenoproteins have been identified as having enzymatic redox activity, structural and transport functions. Thereby it is suggested that Se adequacy is crucial to human and animal health. A detailed review of Se deficiency symptoms, pathology and biochemical mechanisms was published by Gibson (2005). Low or diminishing Se status in some parts of the world, notably in Scandinavian and some other European countries, such as the UK, Baltic States, Croatia, Poland, Hungary, influences human and animal health. There is evidence that Se deficiency may contribute to development of a form of heart disease, hypothyroidism, and a weakened immune system (Combs, 2000; Zimmerman, Kohrle 2002). There is also evidence that Se deficiency does not usually cause illness by itself, because no one specific disease has been found, but it can make the body more susceptible to illnesses caused by other nutritional, biochemical or infectious stresses (Beck et al. 2003).

Epidemiological evidence in humans suggests a role for selenium in reducing cancer incidence and mortality, especially from prostate and colorectal cancer (Mantovani et al. 2004; Luty-Frackiewicz 2005; Finley 2006). The latest investigations show that Se administration decreases the toxicity of inorganic and organic forms of mercury (Cabanero et al. 2006). There are three arguments for increasing the Se intake: (i) Se deficiency may leave, than optimally protected against a number of adverse health conditions; (ii) Se intakes above those required to replete glutathion peroxidases and other selenoenzymes appear to confer additional health benefits and (iii) Se intake is low or marginal in many countries. Se enters the food chain through plants, but its incorporation is dependent not only on soil content, but also on the soil pH, rainfall, land profile, and activity of microorganisms (Combs 2001). Increasing Se intake from normal food sources is difficult to achieve. Meat and dairy products, eggs, Brazil nuts and wheat products are natural Se sources, but it is difficult to achieve the EU recommended 55 μg day^{-1} dose. Therefore it is necessary to perform food enrichment with dietary supplements of Se. Today situation is even more complicated because since August 1, 2005 dietary supplements containing organic Se forms are prohibited in the EU.

Our new project deals with the development of a novel type of functional food – Se enriched yogurt using probiotics able to concentrate Se intracellulary. It has been demonstrated that *Lactobacillus* accumulates some inorganic Se compounds in the form of selenocysteine (Calomme et al. 1995). Our experiments showed that supplementation of MRS broth (Sifin, Germany) with Bioenergostims Ultra Top (five inorganic Se compounds) promote yogurt starter cultures (*Lactobacillus bulgaricus* + *Streptococcus thermophilus*) growth at the Se concentration 100 mg l^{-1} till 15 % (Toma et al. 2006). Also, yogurt starter cultures become treatable to low pH in comparison with the control (Table 4). Supplementation with Se may stabilise membranes against the rigidity due to aging (Garcia et al. 2005). Preliminary experiments with fluorescent probe ABM (Kalnina et al. 2000) suggested an idea that the membranes of bacterial cells are selectively strengthened.

The combination of probiotics with Se in one product could confer benefits beyond those of either on its own.

Results with probiotic bacteria *Enterococcus faecium* demonstrate that the micronutrient selenium enhances the antimutagenic activity of probiotic bacteria (Križkova et al. 2002). It shows a potential benefit for the future development of new Se-enriched probiotic exhibiting higher antimutagenic properties.

Table 4. Effect of selenium on viability (log CFU ml^{-1}) of yogurt starter cultures after exposure to 0.2 M HCl-KCl buffer pH 2.5 (Toma et al. 2006)

	Time of exposure (h)		
	0	**1**	**2**
Control	9.9	6.1	3.5
Selenium (100 μg ml^{-1})	10.3	7.6	5.8

Probiotics and prebiotics as functional food

Probiotics and prebiotics simultaneously present in a product are called synbiotics. Such a combination aids survival of the administered probiotics and facilitates its inoculation into the colon. Additionally, the prebiotics induce growth and increase activity of positive endogenic intestinal microflora (Tomasik, Tomasik 2003). It was experimentally demonstrated that synbiotics protect the organism from carcinogens significantly better than do either probiotics or prebiotics separately (Gallaher, Khil 1999). Several foodstuffs with probiotics and prebiotics are available in the Latvian marketplace. One of the best is synbiotic yogurt Oat Bio Lacto (Bekers et al. 1999).

Summary

Probiotics can be considered functional foods because they provide health benefits beyond the traditional nutrition function. With few exceptions, most probiotic products currently available contain lactic acid bacteria, which mainly belong to the genera *Lactobacillus* and *Bifidobacterium*. The scientific papers published in major microbiological and nutrition journals suggest evidence of the following beneficial effects of probiotics: normalisation of the intestinal microflora, which both preserves and promotes wellbeing and the absence of disease (not only in the gastrointestinal tract), the ability to block the invasion of potential pathogens in the gut, prophylactic or therapeutic treatment for several types of diarrhoea (independently from aetiology), relief of symptoms of irritable bowel syndrome and inflammatory bowel disease, amelioration of lactose intolerance, prevention of colon cancer, inhibition of *Helicobacter pylori*, reduction of blood cholesterol level, hypertonia. Correction of the properties of unbalanced indigenous microbiota forms the rationale of probiotic therapy. However, an important part of the beneficial effects of probiotics are related to their immunomodulatory effects: immune chancing as well as anti-inflammatory activity. Bearing in mind the need for further evaluations, dietary modification towards a balanced dietary intake of nutrients and probiotics may offer a tool for both the management and risk reduction of allergic and autoimmune diseases.

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Probiotikas – funkcionālās pārtikas veids: mikrobioloģiskie un medicīniskie aspekti

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Kopsavilkums

Probiotiskās baktērijas satur galvenokārt fermentēti produkti, un tieši piena produkti kalpo kā probiotiku nesēji. Funkcionālie piena produkti ir ļoti piemēroti, lai sekmētu probiotiku atzīšanu veselības stiprināšanai šādu iemeslu dēļ: 1) fermentēta pārtika un sevišķi skābpiena produkti tiek lietoti veselības stiprināšanai jau gadsimtiem ilgi; 2) cilvēkiem nav iebildumu, ka skābpiena produkti satur dzīvus mikroorganismus; 3) piens, kas pats par sevi ir veselīgs produkts, plus probiotikas, kas tiek lietotas kā starta kultūras. Probiotikas ir dzīvu baktēriju preparāti (pārtikas produkti vai zāles), kam piemīt klīniski apstiprināta veselību uzlabojoša darbība. Vairumam probiotiku piemīt gļotādas barjeru un imunitāti uzlabojošas īpašības. Cilvēka zarnu traktā un zarnu mikroflorā probiotikām ir īpaši mērķi un uzdevumi. Probiotiku darbības mehānismu izpratne (normālas zarnu mikrofloras uzturēšana, aizņemot savu nišu) var sekmēt probiotiku plašāku izmantošanu uzturā, samazinot atsevišķu saslimšanu risku. Daudz pētījumu liecina, ka ne tikai pro- un prebiotikas uzlabo imūnsistēmas darbību, bet arī atsevišķu mikroelementu iekļaušana funkcionālās pārtikas produktos var uzlabot imūnatbildi. Rakstā apkopoti jaunākie dati par funkcionālo pārtiku, probiotiku darbības mehānismiem un iedarbību, akcentējot labvēlīgo ietekmi uz veselību un īpaši uz imūnsistēmu.

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Comparison of anther culture response among *Linum usitatissimum* L. cultivars and their hybrids

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Abstract

Linseed (*Linum usitatissimum* L.) is an important crop in Lithuania for production of both oil and fibre, but a Lithuanian cultivar of linseed has not previously been developed yet. Identification of responsive genotypes and development of efficient culture protocols are a prerequisite to initiating an effective doubled haploid production system in applied breeding programme. Anther culture response in *L. usitatissimum* was studied in the hybrid populations from responsive and poor/non-responsive cultivars and their parental forms. Two different growth regulators combinations and three concentrations of sucrose were studied. Variation for anther culture response was significant both within and between the hybrids and their parents. F_1 hybrids, produced from crosses between responsive genotype 'Lirina' and poor/non-responsive genotype 'Barbara', showed dramatic increases in callus induction in comparison with the parental form on medium supplemented by 1mg l^{-1} 6-benzylaminopurine and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid containing 6 % sucrose.

Key words: anther culture response, cultivars, hybrids, linseed.

Introduction

Flax (*Linum usitatissimum* L.) is an important oil crop in Europe and is also used as a protein source for animal rations.

Haploid technique is considered to be a much faster and more efficient tool for breeding new varieties in a comparatively short time. Rapid breeding techniques could help in producing new linseed varieties with characters that are adapted to the current demands of industry, which would therefore gain new markets for this crop. Another advantage of doubled haploid lines in a breeding programme is, as a result of gametic-segregation ratios and homozygosity of lines, the smaller size of population necessary to select a desired genotype (Friedt et al. 1995).

Using F_1 hybrids instead of homozygous genotypes would produce the regenerated plants with possibly novel and beneficial gene combinations. Doubled haploid plants and the somatic diploids derived from anther culture can also be distinguished by morphological, biochemical and molecular markers. Furthermore, the overall efficiency of regeneration in anther/microspore culture of F_1 hybrids has been found to be higher than of their homozygous parent genotypes in flax (Chen et al. 1998; Chen, Dribnenki 2002).

Anther culture response is controlled by the genotype of donor plants and non-genetic factors (Kurt, Evans 1998; Rutkowska-Krause, Mankowska 2002; Obert et al. 2004). While

studies on the non-genetic component have produced some dramatic increases in anther response, such factors are more difficult to fix than the genetic component. Changing the genetic component has the added advantage that it can be manipulated in a desired direction (Deaton et al. 1987). As most of the anther-culture responsive maize materials have been found in non-commercial germ plasm, it is likely that culturability can be transferred from the responsive, non-commercial, exotic maize germ plasm into elite types (Afele, Kannenberg 1990). Both nuclear and cytoplasmic factors have been shown to have an affect on wheat (*Triticum aestivum*) anther culture (Sopory, Munshi 1997).

The objectives of this study were to estimate responsibility of F_1 hybrids in anther culture in comparison with parental forms.

Materials and methods

Plant material and anther culture

The experiments were carried out with the following flax cultivars: 'Lirina', 'Barbara', 'Mikael' and their hybrids: F_1 'Barbara' × 'Lirina', F_1 'Lirina' × 'Barbara', F_1 'Barbara' × 'Mikael', F_1 'Mikael' × 'Barbara'. Seeds were germinated and grown in a growth chamber with a 16 h photoperiod, temperature (22/18 °C, day/night) and 75 % humidity. All plants were grown in mixture of peat, vermiculite and sand in a 3:1:2 ratio in 16.5 cm pots. The plants were watered and fertilized with diluted 20-20-20 ($N:P_2O_5:K_2O$) at the rate of 4 g l^{-1} as required.

Flower buds (3.5 to 4.0 mm in length) were collected when the microspores were at the mid uninucleate stage previously determined by microscopic observation of anthers (0.9 to 1.1 mm in length) and stained with 1 % acetocarmine. Harvested buds were surface sterilized in 70 % ethanol for 1 min, then in 2 % sodium hypochlorite for 10 min and rinsed three times with sterile distilled water. Five anthers from each of two buds (total 10) were inoculated onto a plastic Petri dish (35 × 10 mm) containing 3 ml of modified (NH_4NO_3 – 165 mg l^{-1}) MS induction medium (Murashige, Skoog 1962) and incubated at 25 °C in the dark. Every four weeks the calli were subcultured to fresh medium and were maintained at (27/24 °C day/night) under a 16 h photoperiod, at a light density of 40 mmol m^{-2} s^{-1}.

Detailed experiment design and data analysis

Experiment 1. Effect of plant growth regulators on callus induction. Two different combinations of auxin and cytokinin [2 mg l^{-1} 6-benzylaminopurine (BAP) + 1 mg l^{-1} α-naphtylacetic acid (NAA), 1 mg l^{-1} 6-benzylaminopurine (BAP) + 2 mg l^{-1} 2,4-dichlorphenoxyacetic acid (2,4D)] on a modified MS medium were tested. All media were supplemented with 6 % sucrose and solidified with 0.6 % agar.

Experiment 2. Effect of sucrose level on callus induction. For investigation of the effect of sucrose level anthers of tested genotypes and their hybrids were cultivated on a modified MS medium containing 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4D.

A complete randomized design was used for all experiments. For each treatment 120 anthers were cultured (10 anthers per Petri dish; 12 replicates per treatment) and each experiment was done in triplicate. The number of anthers producing calli was scored at 28 days after initial inoculation. The percentage of anthers with calli was calculated as the number of anthers producing calli per 100 inoculated anthers.

The data of the investigations were analysed using the computer programmer STAT 1.55 from "SELEKCIJA" (Tarakanovas 1999) and ANOVA for EXEL, vers. 2.1. Mean values and SE's for each genotype were calculated based on the number of independent replications.

Results

Experiment 1. Effect of plant growth regulators on callus induction

The whole androgenetic process of anther culture in this study was similar as described previously (Burbulis et al. 2005). Formation of a callus was observed within two weeks after culture initiation. Results of the effect of two tested growth regulator combination on callus induction in three flax genotypes and their hybrids are summarized in Fig. 1.

Each genotype responded differently according to the medium. Anthers of 'Lirina' showed the highest value of induced anthers on medium supplemented by 2 mg l^{-1} BAP with 1 mg l^{-1} NAA while the combination of 1 mg l^{-1} BAP with 2 mg l^{-1} 2,4D significantly reduced the number of anthers producing callus. In contrast, anthers of 'Mikael' showed a better response on medium with 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4D, whereas callus formation in this genotype was strongly reduced by the 2 mg l^{-1} BAP with 1 mg l^{-1} NAA combination. Anthers of 'Barbara' cultured on both tested media did not show any response even after six weeks of culture and subsequently became necrotic.

For F_1 'Barbara' × 'Lirina' hybrid, the combination of 1 mg l^{-1} BAP with 2 mg l^{-1} 2,4D in induction medium significantly increased callus formation in comparison with superior

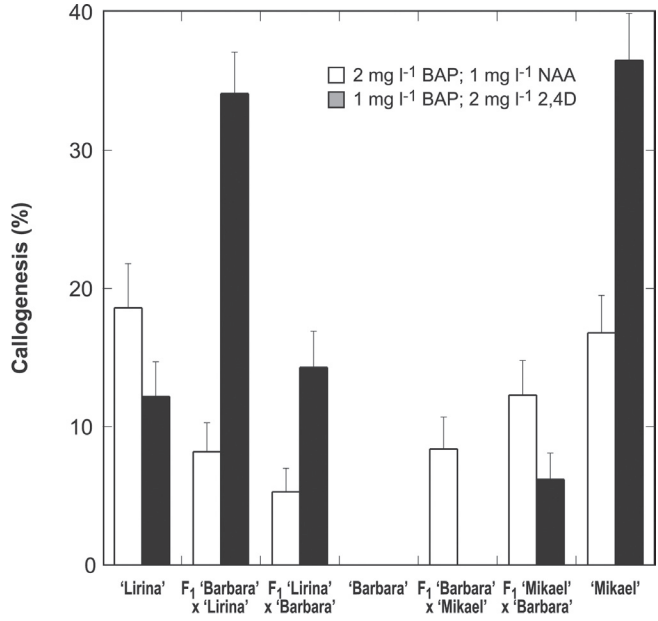

Fig. 1. Effect of plant growth regulator combinations on callus induction in three flax genotypes and their hybrids in anther culture on medium with 6 % sucrose. BAP, 6-benzylaminopurine; NAA, α-naphtylacetic acid; 2,4D; 2,4-dichlorophenoxyacetic acid.

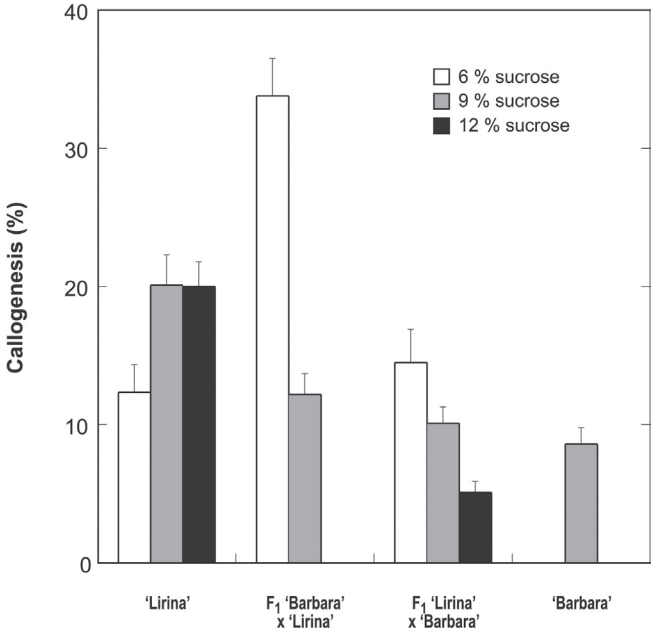

Fig. 2. Effect of sucrose level in culture medium containing growth regulators, 1 mg l^{-1} 6-benzylaminopurine and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid on callus induction in anther culture of cultivars 'Lirina' and 'Barbara' and their hybrids.

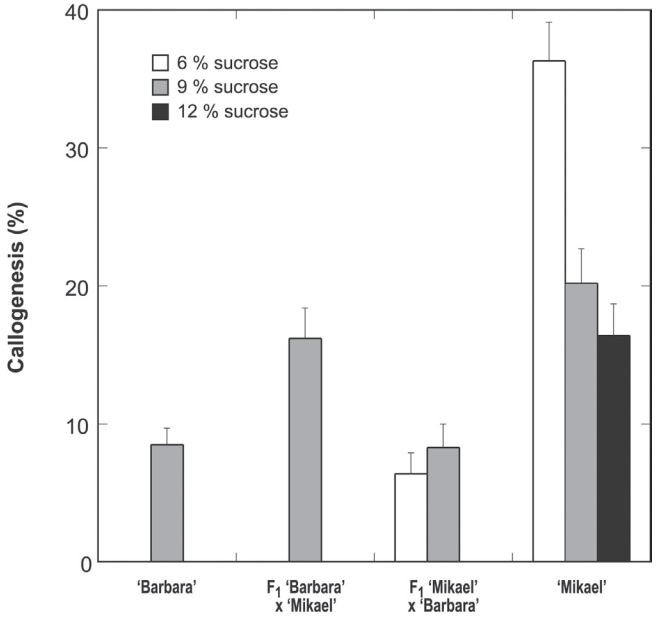

Fig. 3. Effect of sucrose level in culture medium containing growth regulators, 1 mg l^{-1} 6-benzylaminopurine and 2 mg l^{-1} 2,4-dichlorophenoxyacetic acid on callus induction in anther culture of cultivars 'Barbara' and 'Mikael' and their hybrids.

parent 'Lirina'. However, there was no significant difference between 'Lirina' and F_1 'Lirina' × 'Barbara' anthers cultured on the some medium.

For F_1 'Mikael' × 'Barbara' hybrid, the combination of 2 mg l^{-1} BAP with 1 mg l^{-1} NAA increased callus induction in comparison with 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4D combination, however callus formation was significantly lower as compared with the superior parent 'Mikael'. The growth regulator combination 2 mg l^{-1} BAP with 1 mg l^{-1} NAA promoted callus development in anthers of F_1 'Barbara' × 'Mikael', while the combination 1 mg l^{-1} BAP with 2 mg l^{-1} 2,4D completely inhibited callus formation from anthers of this genotype.

Although cultivar 'Barbara' was not responsive for anther culture it is important to note that F_1 hybrids from crosses 'Barbara' with 'Lirina' and 'Mikael' were more or less responsive, except F_1 'Barbara' × 'Mikael' anthers cultured on medium supplemented by 1 mg l^{-1} BAP with 2 mg l^{-1} 2,4D.

Experiment 2. Effect of sucrose level on callus induction

The results of the effect of sucrose level on callus induction in linseed cultivars 'Lirina', 'Barbara' and their hybrids are presented in Fig. 2.

An increasing level of sucrose from 6 % to 9 % increased the frequency of responding anthers of 'Lirina' (from 12 % to 20 %) and 'Barbara' (from 0 % to 9 %), but significantly reduced callus formation in their hybrids. Especially sensitive to higher sucrose content was the F_1 'Barbara' × 'Lirina' hybrid. Cultured anthers of this hybrid on medium supplemented with 9 % sucrose caused a decrease in frequency of responding anthers from 34 % to 12 % in comparison with the 6 % sucrose level.

Increasing the sucrose level from 9 % to 12 % completely inhibited callus formation in anthers of 'Barbara' and F_1 'Barbara' × 'Lirina' hybrid, and significantly reduced callus formation in F_1 'Lirina' x 'Barbara'. For cultivar 'Lirina' there was no significant difference between 9 % and 12 % sucrose levels in callus induction.

Callus formation from F_1 'Barbara' × 'Mikael' hybrid was obtained only when anthers were cultured on medium with 9 % of sucrose, as well as from parent 'Barbara' (Fig. 3). However, there was no significant difference between 6 % and 9 % of sucrose for F_1 'Mikael' × 'Barbara' hybrid.

In contrast, 9 % of sucrose significantly reduced the number of anthers producing callus in 'Mikael'.

Increasing the level of sucrose from 9 % to 12 % completely inhibited callus induction from anthers of F1 'Barbara' × 'Mikael' and 'Mikael' × 'Barbara' hybrids. However, for cultivar 'Mikael' there was no significant difference between the 9 % and 12 % sucrose level.

Discussion

The presence of an appropriate concentration of growth regulators in the medium plays a critical role in callus formation in anther culture. The reports available so far on anther culture suggest that, in the majority of cases, auxin or/and cytokinin has been required as a component of the medium. Growth regulators have widely been used for callus enhancement in anther culture of flax. A significant effect of the combination of 1 mg l^{-1} BAP and 1 mg l^{-1} NAA on callus formation in flax anther culture has been reported by Obert et al. (2005), while higher callogenesis in flax anther in medium with 1mg l^{-1} BAP and 2 mg l^{-1} 2,4D was observed in experiments reported by Chen et al. (1998a). In the

present study, the combination of 1 mg l^{-1} BAP with 2 mg l^{-1} 2,4D in induction medium produced a higher percent of calli in the cultivar 'Mikael' (37 %), F_1 'Barbara' × 'Lirina' (34 %) and F_1 'Lirina' × 'Barbara' (14 %). However, anther of 'Lirina', F_1 'Barbara' × 'Mikael' and F_1 'Mikael' × 'Barbara' showed a better response on medium supplemented with 2 mg l^{-1} BAP and 1 mg l^{-1} NAA.

Response to androgenesis for a number of crops including flax is known to be strongly genotype dependent and influenced by numerous egzogenous factors. Our previous study has shown significant variation in callus producing ability between the genotypes. The cultivar 'Mikael' had the highest callus induction rate, while cultivar 'Barbara' was only able to produce calli in medium supplemented with 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4D containing 9 % sucrose. This results corresponds with that obtained by Nichterlein et al. (1991), Chen et al. (2002), Obert et al. (2005) who found that anther induction rate of linseed varied according to the plant genotype. This difference in the level of culturability is strongly indicative of genetic components to the response rate. To confirm this, crossing experiments in involving responsive ('Mikael' and 'Lirina') and poor/non-responsive ('Barbara') genotypes were made. The current study showed that the appropriate growth regulators combination for hybrids and their parental form differs. Anther of cultivar 'Lirina' showed a higher level of callogenesis on medium with 2 mg l^{-1} BAP and 1 mg l^{-1} NAA, while reciprocal hybrids showed a better response on medium containing 1mg l^{-1} BAP and 2 mg l^{-1} 2,4D. In contrast, cultivar 'Mikael' showed the higher value of induced anthers on medium with 1mg l^{-1} BAP and 2 mg l^{-1} 2,4D, whereas the combination 2 mg l^{-1} BAP and 1 mg l^{-1} NAA promoted callus formation in anthers of reciprocal hybrids of this genotype.

It has been documented that concentration of sucrose is also a very important factor for callus induction in anther culture. Our study results show a different influence of sucrose level on callus induction of cultivars and their hybrids. An increased concentration of sucrose (9 %) promoted higher callogenesis of the cultivars 'Lirina' and 'Barbara', while a lower sucrose level (6 %) was more suitable for their reciprocal hybrids. In contrast, 'Barbara' × 'Mikael' hybrid showed a better response in medium supplemented with 9 % of sucrose, as also did cultivar 'Barbara', while the some sucrose level significantly decreased callus induction of cultivar 'Mikael' in comparison with 6 % of sucrose.

The current study indicates that here is a strong genotype-dependent effect of growth regulators combination and sucrose level on callus production from anthers in flax, and therefore the induction medium must be designed for each genotype. F_1 hybrids, produced from crosses between responsive genotype 'Lirina' and poor/non-responsive genotype 'Barbara' showed dramatic increases in the callus induction in comparison with the parental form on medium supplemented by 1 mg l^{-1} BAP and 2 mg l^{-1} 2,4D containing 6 % sucrose. The high heritability for anther response estimate in our study suggests that a relatively rapid genetic gain can be made in transferring this trait from responsive to poor/non responsive germ plasm.

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Putekšņu kultūras reakcijas salīdzinājums dažādām *Linum usitatissimum* L. šķirnēm un to hibrīdiem

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Kopsavilkums

Lini (*Linum usitatissimum* L.) ir svarīga lauksaimniecības kultūra Lietuvā gan eļļas, gan šķiedru ieguvei, bet vietējās linu šķirnes vēl nav izveidotas. Kompetentu genotipu atlase un efektīvu kultivēšanas protokolu izstrādāšana ir priekšnoteikums dubulto haploīdu iegūšanas sistēmas izveidošanai praktiskās selekcijas programmā. *L. usitatissimum* putekšņu kultūras reakciju pētīja hibrīdu populācijās no reaģētspējīgām un slikti reaģētspējīgām šķirnēm un to vecāku formām. Pētījumos izmantoja divas dažādas augšanas regulatoru kombinācijas un trīs saharozes koncentrācijas. Putekšņu kultūras reakcija būtiski atšķīrās gan starp hibrīdiem, gan to vecāku formām. F_1 hibrīdi no krustojumiem starp reaģētspējīgo genoptipu 'Lirina' un slikti reaģētspējīgo genotipu 'Barbara' parādīja būtisku kallusa veidošanās indukcijas pieaugumu vidē ar 1 mg l^{-1} benzilaminopurīnu un 2 mg l^{-1} 2,4-dihlorofenoksietiķskābi salīdzinājumā ar vecāku formām.

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Internal regularity and quantization of gene parameters

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Abstract

The origin and function of introns in eukaryotic cells has rised much attention since their discovery in 1977. However, none of the opposing theories is universally recognized today. We have elaborated a new approach and methodology to study the origin of genes and introns using analysis and comparison of gene numerical parametres and their internal regularity. Exon dimensions, intron coordinates, sizes of gene exon rows, etc. were investigated. Our aim was to demonstrate that the structures of primeval gene precursors originating at the very early stage of evolution were regular and periodic, and that this regularity was retained partially also in the structures of modern contemporary genes and the corresponding proteins. The ability to determine the size of the gene quantum and to quantize the gene numerical parameters for the most regular modern gene structures gave support for this new approach. In this article we describe new principles and a method of analysis of gene internal regularity, demonstrate the internal regularity of some gene segments, and determine the numerical values of their quanta.

Key words: gene quanta, *ras*-related genes, regularity of exon dimensions and intron coordinates, triosephosphate isomerase genes.

Introduction

Two opposite theories well known as "introns early" (Gilbert 1978; Gilbert 1987; de Souza et al. 1988) and "introns late" (Palmer, Logsdon 1991; Logsdon 1998) have been elaborated to investigate gene and intron origin, but none of them is universally recognized today, as there is insufficient corroborative evidence. We attempted to elaborate and verify a new approach and method to study the origin of genes and introns using new methods of analysis of gene codon root and amino acid sequences (Chipens 1996; Ievina, Chipens 2004), as well as comparison of the internal regularity of gene parameters. We hypothesize that introns and exons are products of gene evolution originating at a definite geological period of time (Ievina, Chipens 2003; Ievina, Chipens 2004; Chipens et al. 2005a,b). We assume that gene precursors were highly regular periodic nucleic acids formed by replication of identical in size and sequence oligonucleotides, named repeat units (RU; Chipens, Ievina 1999). According to our model, exons and introns in gene structures originated from periodic nucleic acids after the emergence of the very first splicing machinery. Introns, in the absence of the constraints imposed by the coding function, as well as natural selection on the level of proteins, accumulated mutations without any limits during billions of years of evolution. Exons, contrary to introns, remained relatively conserved. If the hypothesis of oligonucleotide multiplication is correct, exons in modern

gene structures, according to the amino acid interaction code and the codon root code (Chipens 1996; Chipens, Ievina 2004a; Chipens, Ievina 2004b), likely retained some more or less expressed structural regularity.

Our present investigation was based on the concept of the translational symmetry, particularly the dimensionality and periodicity of lineland (Hargittai, Hargittai 1995). The aim of this research was to demonstrate that the regularity of gene ancestors partially was retained also in the structures of contemporary genes and the corresponding proteins. Support for these ideas was raised by the investigation of the amino acid interaction code (Chipens, Ievina 2004a; Chipens, Ievina 2004b), as well as the ability to quantize gene numerical parameters (Chipens et al. 2005a; Chipens et al 2005b). With the aim to determine elements of regularity in contemporary gene sequences in accordance with the model of gene origin by nucleotide repeat unit multiplication reactions (Ievina, Chipens 2003) in this article we focus on the dimensions of numerical parametres of gene and protein structural elements. In the course of our work new notions and definitions of biopolymer internal regularity as well as methods of their analysis were suggested and elaborated.

The theoretical basis of the investigation was the concept of the singular point and the translational symmetry, particularly the dimensionality and periodicity of lineland (Hargittai, Hargittai 1995), a one dimensional space group which describes the symmetry involving definite repetition or periodicity in one direction (Fig. 1). According to our model (Ievina, Chipens 2003; Chipens, Ievina 1999) gene precursors were formed by reactions of spontaneous saltatory replications of oligonucleotides or nucleotides. In analogous reactions of DNA satellite formation the number of repeat unit monomers in a nucleotide chain is very large [105-107 repeats, e.g. in *Xenopus laevis* or *Drosophila virilis* (Lewin 1995)] and correspond to the definition of lineland.

Simple translation symmetry is the most obvious symmetry element of the space groups. It brings the pattern into congruence with itself over and over again. The absence of a singular point leads to regularity expressed in infinite repetition which characterizes translation symmetry (Hargittai, Hargittai 1995). Real objects are not infinite. For symmetry consideration, it may be convenient to look only at some portion of the whole where the ends are not yet in sight and extend them in thought to infinity (Hargittai, Hargittai 1995). The concepts "lineland" and "translational symmetry" were the basis for elaborating a new term – gene "knot points" (Fig. 2) and a new method for investigation of biopolymer regularity – design and analysis of repeat unit piles (Fig.1 E, F; Chipens, Ievina 1999; Ievina, Chipens 2004). Analysis of gene knot points and intron coordinates led to the discovery of discreet values of gene parameters and the ability to calculate gene quanta Q.

Materials and methods

We investigated the dimensions and numerical parameters of gene and protein structural elements and their internal regularity. Protein and gene parameters (exon length, intron coordinates, etc.) as well as sequences, were taken from the GenBank and SwissProt databases or literature. All parameters of genes were expressed using as a unit of measure one nucleotide (nt). Prime number (a prime number can only be divided exactly by itself or one) multipliers of the obtained parameters were calculated to determine their common

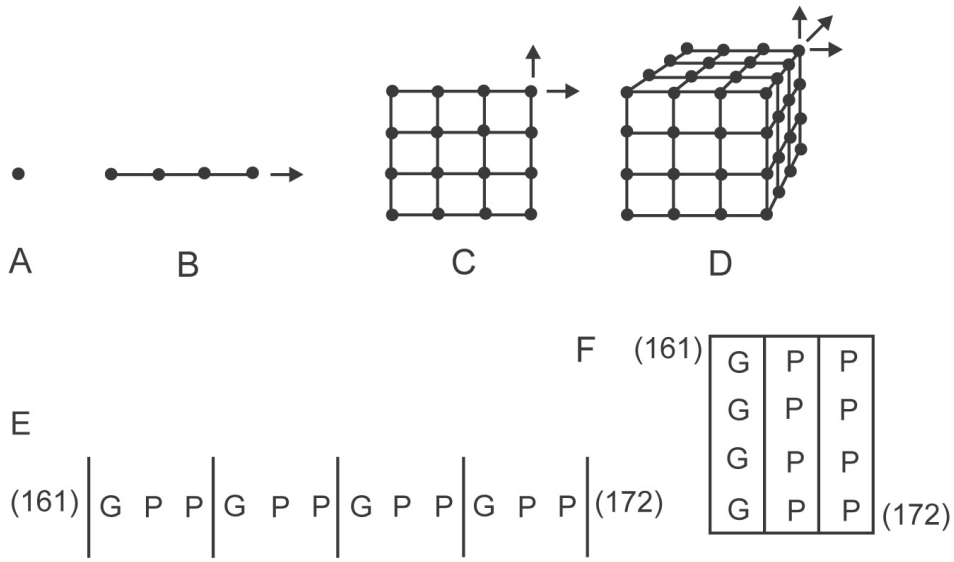

Fig. 1. Dimensionality and periodicity in point groups and space groups. A, pointland; B, lineland; C, flatland; D, spaceland (Hargittai, Hargittai 1995); E, a small fragment of human collagen *COL2A1* gene with amino acid sequence 161-172 (GenBank, accession Q14047) show translation symmetry (the repeat unit is 9 nt or 3 amino acids (GlyProPro); F, translation symmetry is demonstrated by repeat unit pile (RUP) structure (Ievina, Chipens 2004). Vertical lines of RUP contain identical amino acid symbols.

internal regularity (common identical prime multipliers, see an example Fig. 2 D) which in accordance with the model of lineland and gene knot points must be identical for regular segments of gene structure. Each numerical parameter of a gene, for example, the exon dimension or intron coordinate (the intron coordinate is the sum of preceeding exon lengths, expressed by the number of nt), can be represented as a product of prime number multipliers. If several numerical parameters of a gene have a set of common prime number multipliers then this phenomenon is named "common internal regularity". Theoretically, if a gene precursor could be analysed immediately after the multiplication reaction (before mutations and other factors change the precursor sequence) then the gene precursor structure would correspond to the structure of the lineland (Hargittai, Hargittai 1995) (Fig. 1 and 2). The most characteristic feature of the lineland-type structures is regularity expressed in infinite repetition which characterizes translational symmetry. Practically, such a structure can be observed in a multimer formed of identical in size and nucleotide sequence repeat units (Fig. 1 E, F). From the formal similarity of linelands and RU multimer linear chain structures we derrived a new term – the "gene knot point". Distances between two neighbour gene knot points determine the size of the repeat unit RU (nt) and the numerical value of the gene quantum Q (nt). In regular gene structures gene knot points determine the structural organization of the exon row. Distances in the atoms of crystals self-evidently are many times smaller than lengths of RU. However, for simplicity and to demonstrate the principal identity of atom location regularity in crystal facets and repeat unit regularity in gene ancestors and also in modern gene structures (partially), the RU

size in Fig. 2 is equalized to interatom distances in crystal cells.

The concept of gene RU and knot points has key significance in our working hypothesis and new method of gene analysis. It is supposed that gene splicing mechanisms measure exon length by whole numbers of codons by an unknown mechanism (Robberson et al. 1990) and, as we suppose, possibly also by whole numbers of RU.

Results and discussion

The model of gene, exon and intron origin

The general principle of the model of gene precursor formation is that nucleotides or fragments of nucleic acids were spontaneously multiplied laterally to generate a large number of identical copies termed repeat units (RU; Ievina, Chipens 2003; Chipens et

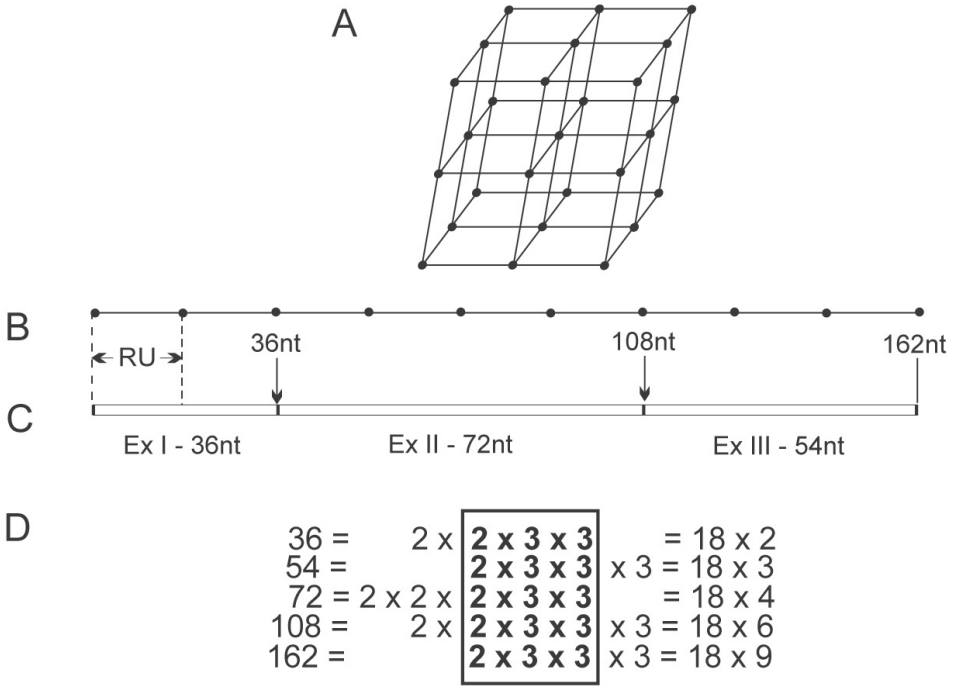

Fig. 2. Principles of calculation of common internal regularity of exon dimensions and intron coordinates. A, a threedimensional crystal cell structure (symmetry G_3^3 or spaceland); B, structure of lineland (G_1^3) derived from a crystal cell; C, scheme of a artificial highly regular model of gene structure. Exons are denoted by ordinal (Roman) numbers, the sizes (nt) of exons (Ex) are shown. Arrows topped with numbers (intron coordinates) indicate gene knot points where introns split the exon row. Repeat unit sizes correspond to the distance between two gene knot points. Exon dimensions are precisely multimers of repeat unit dimensions; D, calculation of prime number-multipliers and identification of common prime multipliers (framed). Common prime multipliers $2 \times 3 \times 3$ determine common internal regularity of the gene model numerical parameters. The product of common prime multipliers $2 \times 3 \times 3 = 18$ allows to determine the potential size of the gene quantum Q – i.e. the number of nucleotides in RU (18 nt), which also coresponds to the repeat unit dimension.

al. 2005a). Exons and introns arose simultaneously in early evolution from common precursors which were highly repetitive simple-structure nucleic acids. It is supposed that the formation of exons and introns was induced by the emergence and action of the very first splicing machinery. Devoid of the coding function, introns accumulated mutations without any limits, did it significantly faster than exons, and lost the regularity of nucleotide and codon root sequences. In accordance to the model intron positions in gene structures were not random – the birth positions of introns were located at internally regular distances between the repeat unit boundaries. These theses are confirmed by contemporary gene and protein structure analysis (Chipens et al. 2005a and this publication). It is necessary to note that reactions of nucleotide multiplication determined the formation of long open reading frames of gene precursors with symmetric exons and all introns in phase zero.

Immediately after the emergence of gene precursors exons and introns were formed of identical in size and sequence repeat units. However, mutations, indels and intron sliding in the course of evolution disrupted this sequence regularity. Introns gradually disappeared or even were lost at once completely. However, as we suppose, contemporary genes may have retained some or several introns in the birth positions. If so, the regularity of location of these introns must be identical with the sequence regularity of exons and exon-coded protein fragments. The latter in separate cases can be demonstrated after translation of gene and protein sequences into codon root symbols (the second codon letter; Ievina, Chipens 2003). However, the concept of gene knot points demonstrates for the first time the discrete structure of large segments of genes and introduces a new concept about gene quanta – the constant number of nucleotides in a given gene repeat unit.

Selection of proteins for studies

Hundreds of G-protein coupled receptors initiate different intracellular signalling chain reactions by the G-protein nanomachine (Clapham 1996). Besides these G-proteins eucariotic cells contain a variety of other structurally related proteins of molecular mass around 20-30 kDa that function as monomers in different regulatory pathways through their capacity to bind and hydrolize GTP specifically (Burgoine 1989). This is the family of small G proteins (SGP), which consists of several subfamilies, such as Ras-, Ypt, Art and others (Ditmaier, Farby 1994). Among SGP, the p21 products of different *ras* genes have attracted the greatest attention, as mutated versions can cause malignant transformation of mammalian cells.

The Ypt proteins (a subfamily of ubiquitous eucariotic SGP) are structurally related to the *ras* gene products and share with them similar biochemical properties (Hanbruck et al. 1989). Several years ago (Ievina, Chipens 2003) we revealed in the basidomycete *Coprinus cinereus* ras gene an identical internal regularity of four neighbour side-by-side exons. Therefore, for analysis we chose other genes of the SGP family – a *ras*-related GTP binding protein encoded by the mice *YPT1* gene (Wichmann et al. 1989), a *ras*-like gene of the edible mushroom *Lentinus edode* (Hori et al. 1991), and the *ras*-related gene of the *Mucor racemosus* (Casale et al. 1990).

The main focus of investigation however was the triosephosphate isomerase (*TPI*) gene (Noltman 1972), as the exon theory arguments suggests that introns are as old as the genes themselves and that apparent correlation of many of the intron sites in plant, animal and fungal *TPI* genes is evidence of their assembly of ancient genes by exon shuffling (Gilbert et al. 1986; Marchionni, Gilbert 1986). The evolution of exon-intron structure of

eucariotic genes from the viewpoint of "introns early" theory is well described in review articles (Gilbert et al. 1977; Long et al. 1995; Fedorov et al. 2001). The data of Gilbert's team indicate that intron positions show non-random distribution in ancient genes (Long et al. 1995), and that only phase zero introns are correlated with structural elements or modules of ancient proteins (De Souza et al. 1998). These data support our concept that in early period of evolution all introns of gene ancestors were in the phase zero (Ievina, Chipens 2003).

Researchers representing the intron insertional theory sequenced the "new *TPI*" genes from three diverse eucariotes – the basidomycete *Coprinus cinereus*, the nematode *Caernorhabditis elegans*, and the insect *Heliothis virescens* (Logsdon et al. 1995). They revealed introns at seven novel positions that disrupt previously recognized gene/protein structural correlation revealed by the "introns early" school. They predicted that, when more *TPI* genes are sequenced, more intron positions would be discovered, and that these sites would fall randomly with respect to *TPI* gene protein structural elements or modules (Logsdon et al 1995).

One of the most studied *TPI* genes is that of chicken (Straus, Gilbert 1985; Banner et al. 1975). Therefore, to compare the results obtained by our model and to analyse the natural gene structure, our choice fell on this sequence. To test the possibilities of "mathematical modelling" of gene structures and to estimate the effects of virtual intron position transfer to the gene knot points (mainly by changing only the intron phase) we selected for analysis the *TPI* gene of *Coprinus cinereus* containing six exons (GenBank accession number U23079) and encoding a 251-amino-acid-residue-long peptide chain. This gene contains no intron in phase zero.

Internal regularity of small G proteins

To determine the potential size of *YPT1* gene quantum and the dimensions of RU, we calculated the common prime multipliers of all exon dimensions and intron coordinates (Table 1 and 2). The obtained values allowed to select a set of common prime multipliers $2 \times 2 \times 3$ (Table 2, framed) and to determine their product – 12 nt. Almost all *YPT1* gene parameters (four intron coordinates and three exon dimensions) could be expressed as multiples of 12 nt. Thus we concluded that the RU size as well as the *YPT1* gene quantum Q is 12 nt. The analysis of this gene structure (Fig. 3 A) showed that most likely during evolution the first intron as a result of mutation had slid off from the gene knot point, i.e.

Table 1. The mouse *YPT1* gene exon dimensions according to Wichmann et al. (1989) and intron coordinates and phases calculated on the basis of exon sizes. The gene exon row structure is given in Fig. 3

Exons ordinal No.	Exon size (nt)	Intron ordinal No.	Intron coordinate (nt)	Intron phase
1	23	1	23	2
2	73	2	96	0
3	96	3	192	0
4	96	4	288	0
5	132	5	420	0
6	195	-	-	-

Table 2. Common prime number multipliers (framed) of exon dimensions and intron coordinates of the mouse *ras*-related gene *YPT1*

Gene parameters	Dimensions (nt)	Prime multipliers	Parameters as multiples of the gene quantum
Exon III and IV dimensions	96	$2 \times 2 \times 2 \times \boxed{2 \times 2 \times 3}$	$96 = 12 \times 8$
Intron No. 2 coordinate	96	$2 \times 2 \times 2 \times \boxed{2 \times 2 \times 3}$	$96 = 12 \times 8$
Intron No. 3 coordinate	192	$2 \times 2 \times 2 \times 2 \times \boxed{2 \times 2 \times 3}$	$192 = 12 \times 16$
Intron No. 4 coordinate	288	$2 \times 2 \times 2 \times \boxed{2 \times 2 \times 3} \times 3$	$288 = 12 \times 4$
Exon V dimensions	132	$\boxed{2 \times 2 \times 3} \times 11$	$132 = 12 \times 11$
Intron No. 5 coordinate	420	$\boxed{2 \times 2 \times 3} \times 5 \times 7$	$420 = 12 \times 35$

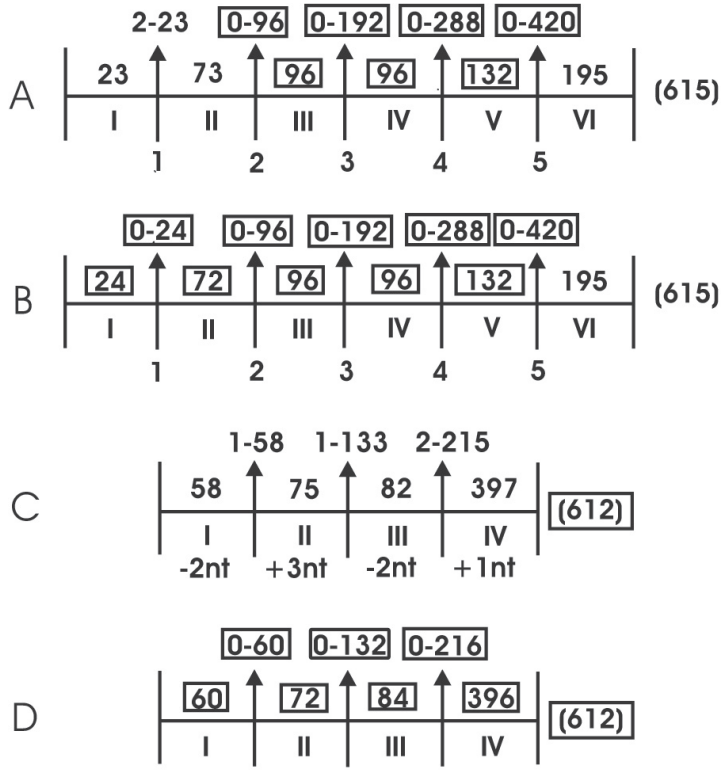

Fig. 3. Schemes of organisation of the mouse *YPT1* gene and *Mucor racemosus* gene *MRAS1* (shown not in scale). Arrows with ordinal numbers are topped with parameters characterizing intron phases and coordinates (nt). The horizontal middle line symbolizes the exon row nucleotide sequence. Exon lengths (nt) are shown above the line, ordinal (Roman) numbers – below. Numbers in brackets show exon row total length including initiation and termination codons. The framed and bold typed numbers and segments of schemes show gene parameters and elements, which can be expressed as gene quantum Q (12 nt) multiples. A, mouse gene *YPT1*; B, the same gene after virtual transfer of the first intron to zero position; C, *Mucor racemosus* gene *MRAS1* (GenBank accession M55175) and changes of exon dimensions (± nt). D, a model of a completely regular *MRAS1* gene.

was shifted upstream by one nucleotide. If the potential intron sliding was corrected, and the intron was virtually shifted back to the birth place, i.e. to the nearest knot point of the gene, then three internally regular structural parameters of the *YPT1* gene appeared (Fig. 3 B) – the length of the first and the second exon (correspondingly 2 × 12 = 24 nt and 6 × 12 = 72 nt) and the first intron coordinate (24 nt). Therefore, 10 numerical parameters of 11 of this gene were internally potentially regular and could be expressed as multiples of Q = 12 nt. The obtained results were unexpectedly good. But not all genes have retained such a high regularity of ancestors, e.g., none of the five *YPT1* intron positions matched those of the H-, K-, or N-ras genes (three introns) or those of the *R-ras* gene (five introns; Wichmann et al. 1989).

However, at the same time, several other genes (Casale et al. 1990) showed striking similarity to human *ras* genes, for example, the *MRAS1* gene of *Mucor racemosus* (GenBank accession M55175) showed the same identical internal regularity (Fig. 3 C, D). The revealed deviations of intron coordinates (1-2 nt) in this case most likely could be explained by changes of intron phases or virtual intron sliding (exact molecular mechanisms of intron phase changes are not yet known). Regardless the length of the exon row in the *MRAS1* gene is precisely internally regular (612 nt = 12 × 51).

The edible basidomycete (mushroom) *Lentinus edode* terminal 5'- and 3'-introns No. 1 (coordinate 12 nt) and No. 6 (coordinate 528 nt; 528 = 12 × 44) are precisely regular and can be quantized (Q = 12nt) indicating that gene structure, at least the nucleotide sequence 1-528 nt, is regular. However, introns No. 2 to No. 5 have slid off from the gene knot points by 1-2 nt (Table 3). Also, *YPT1* and *MRAS1* genes, and several other *YPT1* genes of the *RAS* family (e.g., *Chlamidomonas reinhardtii*, *Schizosaccharomyces pombe*, etc.; Chipens et al. 2005b) show partial regularity corresponding to the gene quantum 12 nt.

Elements of internal regularity of triosephosphateisomerase (TPI) genes

Our viewpoint is that a stable principle of the new model of gene origin (Ievina, Chipens 2003) is the possibility to calculate theoretical sizes of exons and exon rows, as well as the potential intron positions in genes, if the size of the repeat unit is known. This allows to

Table 3. The *Lentinus edode ras*-like gene exon dimensions (GenBank D00742), calculated intron coordinates, and intron position deviations from the nearest gene knot point coordinates calculated using the gene quantum value Q = 12 nt

No.	Exon coordinates (nt)	Exon dimensions (nt)	Intron coordinates (nt)	Intron phase	Calculated nearest gene knot point (nt)	Deviation of intron position from the knot point
1	56 - 67	12	12	0	12	0
2	122 - 167	46	58	1	60	-2
3	224 - 298	75	133	1	132	-1
4	351 - 432	82	215	2	216	-1
5	491 - 684	194	409	3	408	1
6	748 - 866	119	528	0	528	0
7	930 - 1055	123	-	-	-	-

Table 4. The chicken *TPI* gene exon row structure (GenBank 11941) and calculated intron coordinates. Gene parameters – multiples of *TPI* gene quantum (Q = 9 nt) are framed

No.	Exon dimensions (nt)	Intron coordinates (nt)
1	112	112
2	124	336
3	85	321
4	33	454
5	86	540
6	88	628
7	119	747

compare the calculated and the natural parameters of gene and protein structures. The first task was to determine the common prime multipliers of the well known chicken *TPI* gene (Straus, Gilbert 1985). The gene parameters, however, were very unregular (Table 4). None of the exon lengths contained a whole number of codons. We found only two internally regular parameters of the chicken *TPI* gene (Table 4): the intron No. 5 coordinate (540 nt) and the exon row length (as a sum of all exon dimensions) – 747 nt:

$$540 = 2 \times 2 \times 3 \times \boxed{3 \times 3} \times 5 \quad = 9 \times 60$$
$$747 = \qquad\qquad \boxed{3 \times 3} \times 83 \quad = 9 \times 83$$

The potential size of the *TPI* repeat unit and the gene quantum was 9 nt. The length of exons VI and VII between two internally regular gene parameters (intron No. 5 and the 3'-terminal nucleotide No. 747) also had to be internally regular. Virtual changes of exon VI and exon VII dimensions (88 nt + 2 and 119 nt – 2) indeed revealed two new internally regular parameters of the potential chicken *TPI* gene ancestor (Fig. 4): the exon VI dimension (90 nt) and the new intron No. 6 coordinate (630 nt). The common internal regularity of these elments was the same: 90 = 9 × 10 and 630 = 9 × 70. *TPI* genes of other species (Table 5) in separate regions showed the same size of gene quantum and RU. The parameters shown in Table 3 include exon dimensions, intron coordinates, and the exon row length.

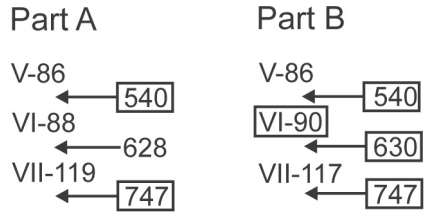

Fig. 4. The *Gallus gallus* gene intron No. 6 (coordinate 628 nt) split two neighbour exons – exon VI (dimension 88 nt) and exon VII (119 nt, Part A; see also Table 4). If the intron No. 6 coordinate is virtually changed by 2 nt, e.g., enlargeing exon VI, but at the same time shortening exon VII by 2 nt (without changing the length of the exon row, Part B), an internally regular unit of *TIM* gene is obtained, containing three new structural elements: exon VI (90 nt or 9 × 10), intron No. 6 in a new position (coordinate 630 or 9 × 70), and exon VII (117 nt or 9 × 13). Numerical values of regular parameters are framed.

Table 5. Examples of internally regular triosephosphate isomerase (*TPI*) gene structural parameters

Species	GenBank accession	Q value	Internally regular parameter of the gene
Drosophila melanogaster	X57576	9	Exon III dimension, 207 nt (9 × 23)
Caneorhabditis elegans	U23081	9	Exon III dimension, 324 nt (9 × 36)
Heliothis virescens	U23080	9	Exon IV dimension, 207 nt (9 × 23)
Oryza sativa	L04967	9	Intron No. 4 coordinate 324 ny (9 × 36)
Gallus gallus	M11941	9	Intron No. 5 coordinate 540 (9 × 60)
Aspergillus nidulans		9	Total exon row length 747 nt (9 × 33)
Heliothis virescens	U23080	9	Intron No. 3 coordinate 297 nt (9 × 33)
Heliothis virescens	U23080	9	Total exon row length 504 nt (9 × 56)
Coprinus cinereus	U23079	9	Total exon row length 756 nt (9 × 84)

Table 6. A short description of the *Heliothis virescens* (tobacco budworm) *TPI* gene exon row structure and intron coordinates (GenBank accession U23080). *, Ordinal numbers of 3'-terminal stop codon nucleotide. **, Changes of intron coordinate dimensions (nt) relative to the natural gene parameters are shown in brackets. Numerical values of the gene parameters which are multiplies of the *TPI* gene family quantum (9 nt) are framed

No.	Exon dimensions (nt)	Intron coordinates	Internally regular parameter of the gene
	a, natural gene GenBank accession U28080		
1	75	75	0
2	112	187	1
3	110	297	0
4	207	504*	-
	b, virtual gene model (the gene quantum 9 nt)		
1	72	72 (-3)**	0
2	117	189 (-2)	0
3	108	297 (0)	0
4	207	504 (0)	0

Our analysis of *TPI* gene parameters demonstrated that the "new *TPI*" genes (Logsdon et al. 1995) have the same internal structural regularity as the "old *TPI*". For example, the *Heliothis virescens* gene contained a small internally regular fragment including the intron No. 3 coordinate (297 nt = 9 × 23), exon No. 4 length (207nt; 207 = 9 × 23) and the sum total of the exon row lengths (504 nt = 9 × 56; Table 6).

Particularly interesting seemed the "new *TPI*" gene of *Coprinus cinereus* (Logsdon et al. 1995). No one of the five introns of *Coprinus cinereus TPI* gene was in phase zero. Particularly this feature determined our choice to analyse the internal regularity of this gene (Table 7). The natural numerical parameters of exon lengths and intron coordinates of the *C. cinereus* gene showed no common internal regularity (Fig. 5 A). Virtual transfer

Table 7. Calculation of structural parameters of the *Coprinus cinereus* triosephosphate isomerase (*TPI*) gene. The exon lengths (nt) are in accordance with GenBank (accession U23079) data. *, this numerical parameter shows that the length of exon row (a sum total of all exon dimensions) – 756 is a multiple of the *TPI* gene quantum Q (9 × 84 = 756). Thus, the exon row contains a whole number of repeat units

Exon ordinal number	The first and last nucleotides of exon	Exon length	Intron coordinate	Intron phase
I	144-255	112	112	1
II	354-477	124	236	2
III	569-612	44	280	1
IV	676-874	199	479	2
V	957-1144	188	667	1
VI	1216-1304	89	756*	-

of intron No. 5 (phase one) with coordinate 667 nt (Table 7) to the nearest supposed knot point (coordinate 666 nt = 9 × 74) at once revealed three internally regular parameters of the gene: the length of exons V (189 = 3 × 3 × 3 × 7) and VI (90 = 2 × 3 × 3 × 5), and the coordinate of intron No. 5 (666 = 2 × 3 × 3 × 37). The length of the exon row of the whole gene including the initiation and termination codons (252 codons = 756 nt; 756 = 2 × 2 × 3 × 3 × 7; Fig. 5 A, C) remained unchanged. The common internal regularity of *C. cinereus TPI* gene parameters was 3 × 3 nt and the gene quantum – 9 nt. These values did not differ from other *TPI* genes (Table 5). Slight correction of the gene parameters in accordance with the gene quantum value (shown in Fig. 5B) revealed that all of the numerical parameters of the gene in such a case were completely internally regular. The sum total of the exon dimensions before and after the virtual intron transfer to phase zero position was not changed (Fig. 5 A, C). The exon rows length corresponded to the whole number of repeat units (756 = 9 × 84). It is necessary to note that two neighbour exon dimensions (IV – 198 nt and V – 189 nt, Fig. 5 C) differed by 9 nt – that is one repeat unit. Comparison of the *Coprinus cinereus* gene-encoded amino acid sequence with intron positions crossing the

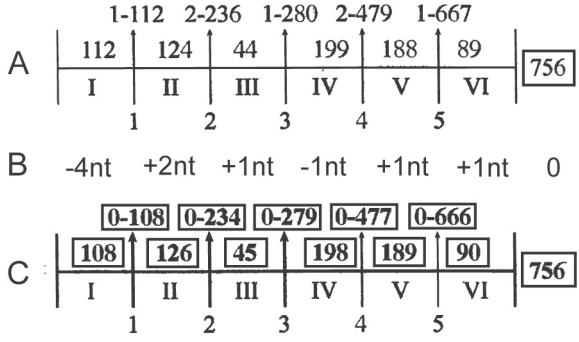

Fig. 5. Comparison of the *Coprinus cinereus TPI* gene numerical parameters. A, exon row of the natural gene; B, changes (± nt) of exon dimensions; C, *Coprinus cinereus TPI* gene after virtual transfer of all introns to phase zero position. Internally regular parameters of the gene are framed.

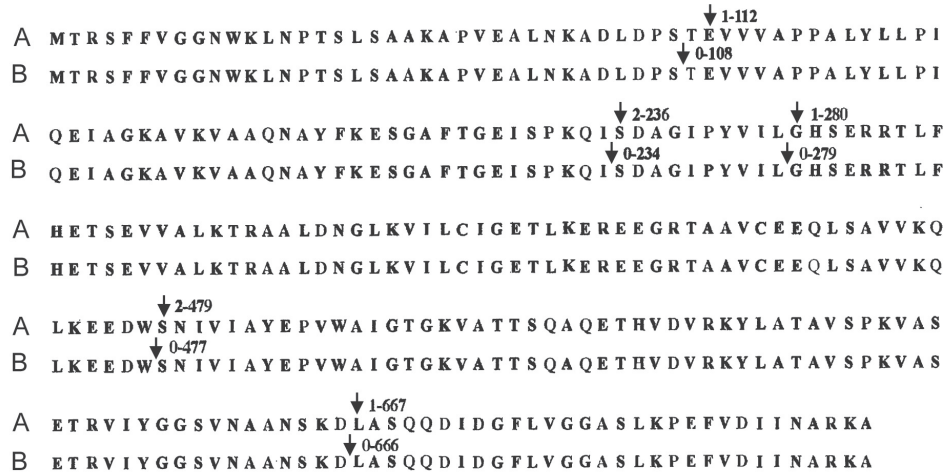

Fig. 6. Primary structure of the *Coprinus cinereus* TPI protein. A, all intron positions shown by arrows and characterised by phase and coordinate cross a codon of the corresponding amino acid; B, the duplicate of the same sequence. Intron positions are shown after their virtual transfer to the phase-zero position (i.e., between two neighbour codons). All coordinates are regular and can be expressed as multiples of the *TPI* gene quantum Q = 9 nt.

peptide chain before and after intron transfer to zero position is shown in Fig. 6 A, B.

In homologous genes we can often find introns in similar but not identical positions between genes separated by large evolutionary distances. In accordance to the exon theory of genes these positions represent the same original intron, possibly moved slightly in position (intron drift or sliding; Gilbert et al. 1997). Supporters of the introns late theory regard that introns can not slide and that intron location diversity in homologous genes is a result of intron loss and insertion or reinsertion in neighbour positions (Palmer, Logsdon 1991). Discrete values of gene intron coordinates and the quantization of gene numerical parameters once again show intron drift or sliding, most likely mainly by changing of intron phases. According to our point of view, assertions that introns whose coordinates in homologous genes differ by 1-2 nt were gained (inserted) independently, or that originally resided within an ancestral gene only one or two base pairs apart, lacks biochemical reasoning and are not logical. Interestingly, the analysis of intron positions splitting *TPI* amino acid sequences reveal the same Q values. Comparison of intron positions of amino acid sequences encoded by three different *TPI* genes – those of *Gallus*, *Heliothis* and *Zea* (alignment data from Logsdon et al. 1995) confirmed (Fig. 7), that the size of TPI family protein quanta and RU is 3aa (or 9 nt). The same parameter value was revealed by comparison of *Caernorhabditis* and *Aspergillus TPI* amino acid sequences – the distance between two phase zero (between codons) intron coordinates in the alignment of protein sequences was 9 amino acids or 3 × 3, i.e. 27 nt = 9 × 3 (Fig. 7).

To further elaborate the new theory and model of gene, intron, and exon origin by oligonucleotide multiplication reactions, we will focus on the similarity existing between the model of G13 (lineland) chrystallography and our models of nucleotide multiplication reactions and gene "knot points" – imaginary points in nucleotide multimers between

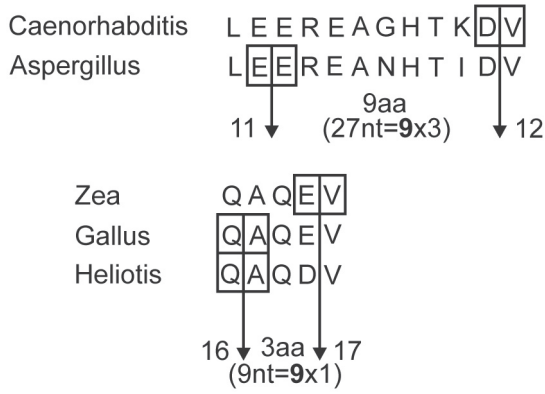

Fig. 7. Fragments of TPI amino acid sequences from genes containing known intron positions. Intron positions are indicated as boxes where they fall between codons (phase zero). Distances between two neighbour introns (shown in form of arrows) are indicated by the number of amino acids (aa). The numbers alongside the arrows correspond to intron designation (Logsdon et al. 1995).

identical in size and related in sequence repeat units. We have elaborated a method of determination of the internal regularity of gene numerical parameters – exon dimensions, intron coordinates, length of coding parts (all parameters are expressed by number of nucleotides, nt) by calculation of common factors and have demonstrated that in regular segments the parameters of gene structures are discrete and can be quantized. The gene quantum of small G protein subgroups *ras*, *MRAS* and *YPT* is 12 nt, and of triosephosphate isomerase genes – 9 nt.

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Gēnu parametru iekšējā regularitāte un kvantēšana

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Kopsavilkums

Par intronu rašanos un funkcionēšanu eikariotu šūnās ir uzdots ne mazums jautājumu jau kopš to atklāšanas 1977. gadā. Tomēr neviena no pretrunīgajām teorijām šodien nav vispārēji atzīta. Mēs esam izstrādājuši jaunu gēnu un intronu izcelsmes pētīšanas metodiku, izmantojot gēnu skaitlisko parametru salīdzināšanu un to iekšējās regularitātes analīzi. Pētot eksonu dimensijas, intronu koordinātes, gēnu eksonu rindu garumus utml., mūsu mērķis bija parādīt, ka agrīno gēnu priekšteču struktūras ir radušās agīnā evolūcijas stadijā, tās bijušas regulāras un periodiskas un, ka šī regularitāte ir daļēji saglabājusies arī mūsdienu moderno gēnu un atbilstošo proteīnu struktūrās. Iespējamība noteikt gēna kvanta lielumu un kvantēt gēna skaitliskos parametrus visregulārākajām mūsdienu gēnu struktūrām kalpo par pieradījumu jaunās pieejas pareizībai. Šajā darbā mēs izskaidrojam gēnu iekšējās regularitātes analīzes jaunos principus un analīžu metodiku, parādām dažu gēnu segmentu iekšējo regularitāti un nosakām to kvantu skaitliskās vērtības.

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Determination of the human epidermial growth factor precursor *hEGFP* gene repeat unit size by quantization of exon dimensions

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Abstract

A new method for determination of eventual regularity and periodicity of contemporary gene exon rows is suggested. The essence of this method lies in the common internal regularity of gene parameters measured by the number of nucleotides (nt) – mainly of gene intron coordinates and exon lengths (or dimensions). For this purpose it is necessary to calculate the prime number multipliers of all these gene parameters, to find a set of common ones, and, finally, to calculate the product of the set of revealed common multipliers. The obtained product shows the potential size (nt) of the gene repeat unit (or gene quantum). Here we demonstrate that the potential size of gene repeats can be determined using only exon dimensions. The method is used for *hEGFP*. The first exson of this gene includes a long and unregular 5'-untranslated region. This does not allow to determine the correct reference point for determination of regularity of intron coordinates. The size of the calculated primary repeat unit of *hEGFP* is 21 nt.

Key words: gene quantum, internal regularity of genes, repeat units.

Introduction

Continuing our studies of the origin and structural organization of genes and proteins (Chipens et al. 2005) we analyzed the precursor of human epidermal growth factor (hEGFP). Epidermal growth factor (EGF) is a 53 amino acid polypeptide that has many different biological properties – it is a potent mitogen for cells *in vitro* and stimulates proliferation and differentiation of cells *in vivo* (Carpenter, Cohen 1990). Human EGFP consists of 1207 amino acids (aa), and the corresponding cDNA coding part – of 3621 nucleotides (nt) (Bell et al. 1986). The precursor is processed to EGF in different tissues. The sequence of hEGFP includes not only EGF, but also eight EGF-like units (egf; Fig. 1) and near the carboxyl terminus a hydrophobic sequence characteristic for an integral membrane protein with its NH_2-terminus external to the cell surface (Doolitle et al. 1984).

The sequence of EGF has been reported to be similar to fragments of several blood coagulation factors, LDL-receptor and tumor growth factor (Doolitle et al. 1984; Bell et al. 1986). It is supposed also that tumor growth factor and EGFP arose as a result of common ancestral gene (Doolitle et al. 1984). According to our viewpoint, to investigate the relatedness of these and other bioregulators and to study the evolution of hEGFP structure itself (Bell et al. 1986), first of all it is necessary to know the potential sizes of

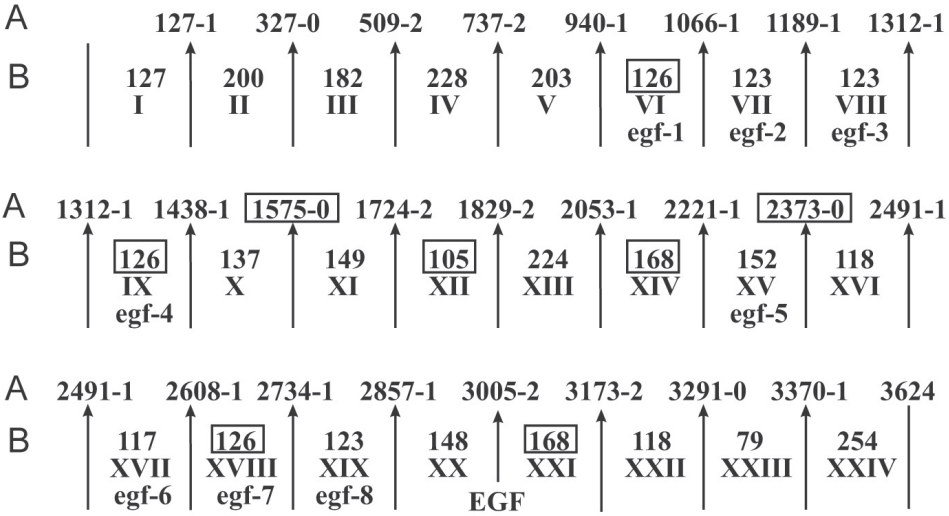

Fig. 1. Scheme of structural organisation and numerical parameters of exon length and intron coordinates of human *EGFP* cDNA, in accordance with the data in the GenBank X04571 and SwissProt P01133. A, arrows topped with intron coordinates (nt) and phases separate exons in the exon row. Intron coordinates are calculated as a sum of preceeding exons length. B, exon length (nt) and ordinal (Roman) numerals of exons. Parameters which can be expressed as multiples of the gene quantum (21 nt) are framed. EGF written in small letters (egf) denote the location of EGF-like amino acid sequences encoded by the given exon.

their repeat units expressed as the number of nt which is termed gene quantum (Ievina, Chipens 2003; Chipens et al. 2005). To quantize means to select a discrete set of values from a continuous range of possibilities.

Methods

The aim of our work was to determine the potential dimensions of the repeat unit (RU) of *hEGFP* gene and protein. For this purpose we use cDNA structure of *EGFP* and a scheme of exon-intron organisation described in the literature (Bell et al 1986). The first exon of the human *EGFP* contains a long unregular 5'-untranslated region about 451 nt (Bell et al. 1986), which due to potential indels in the nucleotide sequence do not allow to determine the exact reference point (the first nucleotide of the whole first exon). As a consequence, this does not allow to calculate correct intron coordinates. Therefore, to determine the potential sizes of *hEGFP* gene quantum and RU we used only the dimensions of exons expressed by number of nucleotides (Fig. 1).

Determination of RU dimension and a gene quantum is based on a model of gene precursor (highly repetitive and periodic nucleic acids) origin by oligonucleotide multiplication reactions (Ievina, Chipens 2003; Chipens et al. 2005). According to this model exons of gene precursors were formed of a whole number of RU, and introns were located between exon and RU boundaries and crossing the gene knot points (the 3'-terminal nucleotides of RU). The gene quantum characterize the size of primary RU of a

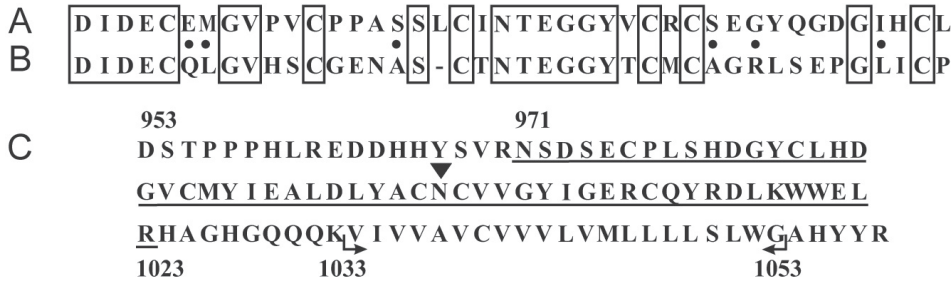

Fig. 2. Two EGP-like amino acid sequences and location of epidermial growth factor (EGF) of the *hEGFP* gene encoded protein. A, EGF-like sequence (amino acids 870-911 encoded by the exon XVIII). B, EGF-like sequence (amino acids 912-952 encoded by the exon XIX). Identical symbols of amino acid residues are framed. Common-root amino acids with identical second codon letters are denoted by dots. C, Amino acid sequence encoded by the human EGFP exons XX and XXI (amino acids 953-1058). EGF structure (971-1023) is underlined. The intron position which crosses the EGF sequence (intron coordinate 2878-1, Fig. 1) is shown by a filled arrowhead. The transmembrane domain (amino acid residues 1033-1053) is marked by broken arrows.

given gene. Both parameters – RU and a gene quantum – can be calculated on the basis of common prime multipliers of exon dimensions and/or intron coordinates. The method of calculations in detail is described in Chipens et al. (2005) and Ievina et al. (2006).

Results and discussion

The human *EGFP* gene consists of 24 exons and 23 introns (Bell et al. 1986). Determination of prime multipliers of exon dimensions allow to select six exons (VI, IX, XII, XIV, XVIII and XXI; Fig. 1) whose dimensions had common prime multipliers 3×7. For example $126 = 2 \times 3 \times 3 \times 7 = 6 \times 21$; $168 = 2 \times 2 \times 2 \times 3 \times 7 = 8 \times 21$; $105 = 3 \times 7 \times 5 = 5 \times 21$. Thus, the potential values of the primary RU and the gene quantum are 21 nt or 7 aa. As we suppose, the *EGFP* gene was formed by multiplication of the secondary RU – 126 nt long nucleotide – a hexamer of primary RU. Among the *EGF*-like sequences (egf; Fig. 1), there are also 123-nt-long repeats (exons VII, VIII, XIX), the size of which changed by intron sliding (drift) or one codon deletion.

The supposed secondary RU had homology of amino acid sequences, e.g., exons XVIII (126 nt/42 aa) and XIX (123 nt/41 aa, Fig. 2 A, B). The human epidermial growth factor (EGF, 159 nt/53 aa; Fig. 2 C) was formed during evolution, most likely from two neighbour exons XX and XI (Fig. 1, Fig. 2 C). The *hEGFP* nucleotide sequence contained also several exon dimensions and intron coordinates whose numerical parameters (as a result of intron drift during evolution) differed from those calculated on the basis of *hEGFP* gene quantum multiples by 1-2 nt, e.g., exon I ($6 \times \underline{21}+1$), exon XI ($7 \times \underline{21} + 2$), exon XX ($7 \times \underline{21} + 1$), intron 1724-2 ($82 \times \underline{21} + 2$), intron 2857-1 ($136 \times \underline{21} + 1$), intron 3005-2 ($143 \times \underline{21} + 2$), etc., supporting our viewpoint, that introns and exons were formed from one and the same regular precursor having identical size and structure of RU. The exon row length of the human *EGFP* gene, including the nontranslated parts of the 5'-terminal and 3'-terminal exons is 4871 nt (Bell et al. 1986), which differs from the gene quantum multiple ($232 \times 21 = 4872$) only by one nucleotide.

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Cilvēka epidermiāā augšanas faktora priekšteča gēna atkārtojuma vienības izmēru noteikšana kvantējot eksonu dimensijas

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Kopsavilkums

Mēs esam izstrādājuši jaunu metodi mūsdienu gēnu eksonu rindu iespējamās regularitātes noteikšanai. Metodes būtība ir kopējās iekšējās regularitātes noteikšana gēnu parametriem, galvenokārt intronu koordinātēm un eksonu garumiem (dimensijām), kas izteikti ar nukleotīdu skaitu (nt). Šim nolūkam jāatrod visu šo parametru pirmreizinātāji un no tiem jāatlasa kopējo faktoru kopa. Faktoru reizinājums parāda iespējamo gēna atkārtojuma vienības lielumu (nt). Šeit mēs demonstrējam, ka atkārtojuma vienības lielumu var noteikt, izmantojot vienīgi eksonu dimensijas. Metode ir pielietota cilvēka epidermiālā augšanas faktora priekšteča gēnam. Pirmais šī gēna eksons ietver garu neregulāru 5'-netranslēto rajonu. Tas neļauj izvēlēties pareizu atskaites punktu intronu koordinātu regularitātes noteikšanai. Aprēķinātais gēna pirmējās atkārtojuma vienības lielums ir 21 nt.

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What has the beaver got to do with the freshwater mussel decline? A response to Rudzīte (2005)

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In this journal, Rudzīte (2005) presented data on the status of the freshwater mussel *Margaritifera margaritifera* populations in Latvia. In it, the author claimed that the Eurasian beaver (*Castor fiber*) presented "a threat for the pear mussel population and therefore [its presence is] unacceptable in streams inhabited by *M. margaritifera*" (Rudzīte 2005; pp. 126). This message was repeated in the discussion, summary and abstract of the paper. This is a strongly worded condemnation of beavers in Latvia and therefore the author should present clear data to back-up this claim. However, the evidence presented by Rudzīte was at best anecdotal.

Rudzīte presented data from seven rivers in Latvia with some detailed information on the body lengths of mussels from two rivers (the Ludze and the Rauza). The latter two rivers were subdivided into two sections ('a' and 'b') of these, the upper section of the Ludze river (Ludze b) is the most heavily affected by beaver activities such as damming. Indeed, this section may be the only one that actually contained beavers at the time of study, however this was not clear from the manuscript. Rudzīte stated that beaver dams cause silting, warm water, increased eutrophication and shading. The inference presented by the author here is that beavers are intrinsically 'bad' for freshwater mussel populations. Indeed, it does seem logical that freshwater mussels would not survive in a beaver-pond since they prefer fresh flowing water and sandy substrate stabilised with large boulders (Vannote, Minshall 1982). However, no evidence to support this statement was provided. Of the various indicators of population quality, Rudzīte only presented one when comparing the 'beaver' and 'beaver-free' sections of the Ludze river (which as an aside, represents a sample size N = 1 and thus can only be regarded as anecdotal). The measure used is body length (a surrogate of age class; Rudzīte 2005; Fig. 1). From this presented data, there appears to be no evidence that the mussel populations in the beaver section of the river are in any worse condition than those in the beaver-free section. Indeed, the four smallest mussel specimens measured in Ludze river were all found in the beaver section. Small mussel specimens are generally considered a good sign since this represents recent recruitment into the population (Skinner et al. 2003; Rudzīte 2005). It seems then that Rudzītes ambiguous claim that beavers are bad for the endangered freshwater mussel is based upon the assumption that since beaver ponds are bad, so must the beaver be.

The last sentence might appear to be logical, but is in fact quite the opposite when we examine the behaviour of the beaver and the consequences of dam building in further

detail. Beavers damming behaviour is affected by habitat characteristics and thus not all beaver colonies will build dams (Collen, Gibson 2000). Furthermore, beaver activity will only affect a proportion of the stream length that can range from < 1 % to 50 %, the latter being recorded in the North American beaver (*C. canadensis*; Rosell et al. 2005). This means that habitat can be available for freshwater mussels between beaver ponds. Freshwater mussels require clean oligotrophic flowing water but severe flooding or very low summer flows have a detrimental impact on populations, as can high sediment loads (Skinner et al. 2003). By slowing river flow and retaining water at ponds, beaver dams can retain sediment, pollutants and nutrients as well as regulate flow so that the water quality downstream is improved, extreme water fluctuations are avoided and stream sediment load is reduced (Gurnell 1998; Rosell et al. 2005). Though the total impacts of a beaver dam will depend on the physical characteristics of each site, it would appear that beaver dams might actually benefit mussel populations, downstream at least.

Freshwater mussels have been experiencing a decline throughout their range (Skinner et al. 2003). Simultaneously, it would appear, both the Eurasian beaver and the North American beaver have been experiencing a rapid increase in range and population (Halley, Rosell 1998; Larson, Gunson 1983). A worrying correlation, one might think. However, the decline of the mussel has been evident in countries which currently have no wild beaver populations, such as the United Kingdom (Halley, Rosell 1998; Skinner et al. 2003). Moreover, beavers of either species were once found throughout temperate Eurasia and North America. One must assume that the range of the freshwater mussel overlapped that of the beaver significantly and that during this period, both species were able to coexist. Correlation is not causation and a much better study is required to determine the real influences of beaver dams on freshwater mussel population.

Acknowledgments

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An answer to Campbell

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R.D. Campbell (2006) in his paper argues on the inconsistency of data and statistics as well as disagrees on the blaming of European beaver *Castor fiber* in the decline of freshwater pearl mussel *Margaritifera margaritifera*. We thank Campbell for his comments on beavers. In addition, we must apologise on a mistake in the article (Rudzīte 2005). In the chapter "Results", labels A and B were in reverse places in Fig. 1.

Campbell (2006) does not use any information on the beaver population in Latvia. His arguments are partly based on the literature on the behaviour of Canadian beaver *Castor canadensis*. However, Canadian beaver has never been found in Latvia (Balodis 1990).

Why do we argue that beaver is a threat for freshwater pearl mussel in Latvia? The freshwater pearl mussel is a highly threatened bivalve. A 85 to 100 % decline in known populations in Central and Southern Europe has been estimated, which may be due to a number of factors, including increasing siltation and eutrophication of rivers, and also the recent declines in migratory salmonids upon which the larvae depend (Skinner et al. 2003). Most pearl mussel populations have lacked successful reproduction for 30 to 50 years. Formerly dense and connected populations have often become fragmented. However, a potential for recovery is offered by the longevity of this species, i.e. a lifespan of more than 100 years, together with the high reproductive potential of adult pearl mussels even in polluted rivers and in extreme old age (Geist 2005).

Freshwater pearl mussels live almost buried in coarse sand and fine gravel in clean, fast flowing and preferably unpolluted rivers and streams. For the successful reproduction of this mussel, the very specific requirements for juveniles are critical. For example, the nitrate level should not exceed 1.0 mg l^{-1}, phosphates 0.03 mg l^{-1} (Skinner et al. 2003), dissolved oxygen 6 mg l^{-1}, water temperature 21 °C, or 10 °C during the breeding period in spring; interstitial water chemistry should resemble the free running water nutrient levels (Moorkens et al. 2000).

Currently, there are only five rivers with viable pearl mussel populations in Latvia. The total number of individuals has been estimated up to 12 000 to 25 000 (Rudzīte 2004). More or less, beavers inhabit all these rivers.

When evaluating the impact of beavers on the pearl mussels, the following factors should be considered: the habitat quality in beaver dams; the water quality below the dams, as well the size and number of dams, and the changes of their locations.

On small rivers, beavers build dams. In floodplain areas, even a low dam can flood a large area. This is especially typically for Latvia where plain landscapes are characteristic. Beaver ponds store significant amounts of nitrogen in sediments. The organic matter is increased also with fallen wood, which is a long-term source of nutrients to the pond water and outflow. Even anaerobic conditions can be reached in beaver ponds (Rosell et al. 1995). Such conditions are incompatible with the recruitment of young pearl mussels, but

the oldest pearl mussels can survive also in dams; this is seen also in Fig. 1 of the discussed paper (Rudzīte 2005).

The influence of beaver dam continues also in areas downstream of the beaver dams where high total organic nitrogen and total phosphorus, and high water temperature can be observed (Rosell et al. 2005). As a result, species composition of downstream sections differs from upstream sections – the macroinvertebrate fauna downstream of the beaver dam is quite similar to the dam itself (Hering et al. 2001). These effects are site-dependent and decrease shortly after the dam (Rosell et al. 2005). A longer-lasting effect is the reduced water discharge because the evaporation in the summer is enhanced by the enlargement of the open water area. This situation can be observed in hot summers in Latvia when beaver-inhabited small rivers become a chain of ponds with no stream connecting them (Rudzīte, unpublished data).

Often, a series of dams are built on small streams. The location of dams changes with the time – old dams become abandoned and beavers build dams in previously intact sites. So, there is no asylum for pearl mussels in areas between the dams.

The above has been observed in River Pērļupe, where one of the most well known and monitored populations of freshwater pearl mussels in Latvia is located. The pearl mussel population in whole river was estimated as 2000 in year 1977 (number based on calculations and not on direct counting; Krišāns 1977), 1400 in year 1984 (here and further – direct countings). Beavers settled in this river between the years 1987 and 1992. Currently, there are no beavers in Pērļupe, the number of pearl mussels is estimated up to 400, and they all are aged. It is expected that this population will die-out within five to ten years because of lack of juveniles (Rudzīte 2001).

Here we give additional information on the studied rivers and pearl mussel populations. River Rauza ir 56 km long, it's inclination is 144 m (2.6 m per km). The catchment area including tributaries is 263 km^2 (Zīverts 1997) Here, 200 to 250 beavers were counted in 2005 (Valka Forestry, unpublished data). There are no beaver dams on the Rauza but beavers live in riverbanks and in tributaries. Along the river, there are a few rural settlements and one small swine farm. Pearl mussel was found in one 7800 m long section of this river; the number of individuals is approx. 3000 (Rudzīte 2005).

River Ludze (tributary of Rauza) is 24 km long, catchment area 80 km^2 (Zīverts 1998), 66 km^2 forests (Rudzīte, unpublished). Upstream in the river there is a section of 17 km with almost a continuous chain of beaver dams. This area is mainly open, and a river has a wide floodplain. The downstream area is beaver-free, relatively pristine, and generally covered with coniferous forests. In this part 20 000 pearl mussel individuals are located. This is considered as the largest and most vital pearl mussel population in Latvia, and this is the only one location where young pearl mussels are found.

R.D. Campbell (2006) argues that pearl mussels and beavers can live together as they did in ancient times. In ancient times, European pearl mussel was one of the most abundant bivalve in rivers (Skinner et al. 2003). Later, both beavers and pearl mussels suffered from overexploitation because of hunting (Rosell et al. 2005) and pearl fishing (Skinner et al. 2003). In 20th century, reintroduction of beavers was started in Europe (Rosell et al., 2005) and also in Latvia where the first beavers were released in 1927 (Balodis 1990). The number of beavers reached 37 000 in 1990 (Balodis 1990), and now their number is estimated at 72 000 according to the monitoring of State forest service, but only forested areas were surveyed for beavers (State forest service, unpublished data).

However, pearl mussels did not recover because their colonies were small, and in the 20th century the continuous eutrophication of rivers due to agricultural development was ongoing.

Currently, there are very many beavers in Latvia, and they are not threatened. However, there are very few pearl mussel populations, and they are small and threatened mainly by river eutrophication, which is partly caused also by beaver. Considering that there are only a few kilometre-long sections of pearl mussel populations, there is a high probability, that beavers can dam these sections just by chance, and therefore the control of beavers is advised in these areas.

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