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Prevalence of various *Borrelia burgdorferi* sensu lato species in *Ixodes* ticks in three Baltic countries

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Abstract

Borrelia burgdorferi sensu lato, a tick-borne spirochete, is the causative agent of Lyme disease, the most prevalent vector-borne disease in Europe and United States. However, the incidence of this disease is variable and the clinical picture depends on the pathogen species. The infectivity of *Ixodes* ticks with *Borrelia*, was 46 % and 35 % in 2000 and 2001 in Latvia, respectively, and 14 % in 2002 in Lithuania, assessed by nested polymerase chain reaction (PCR) amplification of the plasmid OspA gene fragment of *Borrelia*. PCR-restriction fragment length polymorphism (RFLP) analysis of the 16S-23S (*rrs-rrlA*) rRNA intergenic spacer was used for typing of *Borrelia* directly in ticks. Species-specific primers and subsequent sequences analysis were used as another approach for *Borrelia* species typing. All three clinically relevant *B. burgdorferi* sensu lato genospecies (*B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto) were detected in the ticks collected in Latvia. The same result was obtained earlier in Estonia. *B. valaisiana*, a possible infectious agent of Lyme borreliosis, was detected only in Latvia. Only *B. afzelii* and *B. garinii* species were detected in ticks from Lithuania. Different subspecies were also identified. This study demonstrates the predominance of the genospecies *B. afzelii* in all three Baltic countries, and the circulation of different *B. burgdorferi* sensu lato subspecies in the environment. This knowledge might have a significant importance for monitoring of Lyme disease in Europe.

Key words: Baltic region, *Borrelia burgdorferi*, Lyme disease, tick.

Introduction

Lyme disease (LD, Lyme borreliosis) is a multisystem and multistage infection caused by tick-borne spirochetes of the *Borrelia burgdorferi* sensu lato genogroup.

Three species of this group have been confirmed as pathogenic for humans. These include *Borrelia burgdorferi* sensu stricto (distributed mostly in North America), *Borrelia afzelii* (distributed in Western Europe, Central Europe and Russia), and *Borrelia garinii* (distributed in Europe, Russia and northern Asia). Symptoms of LD include arthritis, carditis, dermal symptoms and neurological symptoms, usually preceded by erythema migrans, a characteristic rash that begins days to weeks and spreading the bite site (Steere 2001). Some new cutaneous (alopecia) and ocular manifestations recently have been described (Schwarzenbach et al. 1998; Kostler et al. 1999; Pleyer et al. 2001;

Krist, Wenkel 2002), and a solitary borrelial lymphocytoma was reported from Slovenia (Maraspin et al. 2002). LD has become the most common vector-borne disease in North America and Eurasia (Wang 1999).

Lyme borreliosis in all locations is transmitted by ticks of the *Ixodes ricinus* complex (Lane et al. 1991; Spielman 1994). These ticks have larval, nymphal, and adult stages, they require a blood meal at each stage. In Europe, the principal vector is *I. ricinus*, and in Asia it is *I. persulcatus*. Notably, both of these species are common in Latvia and Estonia, and in the Baltic region of Russia (St. Petersburg region). *I. ricinus* is the main vector of Lyme disease in Lithuania.

PCR-based methods have became the most popular methods in detection and typing of *Borrelia burgdorferi* sensu lato in different biological samples and clinical materials in the world. Sensitive nested PCR method targeted *B. burgdorferi* specific OspA gene was used for the detection of the pathogen in field-collected ticks in our study. Molecular typing of *B. burgdorferi* from infected ticks was performed by restriction fragment lenght polymorphism (RFLP) analysis of PCR-amplified fragments of 16S-23S (*rrs-rrlA*) rRNA intergenic spacer. For PCR-RFLP analysis the restriction enzyme *Hinf* I was used. This typing method is fast and sensitive, and allows the differentiation of *B. burgdorferi* species directly in tick material without the need for isolation and culture of the microorganism.

The prevalence rates of *B. burgdorferi* sensu lato species in ticks was suspected as a major factor in assessing the transmission risk of Lyme borreliosis in endemic areas (Matuschka et al. 1992; Hubalek et al. 1996). The aim of this study was to perform molecular typing of *B. burgdorferi* sensu lato, to investigate the prevalence of clinically relevant *B. burgdorferi* species in host-seeking *Ixodes* tick populations in Latvia and Lithuania, and to compare these data to those obtained in Europe. This type of data can show the relative risk of infection with Lyme disease in the Baltic countries and offer a basis for comparative clinico-epidemiological studies of Lyme borreliosis in Europe.

Materials and methods

Collection of ticks

Questing ticks were collected by flagging of low vegetation in Latvia in 2000 and 2001 and in Lithuania in 2002. A total of 210 and 450 ticks were collected in different regions of Lithuania and Latvia, respectively. All ticks were identified for species, sex and stage; then each tick was placed in a separate plastic tube and frozen at -20 °C until further use.

DNA preparation

Each tick was mechanically crushed with a sterile plastic rod in a tube with 100 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6), and DNA was extracted by phenol-chloroform extraction method. Briefly, buffer with tick was extracted with a 100 µl phenol/chloroform/isoamyl alcohol mixture (25:24:1, pH 8.0) by vortexing and subsequent centrifugation to separate DNA from proteins. The upper layer after centrifugation was transferred to a new tube and the DNA was reextracted with 100 µl chloroform by vortexing and centrifugation. The upper layer was again transferred to a new tube, and heated for 15 minutes (90 °C). Aliquots were frozen and stored at -20°C until further use.

Reference DNA

DNA samples isolated from five reference strains (*B. burgdorferi* sensu stricto B31, *B. afzelii* ACA I, *B. afzelii* VS-461, *B. garinii* Ip90, *B. valaisiana* VS-116), kindly donated by S. Bergstrom, Umeå, Sweden, and D. Postic, Pasteur Institute, France, were used as positive controls in all PCR-based methods.

B. burgdorferi *detection by PCR amplification*

DNA amplification targeting the *OspA* gene located in linear plasmid lp54 was performed as described by Priem et al. (1997); reaction conditions were modified. Briefly, a 50-μl PCR reaction volume contained a 3 μl aliquot of isolated DNA, 100 mM (each) deoxynucleoside triphosphates (NBI Fermentas, Lithuania), 1.5 U of Taq DNA polymerase (NBI Fermentas, Lithuania), and 30 pmol of each primer. First-round amplification employed primers PrZS7 (5'-GGGAATAGGTCTAATATTAGCC-3'; positions 18-39 of the *OspA* gene) as the forward primer and Osp5 (5'-CACTAATTGTTAAAGTGGAAGT-3', positions 660-682 of the *OspA* gene) as the reverse primer. The amplification profile for the first-round PCR consisted of 35 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 20 s, and extension at 72 °C for 60 s. Three microlitres of the first-round PCR product was employed as a template in a second-round PCR with primers Osp6 (5'-GCAAAATGTTAGCAGCCTTGAT-3'; positions 54-75 of the OspA gene) as the forward primer and Osp8 (5'-CGTTGTATTCAAGTCTGGTTCC-3', positions 423-444 of the *OspA* gene) as the reverse primer. The amplification profile for the second-round PCR consisted of 25 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 20 s, and extension at 72 °C for 30 s. PCR amplification resulted in a 391-bp product. Amplicons were visualized on a 1.5% agarose gel stained with ethidium bromide.

To monitor for the occurrence of false-positive PCR results, negative controls were included during extraction of the tick samples: one control sample for each twenty tick samples. Negative and positive control samples were included each time that the PCR was performed. To avoid cross-contamination and sample carryover, pre- and post-PCR sample processing and PCR amplification were performed in separate rooms, and plugged pipette tips were used for all fluid transfers.

B. burgdorferi *typing by 16S-23S rDNA spacer PCR-RFLP*

OspA gene-positive DNA samples were used in further analysis. Nested PCR targeting the 16S-23S (*rrs-rrlA*) rDNA spacer region was performed as described by Liveris et al. (1999). Ten-microlitre aliquots of the nested-PCR amplification products were subjected to RFLP analysis by digestion with 2 U of ***Hinf* I** (NBI Fermentas, Lithuania) according to the manufacturer's instructions. *Hinf* I-digested fragments were analyzed by electrophoresis in a 1.5 % agarose gel stained with ethidium bromide.

Species-specific PCR

Species-specific PCR targeted 16S rRNA gene with *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* specific primers were performed as described elsewhere. (Liebisch et al. 1998)

PCR-RFLP of 5S-23S rDNA spacer amplicons

In order to compare *B. garinii* strains detected in ticks from Latvia to those typed in

Europe, the 5S-23S rDNA PCR-RFLP typing method was used as described elsewhere (Postic et al. 1994).

DNA sequencing

PCR amplicons were purifying with a DNA extraction kit (NBI Fermentas, Lithuania) according to the manufacturers instructions. For DNA sequencing reactions, the fluorescence-labeled dideoxynucleotide technology was used (Perkin-Elmer, Applied Biosystems Division). The sequenced fragments were separated, and data were collected on an ABI automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division).

Nucleotide sequence accession number

The 5S-23S rDNA intergenic spacer sequence of the *B. garinii* that was found in this study is available in the GenBank database under the accession number AY163784.

Statistical methods

Statistical insignificant differences were calculated using the x^2 test.

Results

Prevalence of Borrelia *in ticks*

Altogether, 450 ticks collected in different regions of Latvia (years 2000 and 2001) and 204 ticks collected in different regions of Lithuania (year 2002) were analyzed. The overall prevalence of *Borrelia* in ticks in Latvia in the year 2000 was 46 % which was significantly higher ($P<0.05$) than in 2001 (35 %). The overall prevalence of *Borrelia* in ticks in Lithuania was 14 %, significantly lower ($P<0.05$) than in Latvia. In Table 1 the obtained results are compared with those from Estonia in 1999.

Typing of B. burgdorferi *directly in ticks by RFLP analysis*

The *rrn* cluster of most *B. burgdorferi* sensu lato strains contains a single copy of 16S rRNA (*rrs*) and tandem repeated 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (Fukunaga et al. 1992; Schwartz et al. 1992; Gazumyan et al. 1994; Ojami et al. 1994). The rDNA gene cluster is located at the center of the linear chromosome and is arranged in the following order: *rrs-rrlA-rfA-rrlB-rrfB*. The *rrs-rrlA* intergenic spacer is about 3,2 kb in *B. burgdorferi* sensu stricto and 5 kb in *B. garinii* and *B. afzelii* (Schwartz et al. 1992;

Table 1. The prevalence of different *B. burgdorferi* genospecies in Latvia, Lithuania and Estonia. *, Data from this study. **, Data from T. Priikk et al. 1999

Country	No. of ticks tested	*Borrelia* positive ticks (%)	% (No.) of ticks infected by different genospecies			
			B. afzelii	*B. garinii*	*B. burgdorferi* sensu stricto	*B. valaisiana*
Lithuania*	204	13.7	68 (19)	18 (5)	0	0
Latvia*	450	46.0 (year 2000) 34.9 (year 2001)	76 (80)	18 (19)	2 (2)	2 (2)
Estonia**	422	5.5	65 (15)	17 (4)	13 (3)	0

Table 2. The number of cases of Lyme disease in North-European countries. Data are from *EpiNorth - Bulletin of the Network for Communicable Disease Control in Northern Europe*

Country or region	1999		2000		2001	
	Total No	Per 100000 inhabitants	Total No	Per 100000 inhabitants	Total No	Per 100000 inhabitants
Norway	146	3.3	138	3.1	124	2.8
Finland	404	7.8	895	17.2	691	13.3
Estonia	321	22.2	601	43.8	342	25.0
Latvia	281	11.5	472	19.4	379	16.0
Lithuania	766	20.7	1713	46.3	1153	33.0
St. Petersburg region	265	5.6	541	11.3	323	6.7

Gazumyan et al. 1994; Ojami et al. 1994). We used the typing method based on the PCR-RFLP analysis of a highly variable 16S-23S (*rrs-rrlA*) rDNA spacer previously described by Liveris et al. (1999). This method can be performed rapidly with small DNA amounts, thus obviating the need for culture isolation. Amplification of the partial *rrs-rrlA* spacer, followed by digestion with *Hinf* I produces species-specific RFLP patterns.

A total of 135 and 28 ticks from Latvia and Lithuania, that were positive after *B. burgdorferi* detection, respectively, were employed for further analysis. The amplicon was obtained from 106 and 24 samples, respectively. PCR-RFLP species-specific pattern analysis showed that in Latvia 76 % of samples belonged to *B. afzelii* (80 samples), 18 % - to *B. garinii* (19 samples), 2% - to *B. burgdorferi* sensu stricto (2 samples), and 2 % were *B. valaisiana* (2 samples, Table 1). Results were confirmed by species-specific PCR analysis (data not shown). Two different *B. garinii* subtypes were identified. Those samples differing from the reference strain Ip90 were employed for the 5S-23S rDNA PCR-RFLP analysis and sequencing of the 5S-23S rDNA PCR amplicon. The obtained sequence was compared with data in the GenBank. The results confirmed these samples as *B. garinii* and closely related to the NT29 isolate from Russia. In Lithuania, 68 % (19 samples) belonged to *B. afzelii*, 18 % (5 samples) to *B. garinii*.

Discussion

In Europe, Lyme borreliosis is widely established in forested areas (Steere 2001). The highest reported frequencies of the disease are in middle Europe and Scandinavia, particularly in German, Austria, Slovenia, and Sweden. The infection is also found in Russia, China, and Japan (Steere 2001). Epidemiological data presented in *EpiNorth - Bulletin of the Network for Communicable Disease Control in Northern Europe* shows that the status of Lyme disease in the three Baltic countries is endemic (Table 2). The number of cases of Lyme disease in this region is higher than in neighbouring Northern Europe countries excepting the St. Petersburg region of Russia.

We investigated the prevalence of *B. burgdorferi* sensu lato genotypes in questing ticks from Latvia and Lithuania, and compared the results with those obtained in Estonia and the Baltic region of Russia (St. Petersburg region). The study shows that there is a

considerable risk of contracting a borrelia infection in all three Baltic countries, and that a large human population is at risk. The extremely high percentage of infected ticks in 2000 in Latvia correlates with the high number of registered cases of Lyme disease that year (Table 2).

Not all strains of the described *Borrelia* species are pathogenic for humans. Currently, only *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* have been cultured from Lyme disease patients in Northern America and Europe (Wang 1999). However, recently two other species, *B. bissettii* and *B. valaisiana*, were reported to have been cultured from a LD patient in Europe (Schaarschmidt et al. 2001; Maraspin et al. 2002). DNA specific for *B. valaisiana* has been detected by PCR from patients with LB (Probert et al. 1995), and *B. valaisiana* specific antibodies have been determined in patients sera (Ryffel et al. 1999). Therefore, we can expect that all *B. burgdorferi* sensu lato species mentioned above can cause Lyme disease in Europe.

It has been proposed that the different species of *B. burgdorferi* sensu lato are associated with distinct clinical manifestations of LB: Lyme arthritis with *B. burgdorferi* sensu stricto infection, neuroborreliosis with *B. garinii* infection, and acrodermatitis chronica et atroficans (ACA) with *B. afzelii* infection (Wang 1999). Litherature indicates that the clinical features of Lyme borreliosis may depend on the species of the causative agent. Nevertheless, in everyday clinical practice, the determination of *Borrelia burgdorferi* species cannot be made. Therefore, the analysis of *Borrelia* species in ticks and interpolation of their relative incidence to the clinical cases is the only single possibility to foresee the pathogenesis of definite clinical cases today. The latter is significant for elaboration of prophylactic measures (including vaccination with the single commercially available vaccine against the *B. burgdorferi* sensu stricto) and for therapeutic strategy in Lyme borreliosis cases in the Baltic region.

This study shows that *B. afzelii* is the most prevalent genospecies of *B. burgdorferi* complex in Latvia and Lithuania. *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* have also been detected in questing ticks from Latvia. This finding is in agreement with a previous study by Kurtenbach et al. (2001), but our study covered a wider region. However, in the former study *B. valaisiana* was detected in 18 % of ticks compared to 2 % in our study. This may be explained with the differences of the collection strategy of ticks, since only ticks from the Riga region were included in study by Kurtenbach et al. (2001).

In summary, *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto and *B. valaisiana* species were detected in *Ixodes* ticks in Latvia. All of these four genospecies were detected also in the St. Petersburg region of Russia (Alekseev et al. 2001). In Estonia, *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto were detected in questing ticks (Postic et al. 1997; Prükk et al. 1999). Only *B. afzelii* and *B. garinii* were detected in ticks from Lithuania. The differences might be explained by an uneven distribution of *B. burgdorferi* sensu lato in Europe, or simply by an unsufficient tick sample size investigated.

The most prevalent *B. afzelii* and *B. garinii* genospecies are the most probable aetiological agents responsible for the more than an 2000 cases per year of Lyme disease in the three Baltic countries.

5S-23S rDNA spacer amplicon sequence analysis showed that the second *B. garinii* subtype is closely related (99 % similarity, BLAST) to the *B. garinii* NT29 variant. The NT29 subtype is most frequently isolated by culture from *I. persulcatus* ticks in Russia

and Estonia, and 7 out of 8 isolates from human skin biopsies in Russia were identified as this type (Postic et al. 1997). Interestingly, this variant, frequently isolated in Japan (Fukunaga et al. 1993; Fukunaga et al. 1996), is absent in Western and Central Europe (Postic et al. 1997). Sequence analysis of variable regions could be a useful tool for understanding the evolution of different species and subspecies, and probably could help to explain the different pathogenesis of different *B. burgdorferi* species. Our results also clearly show that the distribution of different *B. burgdorferi* genospecies in Europe depends on the distribution of the *Ixodes* vector. With the exception of the most northern regions, the distribution area of ixodid ticks covers all of Europe. The study in Finland demonstrates that even very urban parks can serve as habitats for *I. ricinus* ticks (Junttila et al. 1999). Interestingly, Postic et al. (1997) suggested that the absence of *B. burgdorferi* sensu stricto from all regions where *I. persulcatus* is the single vector could be explained by the inability of *I. persulcatus* to harbour and to transmit this genospecie. Further investigations of this observation are required, and knowledge of the epidemiology of the LB vectors and their infestation rate is essential for understanding the risk of LB in a local setting.

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Dažādu *Borrelia burgdorferi* sensu lato sugu prevalence *Ixodes* ērcēs trijās Baltijas valstīs

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Kopsavilkums

Laima slimību (Laima boreliozi) izraisa *Borrelia burgdorferi* sensu lato spirohetas, ko dabā pārnēsā ērces. Pētījumā ar 16S-23S rDNA starpgēnu rajona polimerāzes ķēdes reakcijas-restrikcijas fragmentu polimorfisma analīzes (PCR-RFLP) metodi tika noteiktas tās *Borrelia* sugas, kas cirkulē Latvijā un Lietuvā. Dati tika salīdzināti ar Igaunijas un Krievijas (Sanktpēterburgas apgabals) datiem. Visas trīs *B. burgdorferi* sensu lato sugas (*B. afzelii*, *B. garini* un *B. burgdorferi* sensu stricto), kas šobrīd tiek uzskatītas par galvenām Laima slimības izraisītājām, ir atrastas Latvijas ērcēs. Līdzīgs rezultāts tika ziņots no Igaunijas. Lietuvā ērcēs tika atrastas tikai *B. afzelii* un *B. garini sugas*. Šis pētījums skaidri parāda *B. afzelii* sugas dominēšanu visās trīs Baltijas valstīs. Pētījumi par dažādu *Borrelia* sugu cirkulāciju apkārtējā vidē var būt ļoti būtiski Laima slimības monitoringam Eiropā.

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Evaluation of the insertion-sequence-6110-based polymerase chain reaction for detection of pathogenic mycobacteria

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Abstract

The insertion sequence IS6110 is a mobile genetic element typical for *Mycobacterium tuberculosis* complex (MTC) bacteria. Due to its narrow host range, this insertion element can be used for rapid identification of pathogenic mycobacteria. The aim of this study was to evaluate the diagnostic value of the IS6110 element in comparison with common molecular and microbiological methods. A total of 170 clinical specimens from tuberculosis (TB) patients were tested for the presence of IS6110 by polymerase chain reaction (PCR). Detection of MTC by IS6110–PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison to commercial LCx assay (27.6 %) and to the commonly used methods for estimating bacteria growth: BACTEC system (20.6 %) and on Lowenstein-Jensen medium (15.3 %). IS6110-PCR was shown to be a rapid and specific method for identification of the *Mycobacterium tuberculosis* complex. It was found to be especially useful for confirming diagnosis in cases of smear- and culture-negative results, when the clinical expansion of tuberculosis was obvious.

Key words: Identification, insertion sequence 6110, *Mycobacterium tuberculosis* complex, polymerase chain reaction.

Introduction

Mobile genetic elements like insertion sequences (IS) and transposons are found in bacteria, archaebacteria and eukaryotes. Insertion sequences are DNA fragments encoding their own ability to transpose within the genome. The function of most mobile genetic elements is unknown and they are usually considered to be genome parasites. In the genus *Mycobacterium*, there are three groups of IS, which are usually host specific. IS are between 880 bp and 2260 bp long and are found in one to more than 20 copies in the chromosome (Guilhot et. al. 1999). One of those, a 1361 bp long insertion sequence named IS6110, first described by Thierry et al (1990), is present in the *Mycobacterium tuberculosis* complex (MTC) only. MTC contains four genetically and serologically related pathogens causing disease in human and in cattle: *M. tuberculosis*, *M. bovis*, *M.*

africanum and *M. microti*. The majority of investigated MTC strains contained between 5 and 20 copies of IS6110 located in various places of the genome (van Soolingen et al. 1993).

Recent achievements in molecular microbiology such as complete genome sequencing of the *M. tuberculosis* H37Rv strain opened new perspectives in the study of mycobacteria (Cole et al. 1998). At the same time, a dramatic increase of tuberculosis (TB) incidence worldwide in the last decades induced an urgent need for new molecular markers enabling rapid detection of MTC, a mean causative agent for TB. It is known that smear examination may not be effective in non-pulmonary, pediatric or paucibacillary disease, or where HIV is prevalent (Rigouts, Portaels 2001). Recently developed molecular methods based on detection of mycobacterial proteins (protein antigen B), 16S rRNA or DNA fragments, are dissolving this problem. Of these methods, the amplification of insertion sequences is most commonly used. IS6110-based PCR enables detection of nucleic acids from MTC directly in clinical specimens: sputum, bronchial washings, urine etc. (Eisenach et al. 1988; Hance et al. 1989). IS6110-PCR is a more rapid, sensitive and specific in comparison to other methods routinely used in clinical laboratories; such as smear microscopy, and culturing on solid and on liquid media (American Thoracic Society Workshop 1997). Detection of MTC in cerebrospinal fluid (CSF) from patients suspected to have tuberculous meningitis is of great value since cultivation and microscopy may often fail to reveal a low amount of bacteria.

In this study, IS6110 was chosen due to several reasons. Firstly, a narrow host range of the IS6110 allows use of this element for precise identification of MTC. Secondly, it is usually present in more than five copies and can be used as a molecular marker for strain differentiation in epidemiological studies (van Soolingen et al. 1993). The aim of our study was to evaluate the sensitivity and specificity of the IS6110 amplification method on smear-negative samples, in comparison with smear microscopy and culturing on solid Lowenstein-Jensen medium. We expected a higher sensitivity of IS6110-PCR in comparison to commercially available systems for TB detection and MTC culturing, such as the Abbott LCx assay and BACTEC. LCx assay is a DNA-related TB diagnostic system based on protein antigen b gene fragment amplification (Andresen, Hansen 1989), but perhaps, is not as sensitive as IS6110-PCR since only one copy of the antigen b gene is present in the MTC genome. BACTEC is a precise radiometric system for mycobacteria growth detection, but it usually requires several weeks to grow a sufficient amount of bacteria.

Materials and methods

In total more than 250 specimens of bronchial washings (76 %), sputum (11 %), cerebrospinal fluid (4.3 %), urine (3.5 %), smear (2.6 %) and stomach washings (2.6 %) were obtained from Latvian patients admitted in 1998-1999 to the State Centre of Tuberculosis and Lung Diseases with suspected and confirmed TB. The patients had clinical symptoms of lung diseases or had suspicion of pulmonary/extrapulmonary tuberculosis shown by clinical and/or X-ray examination. Each patient was represented by one specimen or, in several cases, by various specimens from different locations. The specimens were directed for TB detection by the Abbott LCx (Ligase Chain Reaction) commercial kit (Abbott laboratories, Illinois, USA), mostly in cases when bacteria growth

was negative on the BACTEC system (Becton-Dickinson, Sparks, USA) or with repeated smear-negative bacterioscopy results. In our study, 157 smear-negative specimens were selected, allowing to test whether IS6110-PCR is more sensitive in comparison to the commonly used methods. A small control group of 13 specimens with smear-positive bacterioscopy results was included. The results of our study were interpreted in the context of the clinical findings, TB history and the efficiency of chemotherapy.

All specimens were collected and processed by the standard *N*-acetyl-L-cysteine-NaOH procedure (Kubica et al. 1963). Smears were analyzed by fluorescent microscopy and the corresponding samples were inoculated on solid (Lewenstein-Jensen) and liquid (BACTEC) mediums. The LCx assay was performed at the Centre of Tuberculosis in accordance with the manufacturer's recommendations (Ausina et al. 1997). DNA isolates for LCx reaction contained 0.5 ml LCx buffer, 27 mM $MgCl_2$, NaN_3 and glass beads. After the LCx analysis IS6110-PCR was directly applied to samples. The DNA concentration method (Boom et al. 1990) was used for extraction of DNA from several smear-negative specimens of CSF and bronchial washings received in year 2001.

The 245 bp fragment (from 633 to 877 nucleotide positions of the IS6110 sequence) was amplified in house using primers 5'-CGTGAGGGCATCGAGGTGGC-3' and 5'-GCGTAGGCGTCGGTCACAAA-3' (Hermans et al. 1990). Initially, the nested PCR method was used. At the first step, a 1224 bp fragment (from 47 to 1270 nucleotide positions) of IS6110 was amplified and was used as a reaction template at the second step, where a 245 bp fragment was produced. Later, the first step of nested PCR was omitted since it did not show any advantages and the 245 bp fragment was directly amplified from specimens. Five μl of DNA isolate in LCx buffer was added to the PCR mixture [containing PCR buffer with $(NH_4)_2SO_4$, 200 μM of each dNTPs, 10 pmoles of each primer and 1 U of Taq DNA Polymerase 9MBI Fermentas, Vilnius, Lithuania)] to a final volume of 25 μl. The optimized cycling protocol was used a *Progene* cycler (Techne, Cambridge, England) with initial denaturation at 95 °C for 2 min, followed by 40 cycles (95 °C for 15 s, 65 °C for 20 s, 72 °C for 1 min) and a final extension (72 °C for 10 min). PCR products were analyzed by electrophoresis in 2 % agarose gel (BioRAD, Hercules, USA) and were visualized with ethydium bromide. A positive PCR signal indicated the presence of the MTC genome. In each set of reactions, one negative control and one positive control (DNA from the *M. bovis* BCG vaccine strain) were included. The reaction specificity was confirmed by digestion with PvuII (MBI Fermentas, Vilnius, Lithuania) and by hybridization with an appropriate ^{32}S-labeled fragment. Testing was repeated on specimens with negative amplification results, with an external addition of control DNA from the *M. bovis* BCG strain in order to evaluate the presence of a PCR inhibitor.

Results

The detection limit of IS6110-PCR, estimated by amplification of serial dilutions of DNA from the *M. bovis* BCG strain containing only one copy of IS6110, was 5 fg per probe. This amount of DNA can be isolated from two bacterial cells, meaning that our PCR method is able to detect at least two bacteria, each containing one copy of IS6110. The fact that MTC usually contains more than one IS6110 copy increases the detection level of our in house method.

The comparative results of MTC detection in 170 specimens are shown in Table 1.

Table 1. Comparison of the in house IS6110-PCR method and TC clinical laboratory results obtained for 170 specimens. *, number of PCR false positive after adjustment with LCx results and samples suspected in cross contamination

Method used	MTC positive (total n=170)	Discrepancy (PCR-positive and 1-4 method-negative)
Bacterioscopy	13 (7.6 %)	56 (32.5 %)
Growth on Lowenstein-Jensen medium	26 (15.3 %)	39 (22.9 %)
Growth in BACTEC system	35 (20.6 %)	44 / 25* (25.8 % / 14.7 %*)
LCx assay	47 (27.6 %)	32 (18.8 %)
IS6110-PCR	69 (40.6 %)	-

Detection of MTC by IS6110-PCR revealed the highest number of MTC positive specimens (40.6 %) in comparison to LCx assay (27.6 %) and to the commonly used methods for estimation of bacteria growth: on BACTEC (20.6 %) and on Lowenstein-Jensen (15.3 %) mediums. Smear microscopy (bacterioscopy), which is reliable in detecting MTC bacteria only when present in high amounts, was the least sensitive with 13 (7.6 %) MTC positive specimens. This group of 13 specimens was MTC positive by all of the remaining methods (100 % specificity for smear-positive specimens).

IS6110-PCR was the most sensitive method in comparison to LCx assay and to other clinical laboratory methods. However, the lack of a "golden standard" for amplification methods does not exclude false positive results. Therefore, cultivation on BACTEC radiometric liquid system is still considered to be the most reliable tool for detection of MTC (Nordhoek et al. 1994). Sensitivity and specificity of PCR was estimated on the basis of BACTEC results (Elder et al. 1997). Thus, 91 PCR negative results and 26 PCR positive results (together 68.8 %) were in accordance with BACTEC system, and were considered as true negative and true positive, respectively. PCR gave positive results in 44 (25.8 %) cases. Of 44 PCR-positive results 12 were correlated with LCx assay results, but not with BACTEC results, a method internationally considered as a standard method for detection of *M. tuberculosis*. Therefore, these PCR results should be considered as false positive, and the overall specificity for IS6110-PCR estimated as 67.0 %. After charging of 32 PCR and LCx discrepant results plus seven suspected cross-contaminated samples, the total number of false positive results was 25 and the PCR specificity was 79.0 %. Nine (5.2 %) discrepant PCR-negative and BACTEC-positive results were observed, thus the estimated overall PCR sensitivity was 72 %. There were nine (5.2 %) discrepant PCR-negative and LCx-positive cases and four (2.3 %) discrepant PCR negative and Lowensten-Jensen culture-positive cases. False positive LCx or BACTEC results may have been caused by laboratory contamination. False negative results may be explained also by the absence of IS6110 fragment in the *M. tuberculosis* pathogen also. Inhibition of amplification reaction by remains of clumps, blood and urine present in a specimen is another probable cause for false negative PCR (Forbes 1997).

Clinical material isolated from CSF was studied as in this case there was an obvious requirement for rapid and precise diagnostics. In our study, seven smear and culture-negative CSF specimens showed negative results by both amplification methods (IS6110-

Table 2. Evaluation of PCR-based methods applied on clinical material isolated by Boom method. SCF, cerebrospinal fluid; L, bronchial washing; -, negative PCR signal; +, positive PCR signal, indicating the presence of MTC genome; ++, strong positive signal; +w, weak positive signal; WT, wild type (no resistance encoding mutation found). H37Rv strain was used as a PCR positive control. B and C types are different groups of genetic patterns, revealed by molecular typing of MTC bacteria

Clinical number	IS6110-PCR	Rifampin suscept. by *Inno-LiPA* kit	Rifampin suscept. by *rpoB* gene sequencing	Isoniazid suscept. by *katG* gene sequencing	Mixed linker PCR typing pattern	Evaluation based on PCR results	Clinical diagnosis
CSF 3024	+	WT	WT	WT	B type	Drug suscept. TB	Tuberculous meningitis
CSF 3025	++	WT	WT	WT	B type	Drug suscept. TB	Tuberculous meningitis
L-202	+	WT	WT	WT	C type	Drug suscept. TB	Lung TB
L- 204	+w	WT	WT	-	Atypical	False positive	Atypical pneumonia
H37Rv strain	++	WT	WT	WT	Typical	-	-

PCR and LCx). The results were correlated with the clinical findings. Later, several specimens were received from patients with suspicion of TB or tuberculous meningitis, although they had smear-negative results. The specimens were analyzed using the Boom (Boom et. al. 1990) method, by increasing the DNA concentration. All of these samples showed positive IS6110-PCR results, confirming the presence of MTC bacteria. In addition to IS6110-PCR, drug susceptibility molecular tests and PCR-based molecular typing were performed in the Biomedical Research and Study Centre. The results of PCR-based methods for MTC detection, molecular drug susceptibility testing and molecular typing of several specimens are summarized in Table 2. In three cases (CSF-3024, CSF-3025 and L-202), our results were in good agreement with the clinical findings and were used for elaboration of therapeutic strategy. In one case (L-204), the PCR methods showed a weak amplification signal and an atypical genetic pattern in Mixed Linker PCR. A suspicion of a false positive result was confirmed by the diagnosis of atypical pneumonia. Unfortunately, Abbott LCx assay was not applied to these specimens, and laboratory investigations, except as smear negative bacterioscopy, were not available. Anti-TB chemotherapy was begun for these drug-susceptible persons.

Interestingly, only two of these methods, IS6110-PCR and Mixed-Linker PCR, were initially designed for a low amount of DNA present in clinical material. The described molecular methods for drug susceptibility testing (Inno-LiPA kit, *rpoB* and

katG gene fragment sequencing) are usually applied for larger amounts of DNA isolated from bacterial culture. In most cases, a low content of DNA is an obstacle for further application of molecular methods in clinical material. In our study, these methods were shown to be sensitive enough to give correct results for clinical material when DNA was concentrated using the Boom method.

Discussion

Nucleic acid amplification gives the best chance of detecting MTC in smear negative samples. IS6110-PCR is rapid and relatively simple method; PCR results are available in 1 or 2 days instead of several weeks needed to growth mycobacteria in the BACTEC system. IS6110-PCR was shown to be able to detect a mycobacterial genome in variable clinical material – sputum, bronchial lavage, CSF etc. The results were comparable with other studies in which PCR was used for diagnosis of (mainly pulmonary) TB, where the sensitivity and specificity ranged between 70-100 % when a culture of MTC was taken as golden standard (Forbes 1997). However, in practice, many problems occur due to inhibition or due to cross-contamination with the products from previous PCRs (Nordhoek et al. 1994). We separated rooms and instruments used at each PCR step: PCR mixture preparation, sample addition and PCR product analysis. Also, specimens with negative PCR results were controlled for the presence of inhibitors. For this aim, PCR-negative specimens were repeatedly tested with a parallel addition of *M. bovis* DNA. Absence or weak amplification of *M. bovis* BCG DNA indicated the presence of inhibiting factor.

As PCR is able to detect both dead and live MTC bacteria, some patients may remain PCR-positive for mycobacterial DNA several months after treatment. Therefore, 44 discrepant PCR-positive and BACTEC-negative samples were evaluated in the context of the clinical findings. Clinical data was available for 38 patients. Fifteen (39.5 %) of them had active TB, confirmed clinically and by X-ray examination. Eight patients had already received a drug therapy course for a period from three days to several months and seven patients have not started treatment. Interestingly, only four of those patients were detected as MTC-positive by LCx assay. Eight (21 %) patients had inactive TB with after-effects, thus confirming that PCR can detect dead or dormant mycobacteria. In general, the clinical findings were in agreement with the shown presence of the MTC genome among 66 % of the false positive samples. Seven (18 %) specimens were, possibly, contaminated during the bronchoscopy procedure. Extrapolation of the PCR results for the remaining six (16 %) patients was problematic.

This study showed the advantages of PCR-based TB detection prior to mycobacteria culturing and admitting of chemotherapy. It is likely that, in the cases of smear positive results, there is no additional need in IS6110-PCR, and it is useful only to distinguish MTC from the other acid-fast bacteria. Therefore, IS6110-PCR can be recommended in cases of smear- or BACTEC-negative results. Invention of PCR-based detection methods may increase a number of recovered TB cases and may dramatically shorten the time required for TB diagnostics.

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Uz inserciju secības IS6110 balstīta polimerāzes ķēdes reakcija: lietojums patogēno mikobaktēriju noteikšanai

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Kopsavilkums

Insercijas sekvence IS6110 ir *Mycobacterium tuberculosis* kompleksam (MTC) raksturīgs mobilais ģenētiskais elements. Saimnieku loks šim insercijas elementam ir šaurs, un tādēļ to var lietot mikobaktēriju ātrai noteikšanai. Darba mērķis bija salīdzināt šā ģenētiskā elementa diagnostisko vērtību ar pazīstamām molekulārām un mikrobioloģiskām metodēm. Dažādus klīniskos paraugus no 170 tuberkulozes slimniekiem pārbaudīja uz IS6110 klātbūtni ar polimerāzes ķēdes reakciju (PCR). MTC noteikšana ar IS6110-PCR deva visaugstāko pozitīvo atbildi (40,6 %), salīdzinot ar LCx testu (27,6 %) un tādām noteikšanas metodēm kā baktēriju kultivēšana BACTEC sistēmā (20,6 %) vai uz Levenšteina-Jensena barotnes (15,3 %). Parādīts, ka IS6110-PCR ir ātra un specifiska MTC identifikācijas metode. Tai ir īpaša vērtība tajos gadījumos, kad ir nepieciešams apstiprināt mikobaktēriju klātbūtni, ja mikroskopiskā izmeklēšana un kultivēšana bijušas nesekmīgas.

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Tumour suppressor gene *CDKN2A/p16* germline mutations in melanoma patients with additional cancer and cancer in their family history

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Abstract

Germline mutations of the tumour suppressor gene *CDKN2A/p16* have been reported in association with familial melanoma, sporadic melanoma and pancreatic cancer. We studied the possibility of mutations in the *CDKN2A/p16* gene in patients with melanoma and additional unrelated cancer and in patients with additional unrelated cancer in their family history as well. Twenty five melanoma patients with additional cancer and twenty six melanoma patients with additional unrelated cancer in their family history were studied. The entire coding region of the *CDKN2A/p16* gene was screened by single stranded conformation polymorphism analyses and direct DNA sequencing. No germline mutations were detected in the observed melanoma patients. The previously described Ala148Thr and 500 C/G polymorphism were detected. It appears that the cancer development in the patients studied is related to a combination of low-risk susceptibility genes and environmental factors.

Key words: Cancer, *CDKN2A/p16*, melanoma, mutations, polymorphism, tumour suppressor gene.

Introduction

During the past several decades the incidence of malignant melanoma has increased throughout the world. The main risk factors for melanoma development are sunlight exposure (Geller, Annas 2003) and hereditary predisposition (Bataille 2000). Cytogenetic, linkage and molecular analyses of familial and sporadic melanoma provided evidence for the involvement of *CDKN2A* or tumour suppressor gene on chromosome 9p21 (Cannon-Albright et al. 1996). This gene encodes a cyclin-dependent kinase (CDK) inhibitor p16 (Serrano et al. 1993) that arrests progression of the cell cycle at the G1 checkpoint prior to S phase. A non-functional p16 protein has lost its regulatory capacity and can not constrain cells from passing through the cell cycle (Ruas and Peters 1998). Somatic *CDKN2A/p16* mutations have been registered in a great variety of human tumours (Foulkes et al. 1997), providing evidence for *CDKN2A/p16* involvement in the development of these malignancies. Germline mutations of the *CDKN2A/p16* tumour suppressor gene have been reported in association with familial melanoma (Hussussian et al. 1994; Yakobson

et al. 2000; Mantelli et al. 2002) and in sporadic melanoma with multiple primary lesions (Auroy et al. 2001). Additional pedigrees with individuals carrying *CDKN2A/p16* mutations who exhibit both melanoma and other cancers, including pancreatic cancer (Bartsch et al. 2002; Lynch et al. 2002), breast cancer (Borg et al. 2000), neurofibroma (Petronzelli et al. 2001), and glioma (Tachibana et al. 2000), have been described. The presence of *CDKN2A/p16* germline mutations in melanoma kindreds with coexisting additional cancers suggest an association of these mutations with increased risk for various malignancies (Platz et al. 2000). It was shown that about 3 % of melanoma patients developed another melanoma or additional unrelated cancer (Retsas et al. 2000). There is a hypothesis that patients with melanoma and additional unrelated cancers may harbour mutations in the *CDKN2A/p16* gene (Alao et al. 2002).

According the data of the Latvian Cancer Registry, the incidence of malignant primary melanoma in Latvian population is about 150 individuals a year. Same of these patients developed another additional cancer and several have an additional coexisting cancer in their family history.

The aim of the present study was to screen for *CDKN2A/p16* germline mutations in patients with melanoma and additional unrelated cancer developed in the same individual, as well as in patients with a family history of melanoma unrelated cancer. Some patients belonged to both groups.

Materials and methods

Patients

Melanoma patients were recruited through the Latvian Oncological Center, where they completed a brief family history, indicating any first and second degree relatives with a clinically confirmed cancer. The including criteria for examined melanoma patients was clinically and histologically confirmed malignant melanoma with i) additional non-melanoma cancer (n=25), or ii) cancer in family history (n=26). All participants enrolled in this study received a brief explanation of the aims of the study, agreed to participate, and signed an informed consent form approved by the Central Medical Ethical Committee of Latvia.

Mutation detection

Genomic DNA was extracted from blood samples by the standard phenol-chloroform extraction method followed by ethanol precipitation.

Polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP) analyses of the entire p16 coding region and an untranslated 3' portion of the gene was carried out by amplifying exon 1ß, exon 2 fragments 2A, 2B, 2C and exon 3 using the primers given in Table 1 and described earlier (Hussussian et al. 1994). The PCR reactions were performed according to the manufacturer instructions (Fermentas). Briefly, the 30 μl reaction mixture consisted of 10 x PCR buffer (100 mM Tris-HCl, pH=8.8 and 500 mM KCl), 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM magnesium chloride, 0.5 U Taq polymerase, 0.5 μM forward and reverse primers, and 100 ng genomic DNA. For exon 1ß and the exon 2 fragments 2A, 2B, and 2C, amplification 5 % DMSO was included. Each sample was denatured at 94 °C for 5 min and subjected to 30 amplification cycles: denaturation at 94 °C for 30 s, annealing (at temperatures specific for each exon as

Table 1. Primers used for PCR amplification of exons of the *CDKN2A/p16* gene. Primer names beginning with X are derived from the intron sequence. The other primers are numbered according to the cDNA sequence. Primer names ending with F and R denote forward and reverse primers, respectively

Exon	Primer	Primer sequences (5'-3')	Annealing temperature (°C)	Product size (bp)
1ß	X1.31F	GGGAGCAGCATGGAGCCG	63	204
	X1.26R	AGTCGCCCGCCATCCCCT		
2A	X2.62F	AGCTTCCTTTCCGTCATGC	56	204
	286R	GCAGCACCACCAGCGTG		
2B	200F	AGCCCAACTGCGCCGAC	56	147
	346R	CCAGGTCCACGGGCAGA		
2C	305F	TGGACGTGCGCGATGC	56	189
	X2.42R	GGAAGCTCTCAGGGTACAAATTC		
3	X3.90F	CCGGTAGGGACGGCAAGAGA	64	169
	530R	CTGTAGGACCTTCGGTGACTGATGA		

given in Table 1) for 30 s and extension at 72 °C for 30 s. The final extension was at 72 °C for 5 min. Following PCR, 10 μl of each reaction product was run on 1.5 % agarose gels to verify the presence of an amplification product. For single stranded conformational polymorphism analysis, 1 to 2 μl PCR product was mixed with 5 μl loading buffer (95 % formamide, 10 mM NaOH, 0.25 % bromophenol blue, and 0.25 % xylene cyanol), denatured at 95 °C for 5 min and chilled on ice. Products were run on 6 % polyacrylamide gel in at least three different conditions (7 °C, 10 °C, 15 °C). The DNA bands were visualised by silver staining.

All samples with aberrant migrating bands found in PCR-SSCP analyses were further investigated by direct DNA sequencing. PCR product was cut out from agarose gel, purified by using a DNA extraction kit (Fermentas, Lithuania) according the manufacturer instructions and subjected to 25 cycles of sequencing reaction by using a terminator cycle sequencing kit (BigDay; Applied Biosystems) and forward and reverse primers separately (Table 1). The precipitated sequencing reaction products were analysed in an automated sequencer (ABI PRISM 3100 Genetic Analyser; Applera).

Results

We analysed 47 cases of melanoma for germline mutations in the *CDKN2A/p16* gene. The characteristics of the patients studied and the results of the *CDKN2A/p16* germline mutation analyses are shown in Tables 2 and 3.

No *CDKN2A/p16* germline mutations were detected in patients with melanoma and additional cancer (Table 2), nor in patients with melanoma and additional non-melanoma cancer in their family history (Table 3).

We detected a nucleotide 500 polymorphism C/G (500 C/G) in the 3' untranslated region of exon 3 in eight patients of the 44 examined, three of whom had additional non-

Table 2. Patients with melanoma and additional cancers screened for *CDKN2A/p16* germline mutations. 500 C/G, *CDKN2A/p16* 3' untranslated region nucletide C at position 500 substituted with G. [a], benign form neoplasm

Case no.	Gender	Additional cancer	*CDKN2A/p16* variants	Cancer in family history (affected relative, cancer)
1	Female	Papilloma[a]		
2	Female	Meningioma		
3	Male	Basalioma[a]		
4	Female	Pancreas		
5	Female	Basalioma[a]		
6	Female	Breast		
7	Female	Uterine		
8	Female	Uterine		Aunt, pancreas Uncle, pancreas
9	Female	Colorectal	500 C/G	Daughter, ovarian
10	Female	Breast, uterine		
11	Male	Lipoma, melanoma satellites	500 C/G	Father, lung
12	Female	Non-Hodgkin's lymphoma		Daughter, breast
13	Female	Ovarian		Father, lung
14	Female	Adenoma, mioangioadenoma		
15	Male	Submandibularis		
16	Female	Breast		
17	Female	Ovarian		
18	Female	Breast		
19	Female	Mioma[a]	500 C/G Ala 148Thr	Cousin, breast
20	Female	Papilloma[a]		Father, prostate
21	Female	Dermatofibrioma		
22	Female	Angiolipoma		
23	Female	Melanoma satellites		Aunt, breast
24	Female	Melanoma satellites		Mother, breast Sister, renal
25	Male	Melanoma dissemination		

melanoma cancer and seven additional non-melanoma cancer in their family history. All three patients with a 500 C/G polymorphism developed additional cancer had a relative with non-melanoma cancer as well (Table 2). One patient with additional non-melanoma primary cancer (case 19; Table 2) and two patients with a non-melanoma cancer in their family history (cases 2, 3; Table 3) were found to have the Ala148Thr polymorphism in addition to 500 C/G. One patient with a cancer in their family history had also a rare G/C polymorphism at position –33 in the 5' untranslated portion of the gene (Table 3).

Table 3. Patients with melanoma and a first-degree relative with non-melanoma cancer screened for *CDKN2A/p16* germline mutations. 500 C/G, *CDKN2A/p16* 3' untranslated region nucleotide C at position 500 substituted with G. -33 G/C, *CDKN2A/p16* 5' untranslated region nucleotide G at position -33 substituted with C. [a], patients also have an additional non-melanoma cancer

Case no.	Gender	Affected realtives	Relatives cancer	*CDKN2A/p16* variants
1	Female	Father	Nase	
2	Male	Brother	Hepatis	500 C/G; Ala148Thr
3	Female	Sister	Micloleukemia	500 C/G; Ala148Thr
4	Male	Father	Cancer (where?)	-33 G/C
5	Male	Mother	Gastric	
6	Female	Mother	Lympholeicosis	500 C/G
7	Female	Mother	Breast	
8	Female	Mother	Skin	
9	Female	Mother	Gastric	
10	Female	Mother	Skin	500 C/G
11	Female	Sister	Skin	
		Father	Bladder	
12	Male	Father	Gastric	
13[a]	Female	Daughter	Ovarian	500 C/G
14	Female	Mother	Breast	
15	Female	Father	Gastric	
16	Female	Mother	Breast	500 C/G
17[a]	Male	Father	Lung	500 C/G
18[a]	Female	Daughter	Breast	
19[a]	Female	Father	Lung	
20	Female	Brother	Gastric	
21	Male	Father	Lung	
22	Female	Father	Larynx	
23	Female	Father	Bladder	
		Brother	Colorectal	
24	Female	Sister	Leucosis	
25	Female	Father	Prostate	
26	Male	Father	Gastric	

Discussion

In our study 25 melanoma patients with additional non-melanoma cancer and 26 patients with cancer in their family history were examined for mutations in the *CDKN2A/p16* gene. The *CDKN2A/p16* is emerging as an important determinant of susceptibility to the development of a malignant melanoma. Genetic alteration of the *CDKN2A/p16* gene has been identified in several different tumour types (Foulkes et al. 1997). Germline mutation of this gene may predispose some family members to an excess risk of melanoma and other

cancers (Borg et al. 2000). It is hypothesised that patients with melanoma and additional cancer may also harbour germline mutations in the *CDKN2A/p16* gene (Alao et al. 2002). However, no such mutations were detected among patients with melanoma and additional non-melanoma cancer in our study. Observation that the presence of *CDKN2A/p16* germline mutations in melanoma kindred with coexisting additional cancers is associated with increased risk for various malignancies (Platz 2000) would indicate that patients with melanoma and unrelated additional cancer in their family history may also have germline mutations in the *CDKN2A/p16* gene. No such mutations were detected among patients with melanoma and an additional non-melanoma cancer in family history in the present study. The determined 500 C/G and Ala148Thr polymorphisms could not be fully evaluated in this study as we have not studied these frequencies in healthy persons. The investigation of the role of Ala148Thr as a low penetrance melanoma susceptibility allele did not support this hypothesis (Bertram et al. 2002). The incidence of 500 C/G polymorphism was associated with increasing familial risk of melanoma (Hayward 2000) and a shorter disease-free survival in melanoma (Kumar et al. 2001). This association was not studied in our cases. In bladder cancers the polymorphism in the 3' untranslated region of the *CDKN2A* gene was related to the mechanism of tumour invasiveness (Sakano et al. 2003). The 500 C/G polymorphism in melanoma patients may be associated with tumour invasiveness as well, through an unknown mechanism (Pjanova, unpublished data). The concurrence of both 500 C/G and Ala148Thr polymorphisms detected in three patients is of uncertain significance. In our study the combination of both polymorphisms in all three cases was obtained in patients with melanoma and an additional cancer in their family history.

There is evidence that patients with two or more primary melanomas appear to have a better prognosis than patients with a single primary lesion (Burden et al.1994). The same would be true in the case with patients with melanoma and unrelated additional primary cancers (Retsas et al. 2000). The impact of mutated genes and polymorphisms on the evolution of the disease is an area of future investigation.

The studied cohort is too small for definite conclusions. However, it would appear that melanoma and additional, apparently unrelated, cancers developing in the same individual and melanoma in context with additional cancer in the family history are related to a combination of low-risk susceptibility genes and environmental factors.

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Audzēju supresorgēna *CDKN2A/p16* iedzimtās mutācijas melanomas pacientiem ar citu audzēju un pacientiem ar audzēju ģimenes anamnēzē

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Kopsavilkums

Audzēju supresorgēna *CDKN2A/p16* mutācijas novēro ģimenes locekļiem ar pārmantotu melanomu un aizkuņģa dziedzera audzēju, kā arī iegūtās melanomas pacientiem. Dotajā darbā meklēja iespējamās *CDKN2A/p16* gēna mutācijas asins paraugos divās melanomas pacientu grupās: 1) 25 melanomas pacientiem, kuriem diagnosticēts cits audzējs, 2) 26 melanomas pacientiem, kuriem ģimenes anamnēzē vienam vai vairākiem pirmās pakāpes radiniekiem ir kāds audzējs. Analīzes veica, izmantojot polimerāzes ķēdes reakcijas, vienpavediena konformācijas polimorfisma analīzi un gēna sekvenēšanu. Nevienā gadījumā melanomas pacientu asinīs mutācijas *CDKN2A/p16* gēnā nenovēroja. Atrada gēna polimorfismu - 500. nukleotīda C nomaiņu ar nukleotīdu G (500 C/G) un 148. kodona aminoskābes alanīna nomaiņu ar aminoskābi treonīnu (Ala148Thr). Rezultāti liecina, ka pētījumā iekļautajiem pacientiem audzēju attīstība nav saistīta ar mutācijām *CDKN2A/p16* gēnā.

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The structure and dynamics of fish communities in the Latvian coastal zone (Pape - Pērkone), Baltic Sea

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Abstract

The fish biodiversity in the Baltic Sea coastal area was analysed in 1998 - 2002. Different ecological groups – marine, diadromous and freshwater – were captured in survey gillnets and beach seine catches. The coastal fish communities revealed a strong seasonal variation. The water temperature increase early in the spring caused migration of juveniles and adult marine fishes from depths to the nursery, spawning and feeding grounds located in the coastal waters. In summer mostly diadromous and freshwater fish species migrated from the adjacent freshwater basins to the sea coastal feeding grounds. In 1998-2002 fish communities comprised representatives of various ecological types. The available information from the monitoring combined with the fisheries data could facilitate an ecologically-focused management of the coastal zone ecosystem.

Key words: Baltic Sea, coastal zone, diversity, fish community, seasonal dynamics.

Introduction

The marine coastal zone plays an important role for many fish and bird species. Some fish species live permanently in the coastal zone and are to varying degrees restricted to specific habitats, whereas other species may be present only as juveniles, migrate seasonally to the coastal zone or just pass through on their way between marine and fresh waters. Environmental conditions in the coastal waters of open sea depend on several hydrodynamic factors the most important being wind direction and velocity, wave height, light and frequent temperature changes (Beyst et al. 2001). Wind-influenced circulation of water masses occurs in coastal zone, creating changes in water temperature and also the structure of coastal fish communities. Wind direction and velocity causes changes in the abundance of certain fish species especially in the warmwater season. Strong land-wind causes a drift of warm waters from the coastal area to the open sea. Warm water masses are replaced with cold waters from greater depths (upwelling effect). The influence of water temperature on coastal fish community structure in brackish waters has been described previously in Scandinavia (Pihl, Rosenberg 1982; Thorman, Wiederholm 1986; Pihl, Wennhage 2002).

The coastal area of Pape – Pērkone is one of four HELCOM Baltic Sea Protected Areas in Latvian territorial waters. Shallow marine waters are generally considered to be important nursery areas, environments where juvenile fishes will experience enhanced

survival and growth (Rozas, Odum 1998). The sea bottom in the study area is covered by *Furcellaria lumbricalis* (Korolev, Fetter 2000) and plays an important role in spawning and fry survival of Baltic herring. Rocky and soft bottoms in near shore shallow waters are the main spawning habitat for turbot and several non-commercial fish species.

Representatives of different ecological guilds (marine, freshwater and diadromous species) are present in the coastal brackish water zone. The coastal areas and fish communities inhabiting them are exposed to anthropogenic pollution risks that can directly affect the food resources, distribution, growth and survival of fish populations. Commercial fishery in coastal as well as open sea areas can also alter the population structure of target species – herring, cod, turbot and flounder.

The health and integrity of different water ecosystems based on fish communities are monitored in different countries. Studies in the river basins of Illinois (Karr 1981; Karr 1986) and lagoons in the Atlantic Ocean in Portugal (Pombo et al. 2002) show that fish are suitable indicators of the ecological state. Monitoring of shallow water fish communities are being carried out in Sweden, eastern and western coast of Baltic Sea (Hansson 1984; Pihl, Wennhage 2002) as well as in the North Sea (Beyst et al. 2001) and various estuaries of Europe (Whitfield, Elliot 2002).

The aim of this study was to investigate the seasonal dynamics and structure of the coastal fish community of the Pape – Pērkone area for the time period from 1998 to 2002. The abundance and distribution of different fish species in different life stages were studied in two depth zones (0 to 2 m and 3 to 5 m).

Materials and methods

Scientific surveys were carried out at two coastal sites in the Liepaja district – Pape (56° 09' N, 21° 02' E) and Jūrmalciems (56° 18' N, 20° 59' E; Fig. 1). These study sites were chosen because the study area is a relatively pristine environment with little influence from anthropogenic disturbances but nevertheless in potential threat of oil pollution from the Butinge oil terminal in Lithuanian waters.

Samples were taken twice a year – in spring (May) and summer (July). Two fishing methods were applied to capture a maximum number of species, at different life stages. In shallow depths (0 to 2 m), fishes were sampled using beach seine with a mesh-size 5 to 10 mm (5 mm in cod-end and 10 mm in wings); the total catch area was approximately 4000 m^2. Details of the sampling methodology are given in Vitinsh (1989). Samples were taken at 5 stations at each site during daylight. The caught fish were preserved in 80 % ethanol and analysed later in laboratory. The number of individuals in each species were determined and fish length and weight were measured. In total, 97 samples were collected from 1998 to 2002. In fish community surveys at medium depth (3 to 5 m), fish were caught using gillnet sets. Two monofilament survey nets (150 m long, 3 m high) with 5 different mesh-sizes (25, 30, 38, 50, 60 mm) and one polyfilament survey net (210 m long, 1.8 m high) with 7 different mesh-sizes (17, 22, 25, 30, 33, 38, 50 mm) were used. Polyfilament survey nets were chosen according to guidelines for coastal fish monitoring (Neuman et al.1997), nets with mesh size 38 and 50 mm were added to catch adult large-size fishes. We used monofilament gillnets to catch fishes more effectively in the 4 to 5 m depth. Fishing was performed between 18:00 - 06:00 in three stations at each site. All fishe were identified to species, weighed (g) and measured for total length after capture.

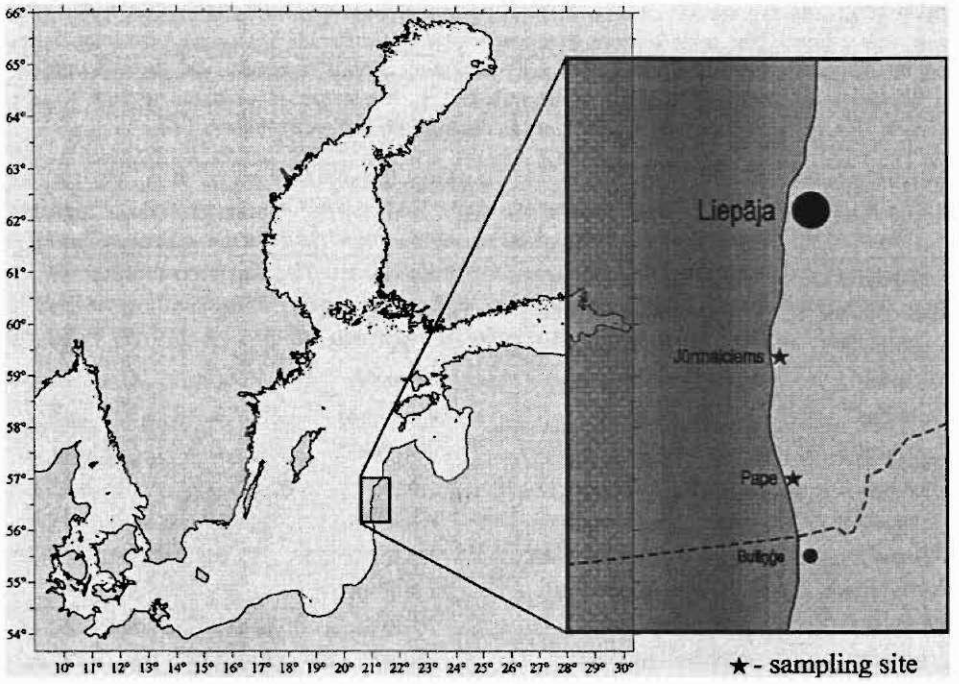

Fig. 1. Location of the survey area. Sampling site in open part of Baltic Sea. Sampling was conducted in two sampling sites in the 0 - 2 m and 3 - 5 m depth.

In total, 57 samples were collected from 1998 to 2002.

Water temperature at the bottom and surface were recorded during each sampling.

Further, all fish species were denoted to one of four ecological guilds (benthic marine; pelagic marine; freshwater and diadromous species) to analyze the functional ecology of the fish assemblages in different seasons. Certain fish species were considered as 'residents' (they were present in more than 50 % of the total number of sampling stations) or 'migrants' (most abundant during a certain period). All other species were recorded 'sporadically' (Clark et al. 1996). Temporal variation in the number of dominant fish species was investigated with a one-way ANOVA using Statgraphics Plus software. The Shannon-Wiener biodiversity index was calculated to analyse temporal and spatial variability of species diversity (Odum 1986).

Results

Fish community structure

In time period from 1998 to 2002 a total of 31 fish species belonging to 19 families were recorded (Table 1). Twenty eight of them are common in the Latvian coastal waters (Winkler 2000), two fish species [black goby (*Gobius niger*) and sabrefish (*Pelecus cultratus*)] can be considered as rare, and one species – twaite shad (*Alosa fallax*) – as very rare. Furthermore, the latter two species are included in the Red Data Book of Latvia, 3rd category.

Table 1. List of fish species caught in the Pape-Jūrmalciems area in 1998 - 2002 with beach seine and gillnets. The table represents the occurrence of different life stages of caught fishes and the assignment of fish species to different occurrence groups and ecological guilds according to Winkler et. al. 2000. A, adult fish; J, juvenile fish; L, fish larvae; B, common species; R, rare species; V, very rare species; M, marine fish; D, diadromous fish; F, freshwater fish

Taxon/Family	Scientific name	Common name	Life stage	Occurrence	Ecological guild
Ammodytidae	*Ammodytes tobianus*	Sandeel	A, J, L	C	M
Ammodytidae	*Hyperoplus lanceolatus*	Greater sandeel	A, J	C	M
Anguillidae	*Anguilla anguilla*	Eel	A	C	D
Belonidae	*Belone belone*	Garfish	A, J	C	M
Clupeidae	*Alosa fallax*	Twaite shad	A	V	D
Clupeidae	*Clupea harengus membras*	Herring	A, J, L	C	M
Clupeidae	*Sprattus sprattus*	Sprat	A, J, L	C	M
Coregonidae	*Coregonus lavaretus*	Whitefish	A, J	C	D
Cottidae	*Myoxocephalus scorpius*	Bullrout	A	C	M
Cyclopteridae	*Cyclopterus lumpus*	Lumpsucker	A	C	M
Cyprinidae	*Abramis brama*	Bream	A, J	C	F
Cyprinidae	*Alburnus alburnus*	Bleak	A	C	F
Cyprinidae	*Blicca bjoerkna*	White bream	A, J	C	F
Cyprinidae	*Leuciscus leuciscus*	Dace	J	C	F
Cyprinidae	*Pelecus cultratus*	Sabrefish	A	R	D
Cyprinidae	*Rutilus rutilus*	Roach	A, J	C	F
Cyprinidae	*Vimba vimba*	Vimba	A, J	C	D
Esocidae	*Esox lucius*	Pike	A	C	F
Gadidae	*Gadus morhua callarias*	Cod	A	C	M
Gasterosteidae	*Gasterosteus aculatus*	Three-spined stickleback	A	C	F
Gasterosteidae	*Pungitius pungitius*	Nine-spined stickleback	A	C	F
Gobiidae	*Pomatoschistus minutus*	Sand goby	A, J, L	C	M
Gobiidae	*Gobius niger*	Black goby	A	R	M
Osmeridae	*Osmerus eperlanus*	Smelt	A, J, L	C	D
Percidae	*Perca fluviatilis*	Perch	A, J	C	F
Percidae	*Stizostedion lucioperca*	Pike-perch	A, J	C	D
Pleuronectidae	*Platichthys flesus trachurus*	Flounder	A, J	C	M
Salmonidae	*Salmo trutta*	Sea trout	A, J	C	D
Scophthalmidae	*Psetta maxima*	Turbot	A, J	C	M
Syngnathidae	*Neophis ophidion*	Straight-nosed pipefish	A	C	M
Zoarcidae	*Zoarces viviparus*	Eelpout	A, J	C	M

Juveniles and small-sized adult fishes dominated in beach seine catches, whereas larger-sized adult fishes dominated in gillnet catches.

Juveniles of marine pelagic and benthic fish dominated (93 % of total number) in beach

seine catches in Pape and Jūrmalciems in the shallow depth (0 to 2 m) in spring (Fig. 2A). They were mainly juveniles of clupeids and flatfishes. A small number of diadromous smelt (*Osmerus eperlanus*) and juveniles of freshwater roach (*Rutilus rutilus*) and bream (*Abramis brama*) were also present. Adult marine benthic fishes such as sandeel (*Ammodytes tobianus*), flounder (*Platichthys flesus*), and greater sandeel (*Hyperoplus lanceolatus*) were more abundant in the coastal zone in Pape whereas smelt were more frequent in Jūrmalciems. Bleak (*Alburnus alburnus*) and three-spined stickleback (*Gasterosteus aculeatus*) were representatives of freshwater species (Fig. 2B).

A marine fish community dominated (94 %) in the medium depth (3 to 5 m) in spring at both sites. Flounder, herring (*Clupea harengus*) and turbot (*Psetta maxima*) were most abundant, and freshwater and diadromous fishes occurred in small numbers (Fig. 2C).

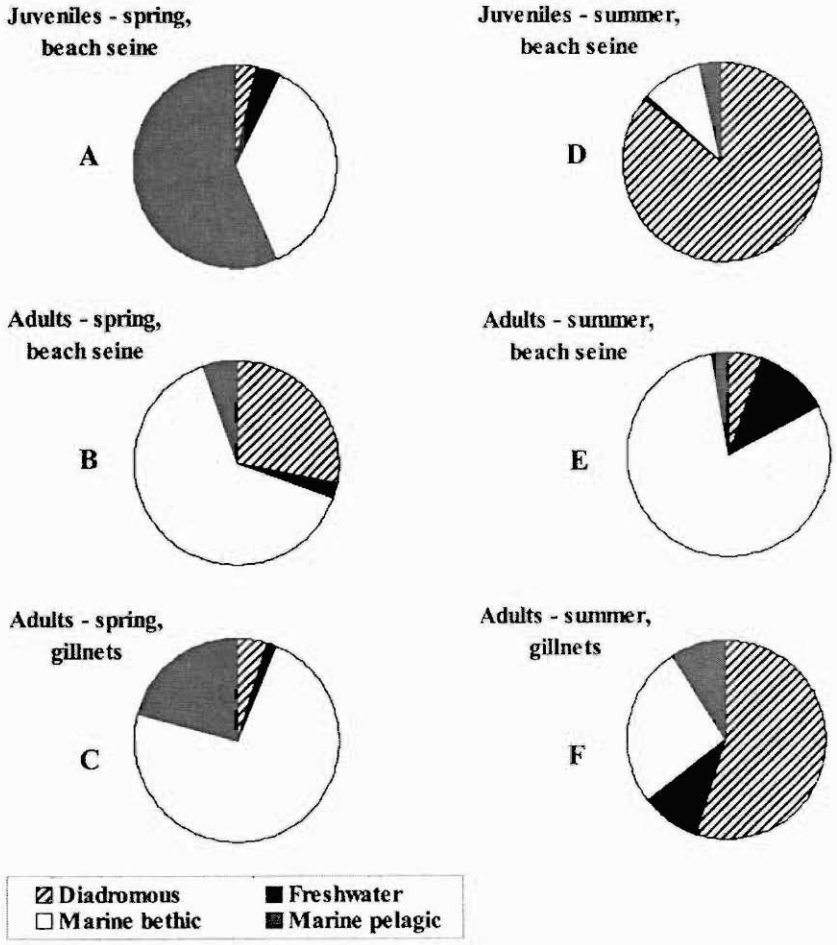

Fig. 2. Percentage of juveniles and adult fishes in ecological guilds in beach seine and gillnet catches. The figure shows comparison of the number of fishe of different ecological guilds and life stages between spring and summer in the survey area.

In summer, in the shallow depth, the proportion of diadromous fishes, mainly juveniles of smelt, increased significantly (86 %), particularly in Jūrmalciems (Fig. 2D). Juveniles of marine benthic species (flounder, turbot and sandeel) were widely distributed but were caught in less number than smelt. The proportion of young freshwater fishes was slightly higher in Pape. The number of adult fishes was lower in summer than in spring. In summer at both sites samples were dominated by marine benthic species such as sandeel, flounder and sand goby (*Pomatoschistus minutus* Fig. 2E).

A similar pattern in species composition was observed in summer in the medium depth, where diadromous fishes, especially vimba (*Vimba vimba*) were the most abundant ecological guild (55 %) in the fish community in Pape. The proportion of freshwater fishes also increased in July, but did not exceed 10 % of the total number (Fig. 2F). The fish community in Jūrmalciems also consisted mainly of diadromous and marine fish (60 and 30 % respectively). The proportion of freshwater fishes there was similar to that in Pape.

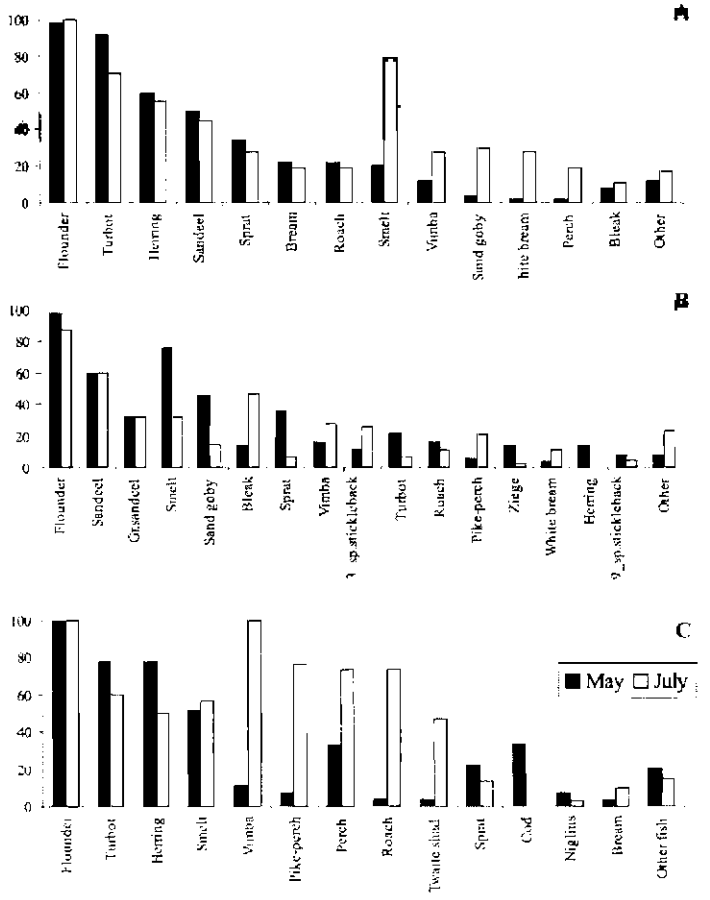

Fig. 3. Fish species occurrence by season. The figure shows the relative occurrence of fish species in sampling stations in May and July. A, juvenile fishes in beach seine; B, adult fishes in beach seine; C, adult fishes in gillnets.

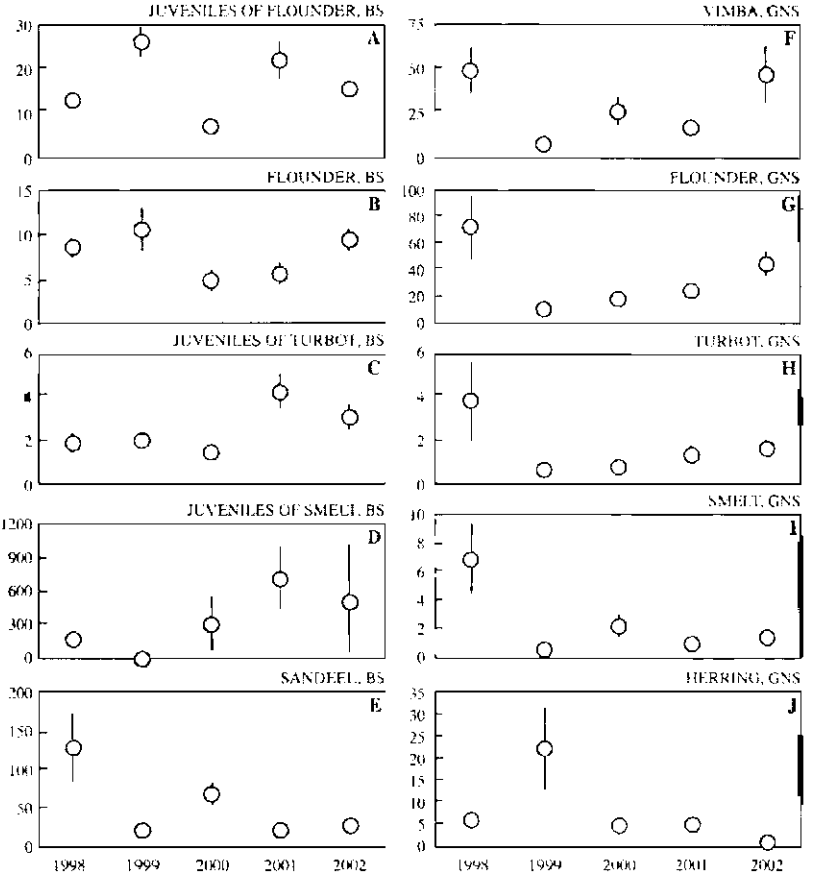

Fig. 4. Density and CPUE (catch per unit effort) of resident species in sampling stations by year. The figure shows the density of resident fishes in beach seine catches (number of fish per sampling station) and CPUE of resident fishes in gillnet catches. BS, beach seine; GNS, gillnets. Values are the means ±SD.

Fish species distribution, density and CPUE in the shallow depth zone

In the shallow depth up to 2 m, six fish species occurred most frequently and were represented by juveniles of flounder, turbot, herring, sandeel and adult flounder, sandeel, great sandeel and sand goby (Fig. 3A, B). These benthic and pelagic fishes occurred in the surf zone more or less throughout both seasons. They were present in 50 to 95 % of all sampling stations depending on time and can be considered as 'resident' species (according to the terminology of Clark; Beyst et al. 2001). The mean number of juvenile flounder was variable without an obvious trend (Fig. 4A). The density of adult flounder was lower, but they were evenly distributed (Fig. 4B). Juveniles of turbot were resident inhabitants of the surf zone with numbers varying between one to four individuals per sampling station. From 2001 on there was slight increase of turbot juvenile number (Fig. 4C). Herring juveniles numbered 20 to 80 individuals per station, however in several stations they were in high numbers. The distribution of sandeel and great sandeel also

aggregated in patches. They occurred in approximately 40 to 60 % of all sampling stations and variation of density was high (Fig. 3; Fig. 4E). The abundance of adult sand goby was high in spring, whereas the number of juveniles increased in summer.

Epibenthic smelt and bleak were classified as 'migrants' (most abundant during a certain period of the year and at several stations). The greatest concentrations of smelt juveniles and fry were encountered in summer (Fig. 4D), but there was sure patchiness observed in their distribution. Bleak was more common in freshwater, but occurred in low numbers also in the surf zone in summer. All of the other species were recorded sporadically.

The Shannon-Wiener biodiversity index in 1998 - 2002 varied between 0.99 to 1.36 and without a tendency. The highest biodiversity index was in 2002 (Fig. 5A), and generally the values were higher in spring.

Fish species distribution, density and CPUE in the medium depth zone

Four fish species – flounder, turbot, herring and smelt – were classified as resident species in the depth from 3 to 5 m. There was a common pattern observed in flounder and turbot CPUE (number of individuals per sampling station) dynamics. They were most abundant in 1998 (70 and 4 individuals per station), but unevenly distributed between depths (Fig. 4G, H). In 1999 their numbers significantly decreased (10 and 1 individuals respectively), but in subsequent years the abundance of both species increased again reaching 45 and 2 individuals per sampling station. In 2002 the distribution between depths was more even.

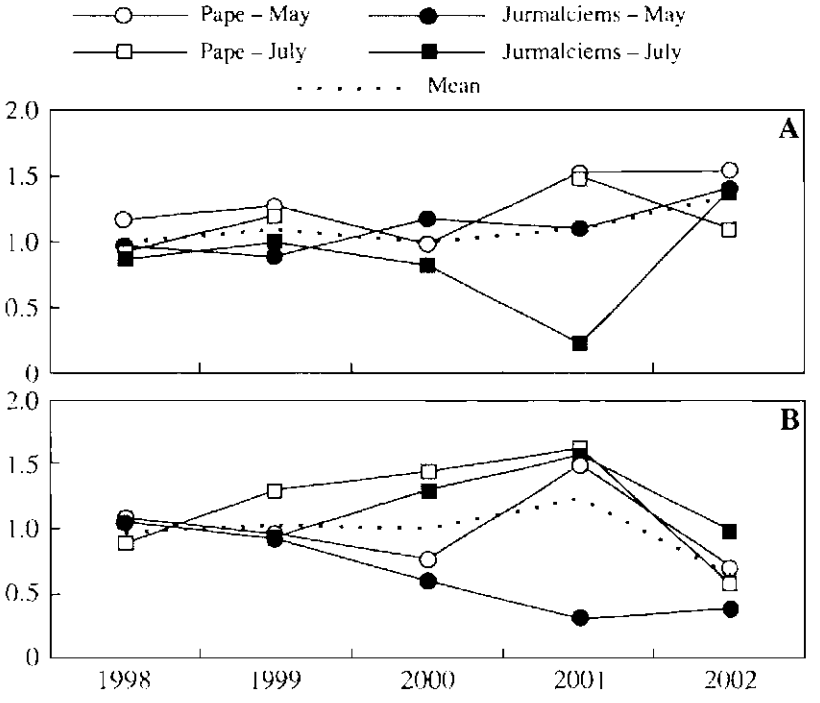

Fig. 5. Shannon-Wiener biological diversity index. Mean annual values by year. A, shallow depth (0 - 2 m); B, medium depth (3 - 5 m).

Baltic herring was very abundant in 1999 – around 20 individuals per station – with a patchy distribution. In later years the CPUE of herring declined (less than 10 individuals per station) and the variability was low (Fig. 4J). Although smelt were resident, their CPUE was low (several individuals per station) and they occurred in equal numbers in samples (Fig. 4I).

Vimba, pike-perch (*Stizostedion lucioperca*), perch (*Perca fluviatilis*) and roach can be considered as migrants and appear in the coastal waters mainly in summer. In Pape-Jūrmalciems coastal waters vimba occurred in great numbers in summer. The highest numbers and variability were recorded in 1998 and 2002 (around 50 individuals per sampling station; Fig. 4F). In time period from 1999 to 2001, vimba was less abundant, with numbers varying from 10 to 25 fishes per station. The perch CPUE in Pape – Jūrmalciems coastal zone was low through the whole period of investigation. A slight increase in perch numbers was observed in summer 2002.

Shannon-Wiener biodiversity varied from 0.66 to 1.24. Generally the values were higher in Pape than in Jūrmalciems. There were also seasonal differences, as biodiversity was lower in spring (Fig. 5B).

Discussion

The environmental conditions in the coastal brackish waters of the study area apparently were more suitable for benthic marine fishes. These species were recorded more consistently and in greater numbers in different seasons through the whole period of study. Other ecological guilds may use the surf and medium depth zones as a transient area when they migrate from the estuary to the open sea and back. Some species may infrequently enter the surf zone to feed, since only adult individuals were found. Coastal waters provide food resources and diversity for different trophic levels.

The fish community structure in coastal waters is dynamic and varies according to season and temperature changes. The period of favourable conditions is relatively short from 4 to 5 months. Pihl and Rosenberg (1982) estimated that 90 % of the annual production is related to warm spring-summer months.

The size and structure of research catches in the coastal zone up to 5 m proved to be dependent on several environmental variables as well as catchability of the sampling gears used. Although no sampling was performed during extreme weather conditions, hydrodynamic variables such as wind speed and direction and wave height are considered to influence the community structure.

A relatively high number of fishes was observed in spring, when water temperature reached 7 to 11 °C. As the temperature in the 40-60 m water layer in May is usually low, about 3-4 °C (E. Jula, unpublished data, 2003), marine pelagic and benthic fish species migrate to warm near-shore waters from the open sea. The pelagic species complex was represented by adult spring-spawning herring and probably by autumn-spawning herring juveniles. The benthic fishes were represented by juveniles of sandeel and flounder that migrate to shallow coastal waters after spending winter at greater depths. Adult flounder in spring perform a feeding migration from Gotland Deep to Irbe Straight along the shore (Vitinsh 1976). Only three (turbot, flounder and herring) of the observed ten fish species were recorded yearly in surveys at a medium depth and they were dominant in numbers. The explanation could be that herring and turbot migrate in spring to coastal areas to

spawn (Korolov et al. 1993). The deeper waters of Pape-Jūrmalciems coastal area play important role in turbot spawning (Ustups 1997). However, the specific selectivity of gillnets used in surveys, do not permit to estimate the actual density of adult turbot. Other fish species were found more inconsistently.

In summer, when the water temperature reached 17 to 21 °C, there were significant differences in fish community structure between shallow and medium depth zones. juveniles and larvae of smelt dominated on depths not greater than 2 m. Another widely encountered ecological group in this area was flatfish juveniles. Shallow waters during the whole season are inhabited by one- and two-year-old flatfishes, but in the case of early spawning also by 0-group fishes. Although the density of flounder juveniles was not as great as in Irbe Straight (main flounder nursery ground; Vitinsh 1976), individuals of different age groups were widely distributed on the sandy bottom of the Pape-Jūrmalciems coastal zone. The density of turbot juveniles was low, but they were equally distributed along the shore in the survey area. Another resident of the shallow surf zone is sandeel, which spends most of the daylight time buried in sand, and feeds aggregated in shoals during night in the water column (Plikšs, Aleksejevs 1998). Adult smelt also inhabit coastal waters (almost year round) though most of the year, and they migrate to the lower reaches of rivers to spawn only in spring (Gaumiga 1967). Of the freshwater species perch, bream (*Abramis brama*), juveniles of white bream (*Blicca bjoerkna*), and bleak were present in the coastal zone in summer. Diadromous fishes were represented by juveniles of vimba.

The number of species at a medium depth was greater in summer than in spring. The species turbot, perch, flounder, vimba and pike-perch encountered in every survey were twice as much as in spring. Vimba and flounder dominated the fish community. In 2000, twaite shad, a rare species in Latvian waters, occurred in significant numbers in coastal waters of Pape and Jūrmalciems. Apparently, this was due to appearance of several strong year classes in Curonian Bay, resulting in expansion of its distribution area (Kesminas et al. 1998).

In summer, the Pape-Jūrmalciems coastal area apparently is significant feeding ground for many fish species. Some of them – pike-perch, smelt, twaite shad and vimba – migrate from Curonian Lagoon. Other migrants are freshwater species like perch, roach, white bream and bream that migrate to coastal waters from the lakes Pape and Liepaja. The abundance of freshwater fishes increased in 2001, like caused by a storm washout of the sluice that regulated water level in Lake Pape, which enabled freshwater fish to migrate unrestrictedly to the Baltic Sea.

There were no significant changes in the number of species and individuals in the coastal zone during the survey period. The variability of biological diversity index showed a seasonal pattern depending on the feeding migrations of juveniles and adult fishes. In the shallow zone in summer, the number of species slightly increased, but the biological diversity decreased due to the occurrence of juvenile and adult smelt in high abundance. The highest variability of biological diversity was in 2001, when in summer in Jūrmalciems smelt juveniles contributed 97 % of total number of fish, causing the lowest biological diversity index (0.23) recorded.

In medium depths, in contrast to the shallow depth zone, biological diversity was higher in summer. In spring flounder was dominant species. As its dominance decreased in summer and freshwater species appeared in the same time, the biological diversity

increased. The lowest Shannon-Wiener biological diversity index was observed in 2002 in Pape, 0.31 in spring, and 0.37 in summer. These low values were caused by a strong dominance of specific fish species – flounder in spring (84 % of total numbers) and vimba in summer (83 %). Generally, Shannon-Wiener biodiversity depended on the biology of the species. Flounder occur in high numbers in the coastal zone in spring during feeding migrations (Vitinsh 1976). Coastal waters are the main feeding grounds of vimba and juveniles of smelt in summer (E. Urtans, unpublished data).

The present study shows that the juvenile fish stay in the coastal waters during the spring and summer seasons, and that coastal habitats provide feeding conditions for different ecological guilds of fishes. During in the adult life cycle, marine flounder, turbot, herring and diadromous smelt utilize different shallow habitats permanently as feeding grounds. Turbot and herring also spawn in the coastal areas. Adult and juvenile diadromous vimba, pike-perch and smelt (juvenile), as well as freshwater species such as perch, roach, and bream were considered as migrants. These fishes migrated from the adjacent water basins to the coastal waters mainly in summer and feeding grounds were utilized for a shorter time.

The study also showed that coastal fish communities are dynamic with complex structure and that fish production of the coastal zone is related to the fluctuating levels of recruitment and stock abundance in the main distribution areas. The monitoring, which focuses on the fish community structure, species distribution, habitat borders and abundance indices could be used to determine the habitats in need of protection and to evaluate the impact of anthropogenic influences (Karr 1981).

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Zivju sabiedrību sastāvs un tā izmaiņas Baltijas jūras Latvijas piekrastē (Pape – Pērkone)

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Kopsavilkums

Zivju sabiedrību daudzveidība Baltijas jūras piekrastē tika analizēta laikā no 1998. līdz 2002. gadam. Piekrastes zivju mazuļu vadiņa un tīklu lomos konstatēti dažādu (jūras, ceļotājzivju un saldūdens zivju) ekoloģisko grupu pārstāvji. Piekrastes zivju sabiedrībās ir novērojamas izteiktas sezonālās svārstības. Agri pavasarī, ūdens masām uzsilstot, notiek mazuļu un pieaugušo jūras zivju migrācijas no atklātās jūras dziļākiem rajoniem uz piekrastes seklajiem ūdeņiem, kur notiek zivju barošanās, nārsts un kas ir mazuļu uzturēšanās rajoni. Vasaras periodā barošanās migrācijas no netāliem saldūdens baseiniem uz piekrastes ūdeņiem veic galvenokārt ceļotājzivis un saldūdens zivis. Zivju sabiedrības laika posmā no 1998. līdz 2002. gadam novērotas dažādas ekoloģiskas grupas. Pamatojoties uz zivju monitoringa datiem, var noteikt aizsargājamās dzīvotnes, kā arī aprēķināt saimnieciskās darbības rezultātā ihtiofaunai nodarīto zaudējumu vērtību.

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Promotion of salmon rearing efficiency by including yeast extract *Aqualase Two* in the diet

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Abstract

The effect of Baltic salmon growth and development treated with ß-glucan yeast extracts *Aqualase Two* was studied in the fish hatchery "Tome" and Latvian Fisheries Research Institute. A total of 30 000 fish from larvae (0+) to smolt stages were used in the experiment. Basic food with and without supplements of *Aqualase Two* (0.5 and 1 g kg^{-1}) was used for feeding. Health of fish was regularly assessed. The investigations showed that testing the preparation decreased mortality during feeding and increased the rate of growth in autumn. The best results – lower mortality and greater increase in weight of smolts, were obtained in the group of fish fed glucan at dose of 0.5 g kg^{-1}. The results of the experimental study showed that immunostimulation with a ß-glucan yeast preparation can be used to improve growth in industrial conditions.

Key words: *Aqualase Two*, feeding, salmon, ß-glucan.

Introduction

Glucan is polysaccharide found in the cell wall of fungi, bacteria and plants. Glucan has multiple effects in animals when administered intramuscular, intravenous, intraabdominal and per rectum (Dalmo, Seljelid 1995; Fuente et al. 1998; Krakowski et al. 1999; Waller, Colditz 1999).

Some studies have shown that intraperitoneal injection of a ß-1,3 and ß-1,6 linked glucan from cell walls of the yeast *Saccharomyces cerevisiae* into Atlantic salmon resulted in increased resistance to several bacterial pathogens and decreased fish mortality (Dalmo, Seljelid 1995). Intraperitoneal injection of glucan increased the resistance of trout (Jorgensen et al. 1993). Intravenous injection of a yeast glucan into mice increased the host antibacterial defense (Kokoshis et al. 1978), but resulted in no gain in weight in penaeid shrimps (*Penaeus vannamei*) on the glucan diet and decreased the host antibacterial defense (Scholz et al. 1999).

Growth efficiency (growth as a percentage of body weight per day) changes with temperature and dietary intake. As both temperature and food amount decrease, the growth efficiency also decreases (Brett 1970).

Healthy fish are more resistant to changes in living conditions and they have better gain in weight. With a change in the environment when the smolt are released in the estuaries or rivers, they do not feed for about two weeks. During that time the fat and protein levels in the body decrease. Therefore the survival of large fish is higher (Stoskopf 1993).

The present work examines the effect of *Aqualase Two* (producer Doxal Italia SpA) on growth and survival of salmon parr.

Materials and methods

The experiment was carried out in the hatchery "Tome". Experiment started in June. A total of 30 000 Baltic salmon (*Salmo salar*) males and females weighing 0.70±0.03 g were divided randomly into three groups (10 000 fish in each group). The fish were kept in 300 litre tanks, later in 800 litre tanks, and supplied with aerated fresh water. The fish were fed daily with commercial salmonid food according to the prescription of the food producer (Table 1). One group (EI) received *Aqualase Two* 0.5 g per kg^{-1} supplements to food, and a second group (EII) had *Aqualase Two* 1.0 g per kg^{-1} additions; a third group served as a control without additions.

Fish samples were placed on the ice and immediately transported to the laboratory for bacteriological analyses. For bacterial analyses smears were made from fish gills and the surface of the body and inoculated on the plates with specific agar and cytophagous medium. All plates were cultivated at +18 - +25 °C for 24-72 h, and representative colonies were reisolated for characterization and identification according to standard bacterial methods (Bergey's Manual of Systematic Bacteriology, 1980).

Mortality was estimated daily. Monthly mortality was calculated as a proportion between a number of dead fish and a number of alive fish at the start of a month. Fish weight was estimated monthly.

All the data analyses were performed using the statistical methods. Standard deviation (SD) of mean length of fish was determined. The experimental groups were compared using the T-test (Arhipova et al. 1997).

Results

There were no significant differences ($P>0.05$) between the mean or range of initial body masses for the two experimental groups and control group in June (Table 2). The mass of parr was similar in the summer months. The differences began in September when the water temperature decreased. The highest growth of fish was seen at the end of November in the group EI (additions 0.5 g kg^{-1}) with a mean mass of 20.4±8.6 g. The mass of the fish from groups EII and K in that time were 16.8±8.0 and 15.0±8.3 g, respectively (Table 2). There was no gain in mass of parr in the winter months. The rate of growth increased at the end of February in all groups, but the differences in mass were maintained. There

Table 1. Diet of fish depending on lengths and mass

Food type	Fish length (cm)	Fish mass (g)	Size of pellets (mm)
Aller Aqua SGP-493	2.0-3.0	0.1-0.3	0
Aller-Aqua SGP-493	3.0-4.3	0.3-0.5	1
Aller-Aqua SGP-493	4.3-7.0	0.5-3.0	2
Aller-Aqua SGP-493	7.0-9.0	3.0-8.0	3
Aller-Aqua SGP-493	9.0-11.0	8.0-15.0	4
Salir	> 10	> 13	5

Table 2. Mass of salmon parr in the experimental groups (mean ± SD)

Date	Experimental groups		
	E I	E II	K
June 1	0.7±0.0	0.7±0.0	0.7±0.0
July 1	1.6±0.5	1.3±0.5	1.2±0.5
August 1	2.9±1.8	2.4±1.7	2.0±1.7
September 1	6.9±3.9	5.6±4.0	5.9±3.0
October 1	11.4±5.3	10.0±5.0	8.5±5.0
November 1	20.3±8.0	16.7±7.5	14.8±7.7
December 1	20.4±8.6	16.8±8.0	15.0±8.3
January 1	20.4±10.0	16.2±8.9	15.1±9.1
February 1	21.2±12.0	17.0±10.6	15.1± 9.8
March 1	22.9±12.0	18.5±11.9	15.9±11.0
April 1	26.3±13.3	21.6±13.2	19.3±13.1
May 1	29.5±13.7	26.9±13.0	24.6±13.2

were significant differences (P=0.000) between body mass for the two experimental groups and the control group in spring.

Fish from each experimental group were divided in to three groups by mass. There were 45 % of fish with a mean mass of 20.0±2.0 g in group EI (Fig. 1), compared with 39 % and 34 % in group EII and K, respectively. The proportion of the group of fish with a medium mass (14.0±4.0 g) in the group EI was 39 %, in group EII – 25 %, in control (K) group – 30 %. The proportion of small fish with a mean mass at 10.0±3.0 g in EI was 16 %, and in groups EII and K – 36 %.

At the smoltification time in group EI there were 97 % smolt, compared to 90 % and 87 % in groups EII and K, respectively (Fig. 2).

There were significant differences in mortality between the EI and K group (P=0.035), but no significant differences between the EII and K group (P=0.796) (Fig. 3). Mortality increased in summer and decreased in autumn. Salmon mortality slightly increased in June, sharply increased in July and achieved in groups EI, EII, and K 16 %, 21 % and

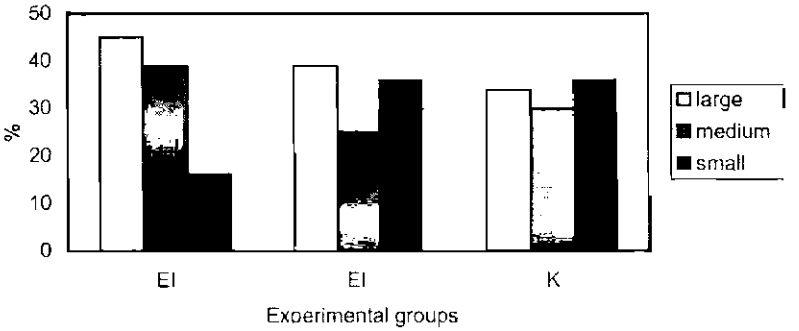

Fig. 1. Mass distribution of fish. Large, fish average mass 20.0±2.0 g; medium, fish with average mass 14.0±4.0 g; small, fish with mass less than 10.0 g.

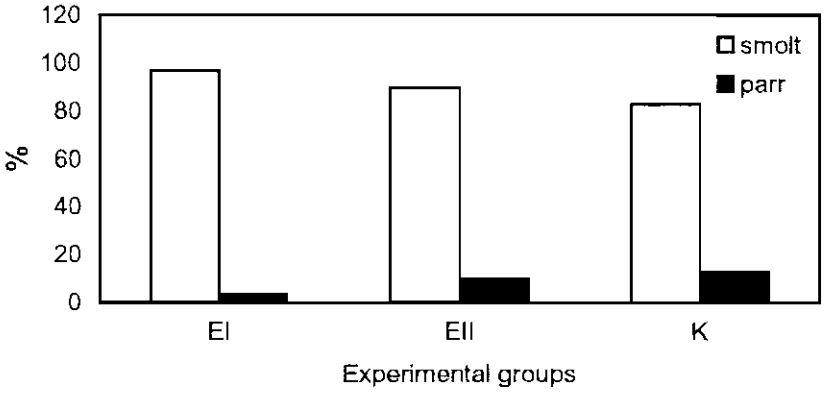

Fig. 2. Relative numbers (%) of smolt and parr in experimental groups in May.

20 %, respectively. Mortality decreased from the beginning of August. In June there was a lower mortality in the group EII – 2 %, in July in the group EI – 17 %. The highest mortality in the summer was in the group K (9 % in June, 19 % in July, 6 % in August; Fig. 3). The highest overall survival of salmon smolt was in group EI. The winter mortality in other groups was similar, due to the beneficial effect of cold water. The highest survival of fish was in the group where 0.5 g yeast glucan per kg of food was added.

Clinical examination showed signs of myxobacteriosis in some fish in every pond. Bacteriological examination confirmed the diagnosis of myxobacteriosis and *Flexibacter* spp. bacteria was isolated.

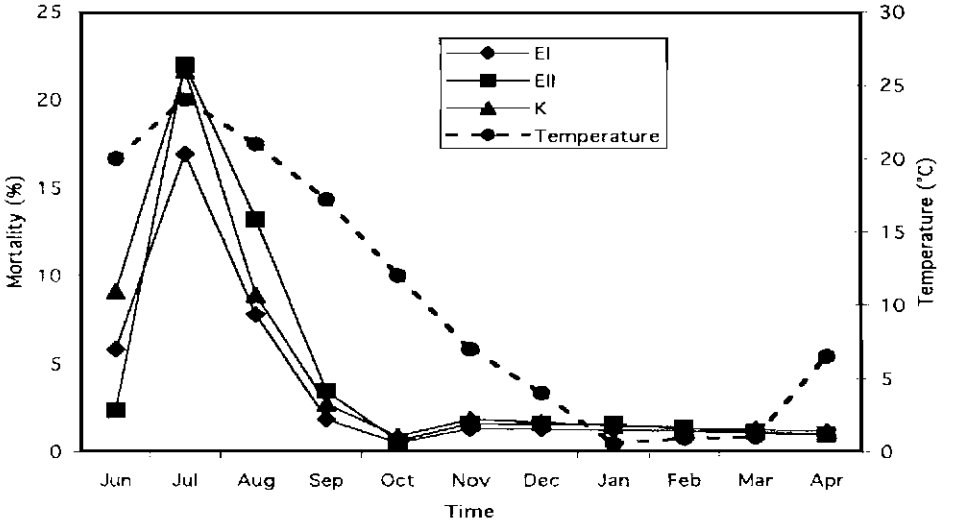

Fig. 3. Fish mortality in experimental groups and water temperature. EI, group with *Aqualase Two* 0.5 g kg^{-1}; EII, group with *Aqualase Two* 1 g kg^{-1}; K, control group. There were significant differences between the EI and K groups in July - September ($P=0.035$), but no significant differences between the EII and K group ($P=0.796$).

Discussion

Sockeye juveniles in fresh water have nearly the same growth efficiency on 3 % of a body mass diet at 5 °C as they have on a 6 % diet at 15 °C (Brett 1970). In our experiment, the rate of growth increased in September, decreased from December to February, and then increased again. Previous investigations have shown that there was no gain in mass in shrimps fed a glucan diet (Scholz et al. 1999). We found significant differences between body mass for the two experimental groups and the control group in spring in our experiment.

In the experiments with crayfish, glucan did not promote mass gain and immunity (Scholz et al. 1999). *Aqualase Two* treatment of salmon in summer months resulted in a rapid gain of mass in autumn when the water temperature approached the salmon comfort level. The obtained difference of gain of mass remained for the whole winter period.

Treatment of salmon with glucan per os decreased the mortality after infection with *Aeromonas salmonicida* by 10 % (Dalmo, Seljelid 1995). The mortality decreased by about 20 % when glucan was injected compared within the control group. In contrast, the resistance of shrimps fed with glucan diet decreased (Scholz et al. 1999). In our investigation when 0.5 g *Aqualase Two* per kg of food was added to the diet, mortality was about 19.7 % lower than in the control group. When 1 g *Aqualase Two* per kg of food was added, mortality was about 2.3 % lower than in the control group.

In conclusion, the results of our study showed that immunostimulation with a ß-glucan yeast preparation in a form of *Aqualase Two* can be used to improve growth in industrial conditions

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Laša mazuļu audzēšanas efektivitātes paaugstināšana, izmantojot barības piedevu *Aqualase Two*

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Kopsavilkums

Valsts zivjaudzētavā "Tome" un Latvijas Zivsaimniecības pētniecības institūta Akvakultūras laboratorijā tika pētīta ar rauga β-glukānu bagātinātās barības piedevas *Aqualase Two* ietekme uz Baltijas laša (*Salmo salar*) mazuļu augšanu un attīstību. Eksperimentā izmantoti 30 000 laša mazuļu no kāpura līdz smolta stadijai. Laša mazuļiem trīs mēnešus (no 1. jūnija līdz 31. augustam) tika izēdināta barības piedeva *Aqualase Two*, aprēķinot divas dažādas devas: 0.5 un 1.0 g uz vienu kilogramu barības. Zivju veselības stāvoklis tika kontrolēts visā to audzēšanas periodā. Mūsu pētījumi parāda, ka izmēģinātais preparāts mazina zivju mirstību tā izbarošanas laikā, bet augšanas tempu paātrina rudenī. Kopumā, salīdzinot eksperimenta un kontroles grupas lašus, vislabākie rezultāti iegūti zivju grupā, kurai barībā ir pievienota piedeva *Aqualase Two* 0,5 g uz kg barības. Šīs grupas zivīm ir viszemākā mirstība, lielākais svara pieaugums un viengadnieku-smoltu iznākums. Eksperimenta rezultāti ļauj ieteikt barības piedevas *Aqualase Two* izmantošanu laša mazuļu audzēšanai rūpnieciskos apstākļos.

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Changes of the secretory system of leaf epidermal and mesophyll cells during stress and programmed cell death

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Abstract

Comparison was made between the effects of ethylene-induced programmed cell death and leaf senescence-caused programmed cell death on the Golgi apparatus and secretory pathway in tobacco leaves. Transgenic tobacco (*Nicotiana tabacum* L.) plants containing jellyfish green fluorescent protein (GFP) fused to rat sialyltransferase located in Golgi bodies and endoplasmic reticulum were used. Forty-day old plants were sprayed with Ethephon at concentrations of $5x10^{-3}$ M or $5x10^{-2}$ M. We observed cells from intact leaves and leaf sections cultivated in medium. Intact control plants and sections from expanding and mature leaves cultivated in Murashige and Skoog basal medium showed a high number of vectorially moving and oscillating Golgi bodies. Programmed cell death caused by leaf senescence or induced by Ethephon treatment caused a decrease of GFP fluorescence. Golgi bodies were located in the basal part of palisade parenchyma cells in senescent leaves but Ethephon treatment caused an increase of the number of Golgi bodies in the apical part of palisade parenchyma cells.

Key words: Golgi bodies, Ethephon, ethylene, Green Fluorescent Protein, *Nicotiana tabacum*, programmed cell death.

Introduction

Ethylene is a plant hormone involved in senescence and stress responses of plants. Wounding induces ethylene biosynthesis and ethylene is produced rapidly, with levels detectable within 30 min. Ethylene is an absolute requirement for the wounding response (Kende 1993; O'Donnell et al. 1996). The ethylene-releaser Ethephon causes an increase in the ethylene level in two ways: due to decomposition of Ethephon penetrating into leaf tissues and also by formation of ethylene from the endogenous pool of 1-aminocyclopropane-1-carboxylic acid by the action of ethylene-forming enzyme (Warner, Leopold 1969; Abeles 1973; Lieberman 1979; Romanovska et al. 1989; Ievinsh et al. 1990). We used Ethephon as a useful tool to increase the ethylene level in tissues and to stimulate programmed cell death in plant leaves.

The plasma membrane, vacuoles, Golgi complex and endoplasmic reticulum are involved in synthesis and binding of ethylene (Goodwin, Mercer 1983; Mayne, Kende 1986; Thompson et al. 1987; Bouzayen et al 1989; Crevecoeur et al., 1990; Hirayama ,

Alonso 2000). An increased level of ethylene causes changes in the number and activity of endoplasmic reticulum (ER) and Golgi apparatus (Osborne et al. 1985; Selga et al. 1985; Selga, Selga 2001).

Golgi apparatus and the secretory pathway have been recently visualised *in vivo* with the help of green fluorescent protein (GFP) targeted to different membrane proteins (Wee et al. 1998; Boevink et al. 1999; Hawes et al. 2001). However, the mechanisms of secretory system changes during programmed cell death are not clear.

The aim of the present work was comparison of the effect of ethylene-induced programmed cell death and leaf senescence-caused programmed cell death on the Golgi apparatus and secretory pathway in tobacco leaves.

Materials and methods

Transgenic tobacco (*Nicotiana tabacum* L.) plants contained jellyfish green fluorescent protein (GFP) fused to rat sialyltransferase located in Golgi bodies and endoplasmic reticulum (Andreeva 2000).

Plant seeds were disinfected with 70 % ethanol and plated on Murashige and Skoog (MS) basal salt medium (Sigma) containing 0.8 % agar Difco (Murashige, Skoog 1962). After incubation for 2 weeks at 20-22 °C, the seedlings were transferred to soil. Plants were grown in a greenhouse with a photoperiod of 17/7 h, 60 to 80 % relative humidity and 21±5 °C.

We compared expanding leaves, full grown leaves, yellowing leaves and leaves treated with Ethephon. We considered expanding leaves to be ones with an area not exceeding 1/4 of the area of full-grown leaves.

Treatment with Ethephon was realised in the following way: 40 day old plants were sprayed with a water solution of Ethephon (Cerone, Rhone-Poulenc Ltd.) at a concentration of $5x10^{-3}$ M or $5x10^{-2}$ M. Two and fourteen days after the treatment segments among veins from full grown leaves were cut and mounted on glass slides in a droplet of MS basal salt medium Sigma M5519 (4.4 g l^{-1}) and observed under a confocal microscope Zeiss CLSM 410. At the same time, we compared morphology of expanding leaves, full-grown leaves, leaves treated with Ethephon and senescent leaves.

The effect of wounding stress and cultivation in the MS basal medium was examined to evaluate the effect of cutting procedure on fluorescence intensity and number of Golgi bodies in cells of the upper epidermis and palisade mesophyll.

Transverse-sections (thickness = 40 to 50 μm) were cut with razor blade and mounted on glass slides in a droplet of MS basal medium M5519 (4.4 g l^{-1}). Sections were analysed by a confocal microscope at 30 min intervals during 5 h and after 48 h and compared with cells from intact leaves.

Confocal imaging with time-lapse scanning was performed on intact plant leaves emerged into the water on glass slides, leaf sections mounted into water or MS medium. Images were analysed with LSM Dummy (Zeiss) or Zeiss Image Browser 5.1 and cell size measured with Scion Image. We measured size of 300 epidermal cells from 10 leaf samples of control plants and 300 epidermal cells from 10 samples cultivated in MS medium for 48 h.

Location of Golgi bodies was analysed in 3 samples from 3 different leaves of each variant. Fluorescence intensity of samples was analysed with a Zeiss CLSM 410 using

the same objective and laser settings in the area of 512x512 pixels with software LSM Dummy (Zeiss).

Results

Comparing fluorescence, size and distribution of Golgi bodies in intact full grown leaves and leaf sections (d=20 mm), differences were not found. Intensity of fluorescence was variable among different leaves and leaf sectors in intact tissues. Cells showed intense fluorescence of ER in the cell cortex, and intense fluorescence of Golgi bodies. Some cells showed weak fluorescence of ER and Golgi bodies. In epidermal cells, most of the Golgi bodies were located among chloroplasts in the cortical region of the cell (Fig. 1A). The nuclear envelope also showed fluorescence and it was possible to observe nuclei in the lateral part of epidermal cells or at the bottom of the cell. Golgi bodies appeared to move between the nuclear envelope and the periphery of the cell.

In fully grown mesophyll cells of the spongy parenchyma, Golgi bodies were observed in the part of the cell close to epidermal cells (Fig. 1A). The view from above allowed to observe only 30 μm from the top of palisade parenchyma cells. This layer contained very few chloroplasts. In the deeper layer Golgi bodies were located among chloroplasts and close to the cell wall or tonoplast. Golgi bodies were randomly distributed in the mesophyll cells (Table 1). Golgi bodies were observed that appeared to move closely along chloroplasts. Movement was both vectorial and oscillatory.

When leaf segments were cultivated in MS medium, fast growth of leaf segments was observed. Area of leaf pieces during 48 h of incubation increased 2.6 times but the

Fig. 1 (in supplement). Cells of palisade parenchyma and upper epidermis of tobacco leaves in different stages of differentiation. Scale bar 20 μm. GFP fluorescence – green, chlorophyll fluorescence – red. A, Upper epidermis and palisade parenchyma cells of intact full-grown tobacco leaves. Intense green fluorescence of Golgi bodies and nuclear envelope (white arrow). B, Upper epidermis of intact mature tobacco leaves cultivated 24 h in Murashige and Skoog basal medium. High number of fluorescent Golgi bodies. Small Golgi body and large Golgi body (white arrows). C, Upper epidermis expanding tobacco leaves. High number of fluorescent Golgi bodies. D, Upper epidermis of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-4}$ M. Cells of upper epidermis with variable fluorescence and decreased number of Golgi bodies. E, Upper epidermis of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M. Cells of upper epidermis with weak GFP fluorescence in the cortical cytoplasm (white arrow), no Golgi bodies are present. F, Upper epidermis of yellowing tobacco leaves. Cells of upper epidermis with weak GFP and chlorophyl fluorescence in the cortical cytoplasm, no Golgi bodies are present. G, Palisade parenchyma cells of expanding tobacco leaves. High number of randomly located fluorescent Golgi bodies (white arrow). H, Palisade parenchyma cells of intact mature tobacco leaves. Basal location of Golgi bodies increases. I, Palisade parenchyma cells of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M. Apical location of Golgi bodies increases (white arrow). J, Palisade parenchyma cells of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-2}$ M. Weak GFP fluorescence in the cytoplasm, small number Golgi bodies are present (white arrow). K, Palisade parenchyma cells of yellowing tobacco leaves. Cells with weak GFP and chlorophyl fluorescence in the cytoplasm, basal location of Golgi bodies increases. L, Palisade parenchyma cells of leaves 2 days after the treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M containing Golgi body in the vacuole (white arrow).

Table 1. The percentage of the total number of Golgi bodies of a cell in the apical, medial and basal part of palisade parenchyma cells of tobacco leaves at different developmental stages. Values are the means ±SE of three different plants with three replicates each

Zone of the leaf	Expanding leaves (%)	Mature leaves (%)	Yellowing leaves (%)	Mature leaves treated with Ethephon ($5x10^{-3}$ M) (%)
Apical	29.3±4.3	24.6±5.2	17.3±2.8	46.2±3.7
Medial	40.1±5.2	42.7±6.3	36.2±4.5	38.1±2.9
Basal	30.6±3.1	42.7±4.1	46.5±3.9	16.7±3.1

average area of epidermal cells increased by 65 %. The fluorescence of these segments gradually increased during the first 3 h of cultivation. The increase was due to an increase of fluorescence of chloroplasts, Golgi bodies and cortical ER. Enhanced GFP fluorescence was stable for a week. Afterwards, it decreased till the level of control plants. This experiment showed that we can use transverse sections of leaves as models to analyse the structure of palisade parenchyma cells.

The number and size of Golgi bodies increased both in mesophyll cells and epidermal cells after 24 h of cultivation (Fig. 1B). Active fusing and splitting of Golgi bodies were typical. Many transvacuolar strands were formed in epidermal cells and small and bright Golgi bodies moved in different parts of both mesophyll and epidermal cells. Movement of Golgi bodies was similar to intact plants.

Expanding leaves were typical with a higher number and fluorescence of Golgi bodies in epidermis and palisade parenchyma than in full-grown leaves (Fig. 1C, G). An active joining and separation of Golgi bodies to nuclear envelope typically occurred. Golgi bodies were randomly distributed in the mesophyll cells (Table 1). Transvacuolar movement of Golgi bodies in epidermis was observed.

Fluorescence of yellowing leaves (Fig. 1F, K) varied. Different stages of loss of GFP fluorescence in cortical ER and Golgi bodies appeared. Fluorescence was observed in guard cells, at the bottom of epidermal cells and at the top of mesophyll (Fig. 1K). Epidermal cells contained a decreased number of Golgi bodies that showed oscillatory movement (Fig. 1F).

Large and disperse fluorescent bodies appeared in mesophyll cells. Sometimes they moved along chloroplasts and through transvacuolar strands. Transverse sections showed that the number of Golgi bodies is higher on the bottom of palisade mesophyll cells in comparison with the top of cells (Table 1). Different cells manifested different levels of decrease in secretory activity.

Treatment of plants with Ethephon in a concentration of $5x10^{-3}$ M did not alter the morphology and anatomic structure of mature leaves but caused gradual senescence of leaves for 3 weeks after treatment.

The response of epidermal cells was different. The first group of epidermal cells gradually lost fluorescence of cortical ER and the number of Golgi bodies decreased (Fig. 1D). In another group the number and size of Golgi bodies increased. Golgi bodies were typical in the apical part of mesophyll cells (Fig. 1D, Table 1). Most of them moved

between the vacuole and plasma membrane. Sometimes, Golgi bodies appeared in the vacuole (Fig. 1L).

The intensity of fluorescence of tissues gradually decreased during the first week but increased during the second week after treatment, due to a change of GFP fluorescence. At that time the number of mesophyll cells with Golgi bodies decreased. Large and loosely structured Golgi bodies moved between the vacuole and cell wall, close to chloroplasts and the inside of the vacuole. Co-localisation of Golgi bodies and chloroplasts became typical (Fig. 1I), appearing as a changing colour of Golgi bodies from green to yellow.

After treatment with a solution of Ethephon at a concentration of $5x10^{-2}$ M, death of leaf segments appeared but some cells survived. These remaining cells started active growth and division three days after the treatment. In this case, two days after treatment we observed weak fluorescence in the ER of epidermal cells, but fluorescence of tissues was weak. The result was loss of GFP in the mesophyll and weak fluorescence of chloroplasts for most of the cells (Fig. 1E, J). Seven days after the treatment fluorescence started to increase and several epidermal and mesophyll cells showed the presence of Golgi bodies in the periphery of cells. The leaf blade expanded actively during two following weeks and cells showed an increase of fluorescence that was similar to that in the control plants. We observed a large number of Golgi bodies in the cortical part of epidermal cells but a small number of Golgi bodies in the mesophyll cells. Golgi bodies moved along transvacuolar strands and in few cases appeared in the centre of the vacuole or moved from the vacuole to the cortical part of the cell.

At the same time, several groups of cells did not lose GFP fluorescence. Few epidermal cells showed an active transport of Golgi bodies along the cell wall and between the nucleus and the cell wall through transvacuolar strands and between the cortical part of the cell and vacuole. In mesophyll cells Golgi bodies were located between the tonoplast and chloroplasts. Sometimes they appeared near the cell wall. After 24 h and 48 h, cells showed the features described above.

Discussion

Location and movement of Golgi bodies in epidermis of control plants is similar to that previously described (Hawes et al. 2001). Observation of palisade parenchyma cells is difficult due to the fact that in thick samples ($l > 50$ μm) a laser beam loses light intensity. Thus, in vivo it is only possible to observe thin leaves or upper and lower epidermis, spongy mesophyll and the top of palisade mesophyll cells.

Mesophyll cell cultures are widely used to study programmed cell death and terminal differentiation (Sheen 1995; Lam et al. 2001). This experimental system permits to observe changes in gene expression and proteins during stress-induced programmed cell death (Kovtun et al. 2000; Tena et al. 2001). The preparation of mesophyll cell cultures involves damage of the cell wall (Sheen 1995). The study of the secretory system of mesophyll cell culture during stress is restricted because Golgi bodies could be more involved in the formation of the cell wall.

Comparison of cells from leaf segments and segments cultivated in Murashige and Skoog basal medium with cells of intact plant leaves shows that wounding stress does not damage Golgi bodies and does not disturb the transport system of these cells. In all cases Golgi bodies are visible, and we observed their vectorial movement, indicating an

unchanged cytoskeleton.

In all observed samples we observed bright GFP fluorescence in the nuclear envelope. In addition, bi-directional moving of Golgi bodies from cortical cytoplasm to nuclei was typical, indicating that the surface of nucleus in tobacco leaves plays an important role in the secretory pathway. In *Toxoplasma gondii*, microtubule inhibitors or dithiothreitol have been observed to disrupt Golgi, causing swelling of the nuclear envelope (Hager et al. 1999).

A typical feature of programmed cell death of leaves either caused by terminal differentiation programme or induced by stress is the activation of proteolytic cascade (Lam et al. 2001; Tena et al. 2001). This can explain the decrease of GFP fluorescence intensity in all cases of observed programmed cell death.

However, 4 to 7 % of epidermal and palisade parenchyma cells retained GFP fluorescence intensity at a level similar to that in mature leaves. This shows that these cells retained high secretory activity and inhibited programmed cell death.

Golgi stacks can actively move along actin filaments and microtubules using motor proteins (Nebenführ, Staehelin 2001). In preparation for cell division, the Golgi stacks redistribute to the perinuclear cytoplasm, but during cytokinesis, this distribution changes and a higher density of Golgi stacks is found near the phragmoplast, the site of cell plate formation (Nebenführ et al. 2000).

Analysis of the distribution of Golgi bodies in cells of expanding and full-grown leaves as well as undergoing programmed cell death showed different locations of Golgi bodies (Table 1). Palisade parenchyma cells of expanding leaves showed random distribution of Golgi bodies. In contrast, yellowing and mature leaves with secretory activity at the basal part of the cell were typical. This suggests secretion of cell products to the apoplast and leaf veins. However, the data from expanding leaves are not precise due to the fast vectorial movement of Golgi bodies.

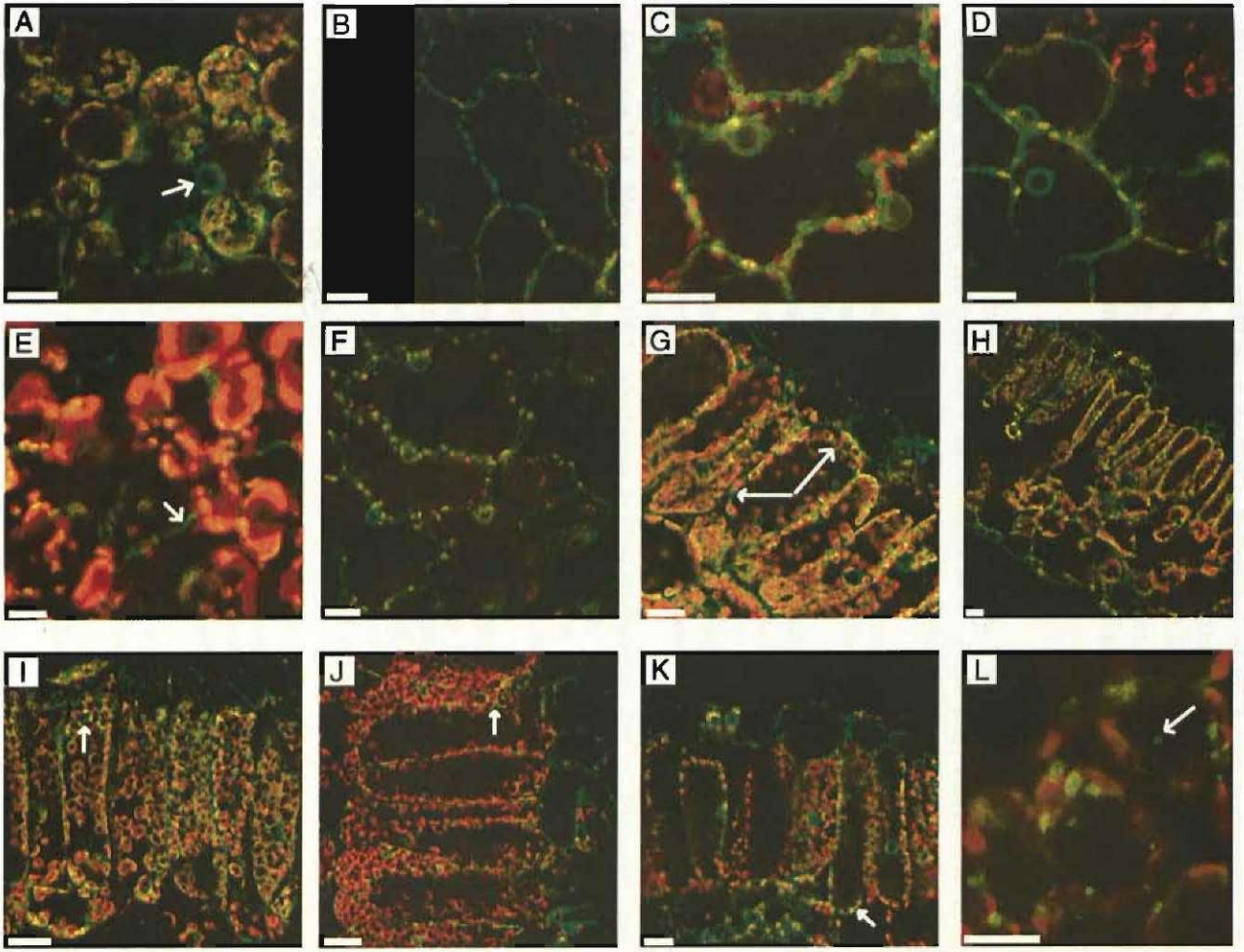
Ethephon in a concentration of 5×10^{-3} M induced activation in the secretory system in the apical part of the palisade parenchyma cells. The number of large Golgi bodies increased, suggesting that cells during stress excrete toxic metabolites into the apoplast and out of leaves. Co-localisation of chloroplasts and Golgi bodies became typical. This supports previous observations that ethylene induces the appearance of transport vesicles in the narrow (20 to 100 nm) space between chloroplasts and both cis and trans faces of Golgi bodies (Selga, Selga 2001).

Ethephon in a concentration of 5×10^{-2} M induced fast disappearance of GFP and chloroplast fluorescence. This can be explained by a hypersensitive response of cells.

The presented calculations of the number of Golgi bodies can show only trends of their distribution. Electron microscopic analysis of the location of Golgi bodies in mesophyll cells is required to diminish uncertainties due to vectorial movement of Golgi bodies and their impact on the number of visible Golgi bodies.

Programmed cell death induced with Ethephon differs from that of yellowing, in the direction of transport of secretory products. Yellowing causes transport towards leaf veins but Ethephon induces secretion towards leaf surface.

All observed cases of programmed cell death suggest that there exists a population of leaf cells that does not enter cell death programme.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Lapu epidermas un mezofila šūnu sekretorās sistēmas izmaiņas programmētās šūnu bojāejas procesā

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Kopsavilkums

Darbā salīdzināja Goldži kompleksa un sekretorās sistemas izmaiņas lapu novecošanas un etilēna izraisītās programmetās šunu bojaejas procesā. Petījumos izmantoti transgēni tabakas (*Nicotiana tabacum* L.) augi, kas satur zaļo fluorescento proteīnu (GFP), pievienotu pie žurku sialiltransferāzes, kas lokalizēta Goldži ķermenīšos un endoplazmatiskajā tīklā. Četrdesmit dienas vecus augus apsmidzināja ar etefonu $4x10^{-4}$ M vai $4x10^{-3}$ M koncentrācijā. Petīja šunas, kas atradās intaktu augu lapās vai lapu griezumos, kuri bija ievietoti Murišiges un Skūga sāļu barotnē. Visvairāk vektorjāli un oscilējoši kustīgu Goldži ķermenīšu bija intaktu augu, barotnē ievietotu augošu un izaugušu lapu griezumu šūnās. Programmēta šūnu bojāeja visos gadījumos izraisīja GFP fluorescences samazināšanos. Goldži ķermenīši visbiežāk bija novecojošu lapu zedeņu parenhīmas šūnu bazālajā daļā, bet etefona apstrāde izraisīja Goldži ķermenīšu skaita palielināšanos zedeņu parenhīmas šūnu apikālajā daļā.

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Possible role of trichomes in resistance of strawberry cultivars against spider mite

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Abstract

The trichome types on leaves of garden strawberry (*Fragaria* × *ananassa*) were analyzed. Two types of trichomes was described. The first were unicellular long and thin simple trichomes, located mainly on leaf veins and leaf margins, mostly on the underside of the leaf. The second were smaller multicellular uniseriate glandular trichomes. These trichomes consisted of one basal epidermis cell, several stalk cells, and a single rounded head cell. Strawberry cultivars with different resistance against two-spotted spider mite (*Tetranychus urticae* Koch) were used to test a hypothesis that higher pubescence in strawberry cultivars is correlated with a higher degree of resistance against the herbivore. Nonglandular trichomes were not the resistance factor for strawberry cultivars against spider mite because trichome density was affected by growth conditions and developmental state. It was concluded that gladular trichome-localized inducible responses are among the potential resistance mechanisms against spider mite in garden strawberry.

Key words: Glandular hairs, polyphenol oxidase, garden strawberry, spider mite, trichomes.

Introduction

Morphological features of plant leaves are known to affect herbivores (Peters, Berry 1980). Among them, foliar trichomes are unicellular or multicellular structures arising from the epidermal tissues (Larkin et al. 1996). From a point of view of constitutive plant defences against herbivores, trichomes can be treated as a structural defence detering herbivores. Due to the large diversity of trichome types on different plant species, it is difficult to generalize any specific role of these structures in herbivore defence. However, it is generally believed that pubescent leaves will be more resistant against herbivores than less pubescent ones of the same species due to mechanical restrictions to herbivore activities caused by a high density of trichomes (Levin 1973). Thus, nonglandular trichomes are expected to act as a physical barrier to herbivores. However, experimental data does not always support this relationship.

Spider mite, *Tetranychus urticae*, is one of the most widespread pests on strawberry foliage. The data on the correlation between pubescence (the number of nonglandular trichomes) and resistance against spider mites are mostly contradictory. Resistance to *T. urticae* is positively correlated with increased pubescence in *Buddleia* L. taxa (Gillman et al. 1999). In addition, removal of trichomes increased oviposition of mites. It has been shown also that an increase in density of nonglandular trichomes for *Fragaria chiloensis*

Duch. decreased the number of eggs laid by *T. urticae* (Luczynski et al. 1990). Further, for cotton plants, cultivars with a higher number of highly branched trichomes had higher resistance against *T. urticae* (Kamal, Elkassaby 1965). However, experiments with other cultivated plants revealed a negative correlation between the density of nonglandular trichomes and the resistance against *T. urticae* (Kishaba et al. 1972; Yiem et al. 1993).

Chemical defence against herbivores may be associated with a different type of trichome e.a., glandular trichomes, usually containing putative defense metabolites (Levin 1973). However, the information in the literature on particular roles of glandular trichomes in plant resistance against herbivores is extremely limited.

Several studies have indicated significant differences in the tolerance levels of garden strawberry cultivars to *T. urticae* (Ferrer et al. 1993; Shanks et al. 1995; Shanks, Moore 1995; Petrova et al. 2000). Recently we have shown that cv. 'Zephyr', partially resistant to *T. urticae*, is characterized by a higher activity of wound-induced polyphenol oxidase and peroxidase in comparison to the more susceptible cv. 'Korona' (Steinite, Ievinsh 2002). Since the main proteinaceous component of glandular trichomes is polyphenol oxidase (Kowalski et al. 1992), it can be to suggested that the higher resistance of certain strawberry cultivars against *T. urticae* is associated with a trichome-localized inducible increase of polyphenol oxidase and peroxidase activities.

In spite of the idea that foliar pubescence might be related to spider mite resistance in strawberry (Kishaba et al., 1972), there is no detailed data available in the literature on trichome morphology in garden strawberry. Therefore, the aim of the present investigation was to analyse trichome types on leaves of strawberry and to test the hypothesis that higher pubescence in strawberry cultivars is correlated with a higher degree of resistance against the spider mite, *T. urticae*.

Materials and methods

Eight commercially grown garden strawberry (*Fragaria* × *ananassa*) with different resistance against two-spotted spider mite (*Tetranychus urticae* Koch) were used for the experiments: 'Zephyr', 'Venta', 'Tenira', 'Induka', 'Bogota', 'Senga Sengana', 'Kokinskaja Pozdnaja', and 'Korona' (Petrova et al. 2000). Plants were propagated in plant tissue culture on Murashige-Skoog micro- and macro-salt medium without hormones supplemented with glycine and vitamins (Steinite, Ievinsh 2002). Plants were grown in a greenhouse under natural light and temperature conditions for 3 months in plastic boxes containing commercial peat mixture with mineral nutrients. The experiment was conducted in June and randomly selected leaves were collected from plants to use in a disk bioassay. A sample for bioassay consisted of three leaf disks (14 mm in diameter) cut from adult leaves at the base of each leaf using a cork borer. All leaf disks were examined under a binocular microscope MBC-10 (magnification 14 x) from both surfaces. An additional leaf disk bioassay was performed with 'Zephyr' and 'Korona' plants in October.

To investigate the morphology of trichomes, fresh plant material from tissue culture as well as from greenhouse-grown strawberry plants was subjected to light microscopy. Epidermal peels from the underside of leaves were examined (magnification 250 x for Fig. 1 and 1000 x for Fig. 2).

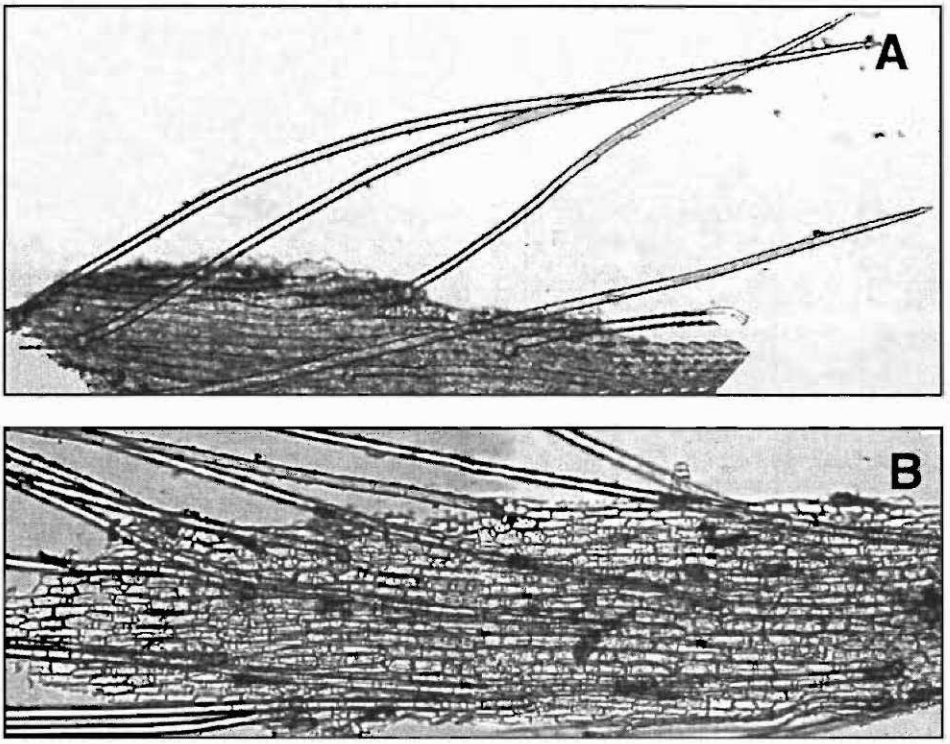

Fig. 1. Unicellular trichomes on leaves of strawberry cultivars 'Korona' (A) and 'Zephyr' (B).

Results

Strawberry leaves had two types of trichomes. The first were unicellular simple trichomes, long and thin, 0.8÷1.0 mm in length (Fig. 1). These trichomes were located mainly on leaf veins and leaf margins, mostly on the underside of the leaf. The second were smaller multicellular uniseriate glandular trichomes, 0.04÷0.05 mm in length, belonging to a type of a capitate glandular trichome (Fig. 2). These trichomes consisted of one basal epidermis cell, several stalk cells (3÷4), and a single rounded head cell.

Glandular trichomes differed in number and colour. Strawberry plants grown in vitro had a low number of glandular trichomes with colourless head cells on their leaves (Fig. 2A and B). For greenhouse-grown plants of the susceptible cv. 'Korona', the glandular trichomes were mostly colourless (Fig. 2C). In contrast, plants of the resistant cv. 'Zephyr' had a high number of glandular trichomes with dense red-brown colouring in their head cells (Fig. 2D).

To test for a relationship between the number of unicellular trichomes and resistance against spider mite, trichome density was measured for different strawberry cultivars grown in the greenhouse. For greenhouse grown plants in June, more susceptible cultivars in general had a higher number of trichomes on the underside of leaves than resistant

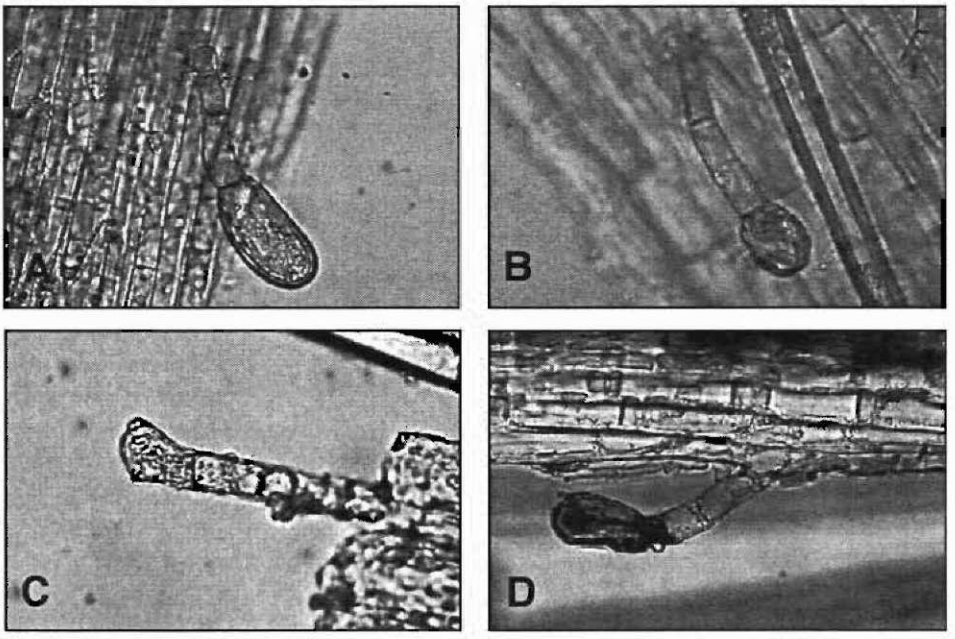

Fig. 2. Glandular trichomes on leaves of strawberry cultivars 'Korona' (A) and 'Zephyr' (B) grown in tissue culture and cultivars 'Korona' (C) and 'Zephyr' (D) grown in the greenhouse.

cultivars (Table 1). However, the relationship was not supported for several cultivars with partial resistance vs. partial susceptibility. When strawberry leaves were collected from greenhouse grown plants in October, the resistant cv. 'Zephyr' had a higher number of unicellular trichomes than the susceptible cv. 'Korona' on both sides of different types of leaves (Table 2).

Table 1. Number of unicellular trichomes on different sides of leaves of strawberry cultivars with different susceptibility vs. resistance against *T. urticae* grown in greenhouse conditions in June. Data are the means from 20 samples, 3 measurements each, for every data point ± S.E.

Cultivar	Susceptibility vs. resistance	Number of trichomes cm^{-2}	
		underside	overside
'Senga Sengana'	susceptible	309±38	0
'Kokinskaja Pozdnaja'	susceptible	289±21	0
'Korona'	susceptible	217±17	<1
'Venta'	resistant	200±26	15±4
'Zephyr'	resistant	188±21	22±4
'Tenira'	resistant	174±18	2±1
'Induka'	partially resistant	85±13	0
'Bogota'	partially susceptible	71±8	2±1

Table 2. Number of unicellular trichomes on different sides of leaves of strawberry cultivars grown in greenhouse conditions and in October. Data are the means from 20 samples, 3 measurements each, for every data point ± S.E.

	'Zephyr', resistant		'Korona', susceptible	
	underside	overside	underside	overside
On the leaflet of the lower leaf	2385±180	51±6	1298±128	9±3
On 1 disk of the lower leaf	244±20	49±5	62±9	3±2
On 1 disk of a growing leaf	132±15	37±4	132±20	<1

Discussion

Spider mites commonly feed in an inverted position on the underside of leaves. Therefore, to fulfill the hypothesis that higher pubescence is correlated with a higher degree of resistance against the spider mite, only trichomes located on the underside of the leaves should to be taken into the account. For strawberry plants grown in the greenhouse and collected in October, the resistant cv. 'Zephyr' had a higher number of unicellular trichomes on both sides of leaves than the susceptible cv. 'Korona' (Table 2). This relationship was not found for strawberry plants grown in greenhouse conditions in June, where more susceptible cultivars in general had a higher number of unicellular trichomes on the underside of their leaves than the more resistant ones (Table 1). However, for other arthropod herbivores, there is evidence of a defensive role of nonglandular trichomes. For example, a study on the role of soybean pod trichomes demonstrated that densely pubescent soybean has the potential to resist bean leaf beetle feeding on pods (Lam, Pediogo 2001).

Based on the present experiments, it appears that growth conditions and developmental state are among the significant factors regulating unicellular trichome density in strawberries. In Arabidopsis, trichome development is under a tight genetical control (Larkin et al. 1996). On the other hand, an increase of trichome density after herbivore attack is one of the induced defence responses (Mauricio et al. 1997; Agrawal 1998).

In light of the present experiments, it appears that nonglandular trichomes are not the resistance factor for strawberry cultivars but rather, the glandular trichomes containing oxidative enzymes. Although the number of glandular trichomes was not measured for strawberry cultivars with different susceptibility against *T. urticae*, plant leaves of the resistant cv. 'Korona' had dense red-brown colouring in head cells of glandular trichomes in contrast to colorless head cells for trcihomes on the susceptible cv. 'Zephyr' (Fig. 2). So far, only a few investigations have focused on the relationship between glandular trichomes and mite life histories. It has been shown that glandular hairs are associated with resistance in tobacco (Patterson et al. 1974) and tomatoes (Rodriguez et al. 1972). In addition, the glandular trichomes on alfalfa appeared to provide the major host resistance factor, with the resistance to adult leafhoffers being chemically based and resistance to nymphs being chemically and mechanically based (Ranger, Hower 2001).

It was shown that the main proteinaceous component of glandular trichomes of cultivated solanaceous plants is polyphenol oxidase, accounting for as much as 50-70 % of the total protein (Kowalski et al. 1992; Yu et al. 1992). Glandular trichomes contain

also peroxidase activity which further contributes to oxidative reactions (Levin 1973). Most likely, oxidative enzymes in trichomes are involved in oxidation and polymerization of phenolic constituents released from herbivore-damaged trichomes, which in turn act as a physical barrier preventing them from feeding. Previously it was thought that trichomes function primarily as a constitutive defense (Levin 1973). However, recently it was demonstrated that rapid trichome-localized chemical changes are part of induced defense against herbivores (Laue et al. 2000; Traw, Dawson 2002). The present data, together with previous results that strawberry cultivars more resistant to *T. urticae* are characterized by a higher inducible activity of oxidative enzymes (Steinite, Ievinsh 2002), indicate that gladular trichome-localized inducible responses are among the potential resistance mechanisms against spider mite in garden strawberry. More experimental evidence is needed to verify the hypothesis that induction of oxidative enzymes in glandular trichomes on strawberry leaves by *T. urticae* feeding is a main resistance factor against the herbivore.

Acknowledgements

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Lapu matiņu iespējamā piedalīšanās zemeņu šķirņu izturībā pret tīklērci

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Kopsavilkums

Analizēja lapu matiņu veidus uz dārza zemeņu (*Fragaria* × *ananassa*) lapām. Aprakstīti divu veidu lapu matiņi. Pirmkārt, gari un slaidi vienšūnas matiņi, kas izvietoti uz lapu dzīslām un malām pārsvarā lapu apakšpusē. Otrkārt, mazāki daudzšūnu dziedzermatiņi. Šie matiņi sastāvēja no vienas epidermālās pamatšūnas, vairākām kāta šūnām un vienas apaļas galviņas šūnas. Zemeņu šķirnes ar dažādu izturību pret tīklērci (*Tetranychus urticae* Koch) tika izmantotas, lai pārbaudītu hipotēzi par to, ka paaugstināts matiņu daudzums korelē ar attiecīgās šķirnes palielinātu izturību pret šo augēdāju. Vienšūnas matiņi nebija zemeņu šķirņu izturības faktors pret tīklērci, jo to biežums bija atkarīgs no augšanas apstākļiem un attīstības stadijas. Secināja, ka dziedzermatiņos lokalizētas inducējamās atbildes reakcijas ir viens no tiem mehānismiem, kas nodrošina dārza zemeņu izturību pret tīklērci.

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A new approach to study the origin of genes and introns

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Abstract

Eukariotic genomes have two main structural components – different types of repetitive nucleic acids and unique, at first glance nonrepetitive sequences of gene coding parts. A new methodology of sequence analysis based on the structure of the second genetic code can be used to reveal molecular relics in protein and gene structures of tubulins and small G proteins. These are sequences formed of repeat units having identical regularity, as well as ancestral immobile introns distributed in the exon row with the same regularity. A new theory is advanced explaining exon and intron origin from common precursors – highly repetitive simple structure nucleic acids – during the early periods of evolution.

Key words: Multiplication of nucleotides, old ancestral introns, origin of introns, repeat units of genes and proteins, small G proteins, tubulins.

Introduction

During the last decades mainly two main concepts – the exon theory of genes (Gilbert 1987) and the insertional theory of intron origin (Stoltzfus et al. 1994) – have been used to find an answer to the question, whether introns were the media of gene formation by exon shuffling or whether they were inserted later. The problem, however, is not yet solved (Logsdon 1998).

The model of the second genetic amino acid interaction code (or the codon root code; Chipens 1991) has given a posibility to elaborate new methodologies for studies of gene and intron emergence mechanisms, based on comparative amino acid codon root analysis (CAACRA). Codon roots – the second codon letters – are much more conservative and less changed during evolution than amino acids. Twenty natural amino acids determined by the genetic code can be subdivided into four groups of the so called common-root amino acids having identical second codon letters C, G, A and U(T). Natural selection accepts amino acid exchanges in proteins (as a result of point mutations) mainly between the common-root amino acids, indicating that such amino acids are potentially tantamount (Chipens 1991). During evolution, as a result of mutations, amino acids in protein structures may change time and time again, maintaining in many cases the same codon root.

Translating gene exon row nucleotide sequences or protein amino acid sequences

to more conservative codon root sequences in separate cases makes it possible to demonstrate the retained ancestral regularity of genes and proteins. For this purpose several new methods of analysis are useful, such as, autoscanning of protein amino acid and codon root sequences, design of repeat unit piles, calculation and analysis of regularity of intron position co-ordinates and others described below.

The general principle of the advanced model of gene and consequential protein formation is that simple structure nucleotides in early period of evolution were spontaneously saltatory multiplied laterally to generate highly regular polynucleotides consisting of a large number of identical copies termed repeat units (RU). Then, RU diverged in sequences as mutations accumulated in them. At some subsequent time, a group of primary RU from the formed polynucleotide chain could be taken for another (the second step) saltatory multiplication, etc. This model is not principially new – it has been used to study the origin of satellite DNA (Southern 1975). We supplemented this model with the following new theses: (i) exons and introns arose from the same RU-multimer, (ii) formation of exons and introns was induced mainly by emergence and action of the very first splicing machinery, and (iii) splicing sites of introns have been encoded in structures of RU precursors.

Introns evolved much more rapidly than exons, notwithstanding the same rate of mutations in both. Mutations from exons were removed partially by adverse selection as well as by lethal mutations, while introns in the absence of the constraints imposed by the coding function during billions of years accumulated different mutations without any limits. Contrary to these mutations exon structures are under strong control of natural selection, which in accordance with the codon root code (Chipens 1991) accept smainly "symmetric" mutations leading to exchanges between common-root amino acids, which have identical codon roots and are located in the 2D-structure of the genetic translational code symmetrically (Chipens 1991).

Considering that periodic nucleic acids are ancesters of modern genes, the emergence of exons and introns may be easily imagined making only one essential inference. Independently of the biochemical mechanisms of the very first splicing machinery, the splicing sites can be determined only by definite nucleotide structures in the polynucleotide chain – the RU-multimer. Such sites can arise spontaneously by mutations, or alternatively they may be already acidentally encoded in structures of RU precursors. It is well known that nucleotide multiplication reactions form high molecular mass products, e.g. mouse sattelite DNA contains 105 – 108 repeat units (Southern 1975), which not undergoing the splicing after translation can form only giant protein molecules unfit for protein evolution. Evidently the driving force for the evolution of the splicing mashinery is first of all a necessity to diminish the lengh of gene ancestor coding parts. Accidental rare mutations forming splicing site structures, as we suppose, can not have effectively diminished the dimensions of gene ancestors. It more likely seems that splicing sites have been encoded in the RU precursor structures. From this key inference follows a chain of logical conclusions, which create the fundamentals of a new nucleotide multiplication theory of exon and intron origin.

If the splicing site structures are encoded in the nucleotide sequence of a RU precursor then: (i) these sites after the first and the following steps of multiplication reactions are distributed along the nucleotide chain regularly; (ii) intron positions in gene ancestors are regular; (iii) this regularity is the same as the regularity of identical nucleotides or identical

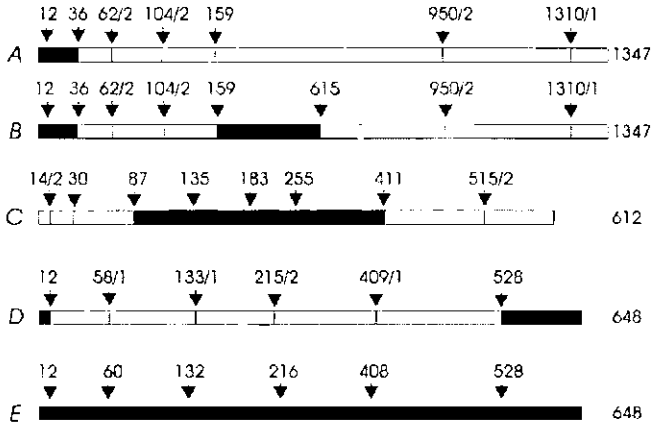

Fig. 1. Regularity of dimensions of intron and exon maps demonstrate the mechanisms of gene emergence by nucleotide multiplication reactions. A and B, intron maps of ß-tubulin genes of the *Aspergillus parasiticus* and *Aspergillus nidulans*. Intron positions are shown as arrows topped with intron co-ordinates/phases (only in cases when the phase differs from zero). Exons containing the whole number of repeat units (nx12nt) are shown as black parts of ribbons. The length of genes coding parts (exon rows, including a stop codon) is shown beside the maps. C, intron map of the green alga *Volvox carteri* gene yptV1. D and E, intron maps of the *Coprinus cinereus* ras gene before and after restoration of intron phases to phase zero (i.e. by changes of intron co-ordinates by ±1 or ±2nt).

amino acids in gene or protein structures; (iv) the birth-positions of introns are strongly determined by the size of RU – introns can be located only between RU (micro-exons) in gene "knot" points. The knot points are situated regularly along the nucleotide chain and denote borderlines of RU; (v) the reactions of nucleotide multiplications determine the formation of long open reading frames of genes with symmetric exons and all introns in a phase-0. Thus, the dominance of symmetric (0,0) exons in natural gene structures (Long et al. 1995) first of all is a signature of gene formation by nucleotide multiplication reactions. And finally (vi), coding parts of gene ancestors have been formed of a whole number of RU. From the essence of the multiplication model, which as a rule postulates formation of only symmetric (0,0) exons follows that during evolution introns can slide and be gradually eliminated, as introns of natural genes in many cases are outside of the gene knot points and have changed phases (Fig. 1).

A stable fundament for the new theory of genes is the possibility to calculate theoretical sizes of exons and an exon row, as well as the potential intron positions in genes, if the size of the repeat unit is known. This allows to compare the calculated and the natural parameters of gene and protein structures and to demonstrate that in many cases contemporary genes have retained some or several introns in the birth positions (ancestral or old immobile introns, OII). The regularity of OII locations is identical with the sequence regularity of exons and exon-coded protein fragments, which can be demonstrated after translation of gene and protein structures into codon-root (second codon letter) symbols and the design of repeat unit piles, or by autoscanning analysis.

The model and nucleotide multiplication theory of exon and intron origin (both further referred to shortly as "the Model") is illustrated by analysis of the tubulin and the small G protein gene families.

Materials and methods

Design and characterization of repeat unit piles

To design a repeat unit pile (RUP), peptide chains and their translations into codon root sequences are cut into fragments corresponding to the repeat unit size. For tabular analysis these RU are laid out horizontally in stacks to form a pile of RU. Immediately after gene formation by multiplication reaction, all the RU in the RUP structure would have identical sequences and the vertical lines (columns) of the RUP would be formed from identical symbols. During the evolution this is disrupted by mutations. The regularity of the repeat unit pile (RUP) structures may be characterized by expressing as a percentage ratio (f) of common-root (CR) and identical (I) amino acids, i.e., f=CR/I. Similarly, the RUP of gene codon root sequences may be characterized by the number of the preserved nucleotide base structures (also expressed as a percentage). The codon root symbols of gene nucleotide sequences and the corresponding amino acid sequences are identical, therefore identical are also their f values, but the amino acid sequence has another f value characterising the preserved identity of amino acid symbols. Thus, the gene RUPs are characterized by a single f value, but the protein RUPs – by two f values as a fraction (the preserved root identity/amino acid identity).

Autoscanning analysis

The protein amino acid and gene exon nucleotide sequence is translated into a sequence of the corresponding codon roots (second codon letters) and moved alongside the sequence duplicate step-by-step (symbol by symbol). The overlapping identical symbols (amino acids and/or codon roots) are counted and registered graphically at each step (a computer-assisted analysis).

For analysis of tubulin structures we used data banks (Dibb, Newman 1989; Liaud et al. 1992) containing information on 109 intron positions and phases in 38 α- and β-tubulin genes, and for analysis of 50 intron positions of SGP – a similar data bank of G-proteins (Dietmaier, Fabry 1994) that included representatives of the following subfamilies: Ras, Rho, Rab/Ypt, Ran/TC4 and Arf. The main attention in accordance with the Model was paid mainly to the regularity of intron positions. To analyse intron regularity it is necessary first of all to transform intron position and phase symbols to intron position co-ordinates. By this term we denote the ordinal number of nucleotides of the gene exon row just before the introns (Table 1).

Results and discussion

Regularity of tubulin intron positions

The family of tubulins is composed of highly conserved proteins, which are the principle structural and functional components of eukaryotic microtubules. Previous studies of tubulin family genes have led to different and conflicting conclusions, e.g., Dibb and Newman (1989) consider that intron distribution patterns in tubulin genes could be

Table 1. Revealed (Liaud et al. 1992) and calculated intron co-ordinates of the α- and β-tubulin families. Numerical values of intron co-ordinates marked by asterisks can be expressed as multiples of 12nt

N	Intron, position/phase and co-ordinate (nt)	Nearest gene knot point and intron deviation (nt)	N	Intron, position/phase and co-ordinate (nt)	Nearest gene knot point and intron deviation (nt)
1	2/0, 3	0/+3	21	90/1, 268	264/+4
2	4/1, 10	12/-2	22	95/1, 283	288/-5
3	5/0, 12*	12/0	23	110/1, 328	324/+4
4	9/0, 24*	24/0	24	126/0, 375	372/+3
5	13/0, 36*	36/0	25	134/0, 399	396/+3
6	16/0, 45	48/-3	26	134/1, 400	396/-4
7	17/0, 48*	48/0	27	177/0, 528*	528/0
8	19/1, 55	60/-5	28	208/0, 621	624/-3
9	20/0, 57	60/-3	29	211/1, 631	636/-5
10	21/2, 62	60/+2	30	224/1, 670	672/-2
11	33/0, 96*	96/0	31	257/0, 768*	768/0
12	35/2, 104	108/-4	32	319/2, 956	960/-4
13	38/2, 113	108/+5	33	327/0, 978	972/+6
14	41/0, 120*	120/0	34	346/2, 1040	1044/-4
15	56/0, 165	168/-3	35	351/1, 1051	1056/-5
16	58/1, 172	168/+4	36	353/0, 1056*	1056/0
17	59/1, 175	168/+5	37	407/1, 1219	1224/-5
18	62/0, 183	180/+3	38	412/1, 1234	1236/-2
19	76/1, 225	228/-3	39	437/0, 1308*	1308/0
20	90/0, 267	264/+3	40	448/2, 1343	1344/-1

understood by intron insertion in proto-splice sites after origin, but Liaud and coworkers (Liaud et al. 1992) are convinced that already primordial tubulin genes were rich in introns in agreement with the exon theory of genes (Gilbert 1987), but intron origin is unknown.

We discovered that a sensitive indicator of the birth position changes of introns was the composition of the prime multipliers of intron coordinates. Introns are regular only when the numerical values of co-ordinates had common prime multipliers, because the prime multipliers characterize an internal regularity of the numbers themselves. The Model postulates that immediately after the gene formation all introns in the exon row were situated regularly. Thus, their co-ordinates had to have common prime multipliers. From this followed also that the co-ordinates of regular introns could be used for prognosis of the size of the gene repeat unit. The potential size of the RU could be calculated also from common prime multipliers of exon length or exon length and intron co-ordinates, because in accordance to the Model, internal regularities of these parameters immediately after gene origin were identical.

Analysis of α- and β-tubulin intron position co-ordinates revealed a large group of introns (10 from 40, 25 %; Table 1) having a common set of prime multipliers, 2x2x3, and as a consequence also common regularity of disposition. These intron co-ordinates could be expressed as multiples of 12nt, e.g. for tubulin intron co-ordinates 12, 48 and 1056nt:

12	=		2x2x3		=	1x12;
48	=	2x2x	2x2x3		=	4x12;
1056	=	2x2x2x	2x2x3	x11	=	88x12.

The co-ordinates of regular introns in Table 1 are marked with asterisks.

The small size of the tubulin repeats, and high density of introns in the 5'-terminal parts of tubulin genes (Fig. 1A, B; Table 1) allowed to suppose that the ancestor of tubuline gene most likely originated in a one step multiplication reaction from a simple precursor – a 12-membered nucleotide designated as 4RU or 12nt/4aa (aa, amino acids). The revealed regular introns evidently had not changed their positions since origin of the gene. They crossed the tubulin gene knot points (situated in the exon row after each 12nt) and could be classified as OII.

The second large group was formed by tubulin introns which had slid off their birth positions, but were still in the zone 3nt around the gene knot points (15 introns from 40, 37.5 %). The co-ordinates of these introns were irregular and no common multipliers could be found. Sliding of an intron from the gene knot point even by one nucleotide radically changes the set of prime multipliers, e.g., in the case of a regular tubulin intron with the co-ordinate 48nt:

48	=	2x2x	2x2x3;
49	=	7x7;	
50	=	2x5x5.	

The number of introns crossing the tubulin gene knot points (10 or 25 %) or located near the knot points (15 or 37.5 %) together formed a large group from the analysed intron positions (25 from 40; 62.5 %), and supported the thesis that in the tubulin gene ancestor introns had been situated regularly.

There presently is no plausible molecular mechanism to account for both frequent intron sliding and the actual patterns of intron distribution in genes. One mechanism proposed by Fink (1987) postulates a normal excision of an intron from pre-mRNA, reverse transcription of the modified pre-mRNA, and homologous recombination of the resulting cDNA with the original gene. An additional event that involves imprecise reinsertion of an excised intron back into the pre-mRNA is also suggested by Martinez et al. (1989). The Fink-Martinez model, if correct, should result in unique gene structures – introns should be concentrated near the 5'end of each gene, because reverse transcription begins at the 3'-poly(A) tract of mRNA, but rarely extends completely to the 5'end, and recombination between the gene and cDNA affects the ends less frequently than the middle. As a consequence, intron sliding should be rarely observed at the ends of genes, especially at the 5'end (Martinez et al. 1989). The *Aspergillus* α-tubulin genes (May et al. 1987) supported this model (Fig. 1A, B).

Repeat unit piles of tubulins

In accordance with the Model, regularity of amino acid symbols in protein primary structures, or codon root symbols in the gene exon row, are identical with the regularity of intron positions of the gene ancestor (including OII in modern gene structures).

Exceptions may be only intron positions of mosaic-like genes formed by exon shuffling. Thus, in primary structures of tubulins the identical symbols of amino acids must have appeared with a periodicity of 4, corresponding to the size of a repeat unit 4RU. However, in the vast majority of cases, amino acid sequences of primary RU after billions of years of protein evolution have diverged beyond recognition, even after translation to the codon root sequences. There are also exceptions – in cases when genes contain molecular relics. By this term we denoted the parts of modern gene and protein structures that may be frozen in time, preserving remnants of ancient events that formed the first living molecules (e.g. structures of primary RU, ancestral intron positions, etc.). For example, fibrillar collagens retain many copies of primary RU GPP (GlyProPro) dispersed alongside the protein amino acid sequence (Huang et al. 1991). Similarly the head domains of cytokeratines (e.g. murine CK-15) have preserved several consecutive primary repeats FGGG (Nozaki et al. 1994; Fig. 2A). We revealed similar structures of the RU-relics also in β-tubulin and tubulin prokaryotic homologue FtsZ (named after the filamenting temperature-sensitive mutant Z; Fig. 2C; Erickson 1995). The structures of these and other repeat-relics allowed to conclude that some (if not all) modern proteins evolved from regular simple structure poly-amino acids containing only a few distinctive amino acid residues, and that many genes containing nearly a full set of codons determining 20 natural amino acids evolved from simple almost monotone codon multimers. The driving force for this type of evolution evidently was a prototropic tautomerism of purine and pyrimidine bases and mispairing of nucleotides during nucleic acid replication reactions (Topal, Fresko 1976). As a result of spontaneous mutations during long evolution, non-canonic nucleotide base

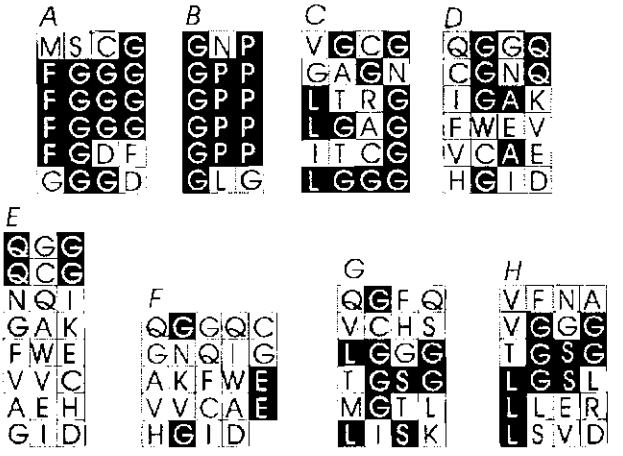

Fig. 2. Repeat unit piles (RUP) of proteins. A, 4RUP of the mouse cytokeratine CK-15 sequence (63-86). Identical symbols in vertical lines are shown against a black background, common-root amino acids are highlighted. B, 3RUP of the human collagen (COL2A1, 158-175). C, three pairs of repeat units 4RU (sequences 43-50, 91-98, and 127-134) from the protein FtsZ (a prokaryotic tubulin homologue). D, 4RUP of the pea ß-1 tubulin, 8-13. E and F, artifically changed sizes of RUP structures (±1 symbol) of the pea ß-1 tubulin diminished the RUP regularity from $f = 67/33$ (D) to 37/16 (E) and 41/16 (F). G, 4RUP of the pea ß-1 tubulin,131-156. H, 4RUP of the *Arabidopsis thaliana* α-1 tubulin, 137-160.

pairings (such as A-C, G-T, A-A, etc.) could transform polyaminoacids or similar simple structure peptide chains to complex modern protein amino acid sequences.

To study the regularity of protein amino acid sequences we elaborated a new methodology based on CAACRA – design and analysis of repeat unit piles (RUP, see *Materials and methods*). The regularity of RUP could be characterized by the content of dominating isosteric identical and common-root amino acids in vertical lines, using the *f*-factor values. Alongside formation of the original gene sequences (by nucleotide multiplication reactions) during evolution also genes-mosaics were formed by exon shuffling (Gilbert 1987) Therefore, it was necessary to investigate the regularity of all exons and to make definite conclusions of their similarity or distinction. In the case of tubulins this was a difficult task, because the primary RU (micro-exons) of tubulins were small (12nt/4aa), very diverged, and no higher-level regularities (e.g. secondary RU) had been found.

Most of tubulin 4RUPs (formed for analysis of regularity, each containing 6 repeat units and covering the whole amino acid sequence of protein) showed low values of the *f*-factor, which was characteristic for biologically highly specified ("nonregular") sequences. However we noticed that some regions of plant *Pisum sativum* and *Arabidopsis thaliana* tubulins, particularly their N- or C-terminal sequences, had an enlarged content of glycine residues, and that separate tetrapeptide fragments of these regions were formed of hydrophobic U-group amino acid (Chipens 1991) and three glycine residues (e.g., LGGG, IGGG, FGGG, etc.). There were also similar dispersed fragments whose structures had been changed by point mutations and common-root amino acids (e.g., VPGG, VGEG, VGGE, IQGG, etc.), indicating that the potential precursor of tubulin primary RU was related to the RU-relic of the cytokeratine ancestor head domain (FGGG).

In this context the structure of the tubulin prokaryotic homologue was of specific interest – the protein FtsZ had similar primary and 3D-structure with tubulins (Erickson 1995). This protein induces constrictions of the cell wall and cell membranes that leads to the formation of two daughter cells during bacterial cell division. The peptide chain of FtsZ is formed of precisely 91 repeat 4RU (364aa), and in three different regions has retained, as we suppose, ancestor protein repeats LGGG or their mutant forms. The distances between these repeats (calculated by comparison of N-terminal amino acid positions of 4RU, shown in Fig. 2C, in this particular case 91 - 43 = 48 = 4 x 12 and 127 – 91 = 36 = 4 x 9) corresponded precisely to whole number of 4RU multiples. Therefore, the alternative explanation that tetrapeptide sequences had been formed by chance or convergent evolution is not very probable. Most likely, in the early period of evolution, tubulins evolved from RU precursors (containing glycines and U-group amino acids) common to other proteins forming protocell structures. This conclusion was supported by the *f*-factor values of tubulin RUP. The high *f* values were revealed only in cases when repeats forming RUP where rich in glycines (Fig. 2D, G, H). Evidently, only such repeats to some extent reflect the RU precursor structure and as a consequence also the regularity.

Regularity of small G proteins and genes

A family of small GTP-binding or G proteins (SGP) is involved in regulation of very different cellular processes such as signal translation, cytoskeletal organisation, organelle trafic in cells and others (Fabry et al. 1992; Dietmaier, Fabry 1994). Based on amino

Table 2. Revealed (Dietmaier, Farby 1994) and calculated intron co-ordinates of the small G protein genes. Numerical values of intron coordinates marked by asterisk can be expressed as multiples of 12 or 24nt, but marked by two asterisks only as multiples of 12nt

N	Intron. position/phase and co-ordinate (nt)	Nearest gene knot point and intron deviation (nt)	N	Intron. position/phase and co-ordinate (nt)	Nearest gene knot point and intron deviation (nt)
1	6/0, 15	12/+3	26	86/2, 257	252/+5
2	13/1, 37	36/+1	27	88/0, 261	264/-3
3	13/2, 38	36/+2	28	88/1, 262	264/-2
4	25/0, 72*	72/0	29	96/2, 287	288/-1
5	26/0, 75	72/+3	30	97/2, 290	288/+2
6	30/1, 88	84/+4	31	104/0, 309	312/-3
7	34/0, 99	96/+3	32	112/0, 333	336/-3
8	37/1, 109	108/+1	33	114/0, 339	336/+3
9	37/2, 110	108/+2	34	122/0, 363	360//+3
10	38/0, 111	108/+3	35	123/1, 367	372/-5
11	38/1, 112	108/+4	36	125/0, 372**	372/0
12	41/0, 120*	120/0	37	128/1, 382	384/-2
13	46/0, 135	132/+3	38	131/1, 391	396/-5
14	47/1, 139	144/+5	39	132/0, 393	396/-3
15	55/0, 162	156/+6	40	135/1, 403	408/-5
16	56/0, 165	168/-3	41	138/0, 411	408/+3
17	57/0, 168*	168/0	42	138/1, 412	408/+4
18	59/0, 174	172/+2	43	151/0, 450	444/+6
19	63/2, 188	184/+4	44	155/1, 463	468/-5
20	65/2, 194	196/-2	45	162/1, 484	480/+4
21	70/1, 208	204/+4	46	163/0, 486	480/+6
22	77/1, 229	228/+1	47	166/2, 497	492/+5
23	80/0, 237	240/-3	48	171/1, 511	516/-5
24	81/0, 240*	240/0	49	178/0, 531	528/+3
25	86/1, 256	252/+4	50	181/0, 540**	540/0

acid similarities and functions, five different SGP subfamilies are recognized (Ras, Rho, Rab/Ypt, Ran/TC4 and Arf). Dietmaier and Fabry (1994) analysed the positions of 125 introns from 28 SGP genes, including representatives of all the tubulin subfamilies, and concluded that most if not all introns in modern SGP genes had arisen by independent insertion events after diversification of the various SGP subfamilies.

We reinvestigated the intron positions of the SGP family genes using the suggested Model and method of analysis, and the same data bank of SGP introns formed by Dietmaier and Farby (1994). About a half of the SGP gene introns were located around the gene knot points in a zone ±3nt (Table 2), confirming the regular intron disposition in a SGP gene ancestor. The green alga *Volvox carteri* gene *yptV1* (Fabry et al. 1992) and

the basidiomycete *Coprinus cinereus* ras gene (Ishibashi, Shishido 1993) can serve as examples of regular organisation of modern SGP. Four exons of the *yptV1* gene have a regular structure and are formed precisely of 4, 4, 6 and 13 repeats (exons 4-7, Fig. 1C):

135 – 87 = 48 (nt); 48 = 4 x 12;
183 –135 = 48 (nt); 48 = 4 x 12;
255 – 183 = 72 (nt); 72 = 6 x 12;
411 – 255 = 156 (nt); 156 = 13 x 12.

The corresponding introns separating these exons all seemed to have "slid off" of the gene knot points (the birth-positions of introns) equally by +3nt, evidently as a result of an indel, because the length of the *yptV1* gene was precisely 51 repeat units (4RU) or 612nt (including the stop codon).

Multiplication reactions of nucleotides, in accordance with the advanced Model, form genes with symmetric exons and all introns in phase-0. Restoration of all introns of the *Coprinus cinereus ras* gene (Fig. 1D) in the birth-positions corresponding to the phase zero (shift by 1-2nt up or down to the nearest gene knot point) revealed a completely regular exon row in which each exon was formed of a whole number of repeats 4RU, but each intron crossed the gene knot points (OII 12, 60, 132, 216, etc.; Fig. 1E). Also in this case the length of gene coding part was formed of a whole number of repeat units (54 x 12nt = 648nt; Fig. 1D, E). Not accidental seemed also the length of the *C. cinereus ras* gene six introns (60, 53, 57, 172, 55 and 61nt), which with one exception all were around 60nt or five repeats (Ishibashi, Shishido 1993).

Visual analysis of long structures of RUP containing many repeats is not handy if large regions of amino acid sequences are investigated. For this purpose we suggested another method termed autoscanning analysis, which was useful for analysis of amino acid as well as codon root sequences. According to this method, a sequence is moved alongside the sequence copy (a duplicate) step-by-step (symbol-by-symbol) and the overlapping identical symbols are counted and registered graphically at each step. When the repeat unit boundaries overlap, the number of overlapping identical symbols is maximal. The scanning graph profiles of regular model sequences resembled a saw and the distances between the teeth of the saw showed the size of the RU.

Natural protein autoscanning graphs, however, in most cases were very complex (as a result of mutations, indels, amino acid sliding, etc.), but there were also exceptions. The autoscanning graph of the green alga *Volvox carteri* Ras protein amino acid sequence encoded by the gene *yptV1* in a small region (steps 38-66) showed maximum positions corresponding to repeat unit sizes 4RU and 8RU (Fig. 3D). The design of Ras protein repeat unit piles (4RUP, 8RUP and 16RUP; Fig. 3) confirmed sequence regularity after 4 as well as 8 symbols. As we supposed, the 8RU was possibly a repeat of the second step multiplication reaction. The *f*-factor values of RUPs were low, but besides this, the density of identical symbols was of special significance for the analysis, e.g., in the 8RUP structure the third column contained four isosteric threonins, but the fourth column – three isosteric isoleucins. There was no possibility of forming such regularities by chance. It is necessary to note that autoscanning analysis of the Ras protein did not show maximum positions which corresponded to the size of 16RU.

The obtained data provided new information about the regular structure of nucleotide and amino acid sequences and is one more step towards understanding intron and gene origin. Does lightening often strike the same place twice? This question was raised during

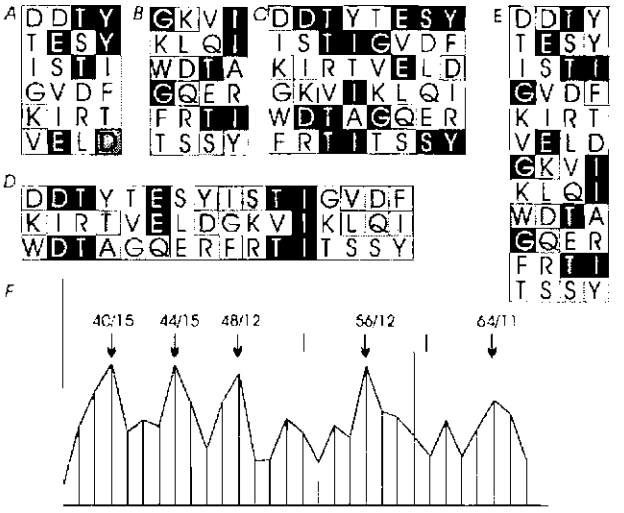

Fig. 3. Analysis of the regularity of the green alga *Volvox carteri* protein SGP encoded by the *yptV1* gene. A-E, repeat unit piles of the protein fragment (sequence 30-77) with different potential sizes of repeats: 4RU, 8RU and 16RU. F, a fragment of an autoscanning graph of the *yptV1* gene encoded amino acid sequence 1-203 (steps 38-66). The bars show numbers of overlapping identical amino acid residues at each step of autoscanning. Maximums are shown by arrows topped with the step number/number of overlapping identical amino acids. Missing maximum positions are shown by vertical dashes.

discussion of origin of the GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene family introns (Logsdon et al. 1994), and characterizes the absence of strong criteria and parameters of the exon theory of genes and the insertional theory of intron origin that are necessary to study the gene structural organisation and to resolve the intron problem. We suggest such parameters: the co-ordinates of intron location, the sizes of repeat units, exons and gene coding parts as well as the regularity of gene and protein sequences characterized by f values of the corresponding repeat unit piles. A stable fundament of a new nucleotide-multiplication theory of exon and intron origin is the possibility to calculate theoretical sizes of exons and an exon row, as well as the potential intron positions in genes if the size of a repeat unit is know. Very important seems also our conclusion that the regularity of ancestral intron (OII) positions is the same as the symbol (amino acids or codon roots) regularity in protein amino acid sequences. Our general conclusion is that introns during evolution have arisen very early, alongside with exons from the same repetitive nucleic acid precursors. Introns are a consequence of gene formation.

Acknowledgements

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