INVESTIGATION INTO GENETIC VARIABILITY OF PARASITIC AND NON-PARASITIC LAMPREYS INHABITING WESTERN RIVERS IN LITHUANIA

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Abstract. The river lamprey (*Lampetra fluviatilis*) is fish ectoparasites that parasitize the herring (*Clupea harengus*), the sprat (*Spattus sprattus*) and other commercial fish species in the Baltic Sea. On the other hand, the river lamprey is a valuable commercial catch itself in the Lithuanian, Latvian, Estonian, as well as northern Swedish and Finnish rivers. According to the latest molecular studies of the parasitic river lamprey and the non-parasitic non-migratory brook lamprey (*Lampetra planeri*) in the populations of Western Europe, there is insufficient evidence to separate the two by molecular markers. This initial study was carried out in order to determine if the hypervariable region of the D-loop could be used as an informative marker for recognising parasitic and non-parasitic lampreys. We established a data set from control region I sequences and identified 21 unique haplotypes unequally distributed among 5 populations. The phylogenetic analysis revealed one highly differentiated lineage among the obtained data set of sequences. This lineage consisted of two haplotypes shared by few individuals from the geographically close populations distributed in two drainages corresponding to the same region. Sequences of these two haplotypes differed by 5.7% ±1.9% (±SE) ($p<<0.01$) from all the rest D-loop sequences belonging to *L. fluviatilis* and *L. planeri* individuals and expressed a greater similarity to the Ukrainian lamprey (*Eudontomyzon mariae)* compared to the *Lampetra* genus. It could be guessed that a part of the non-parasitic lamprey population inhabiting some rivers in northern Lithuania harbour forms of mtDNA considered as belonging to an undescribed species which is the most closely related to *E. mariae*. A lack of highly differentiated clades in *L. planeri* and *L. fluviatilis* representing different drainages suggests possible intensive hybridisation or recent divergence of the two species.

Keywords: *Lampetra*, *Eudontomyzon*, idenfication, D-loop, Lithuania

Introduction. Currently, there are two lamprey species, the river lamprey and the brook lamprey that are officially recognized as native to Lithuanian rivers. The first species represents a parasitic and anadromous life form, which after spending several years in ammocoete beds, undergo metamorphosis and start active downstream migration to the Baltic Sea. In the marine environment, river lampreys feed on fish (herring *Clupea harengus*, cod *Gadus morhua*, sprat *Sprattus sprattus*) tissues, blood and body fluids (Eglite, 1958; Hardisty, 1986; Renaud 2011) till they reach maturity and start upstream migration to spawning grounds. The other species – the brook lamprey – is non-parasitic and stationary form that never leaves natal rivers (Maitland, 2003).

In recent years, much information has been accumulated on the diversity and the taxonomic status of these two nominal species in Europe. In addition to the lack of morphological differences at the larval stage (Loman, 1912; Gardiner, 2003), there was found no support of taxonomic differentiation between parasitic and non-parasitic species in mitochondrial DNA studies (Espanhol et al., 2007; Blank et. al., 2008; Pereira et al., 2010). Due to this similarity, both species are usually described by the term of satellite species (Vladykov and Kott, 1979), which refers to the non-parasitic species that have supposedly evolved from parasitic stems and form a series of species pairs with the parasitic ones and exist in sympatry. Moreover, recent observations of commune spawning of both species in France and Lithuania (Lasne et al., 2010; Staponkus and Kesminas, 2014) and *in vitro* produced hybrids (Hume et al., 2013) suggest possible hybridization and gene flow between these species.

Recent mitochondrial mtDNA studies also reveal the existence of highly divergent allopatric evolutionary lineages of *L. planeri* in the Iberian Peninsula and those of *Lampetra sp.* in Oregon and California (Mateus et al., 2011; Boguski et al. 2012). In the Iberian Peninsula three lineages were described as new cryptic species of *L. alavariensis* in the Esmoriz River, *L. auremensis* in the Nabão River and *L. lusitanica* in the Sado River. These findings were supported by several diagnostic synapomorphies (Mateus et al., 2011; Mateus et al. 2013). It is thought that the Iberian Peninsula as a refuge area during several ice ages provided suitable conditions for multiple ancestral lamprey populations (Espanhol et al., 2007). To the best of our knowledge, prevalence of cryptic species is most probable in the rivers of southern Europe, although similar cryptic populations could also be expected in a wider range of European watershed.

During this study, five *L. fluviatilis* and *L. planeri* populations inhabiting the western Lithuanian rivers were studied using mtDNA D-loop region genetic markers. Thus far there has been very little data on the genetic structure of the lamprey inhabiting the south-eastern Baltic region, therefore, the main aims of the study were to explore genetic diversity of the satellite species of the parasitic and non-parasitic lampreys in Lithuanian rivers and to find out whether the findings indicating the absence of genetic differences between satellite species *L. fluviatilis* and *L. planeri* in Western Europe corresponded to the local population.

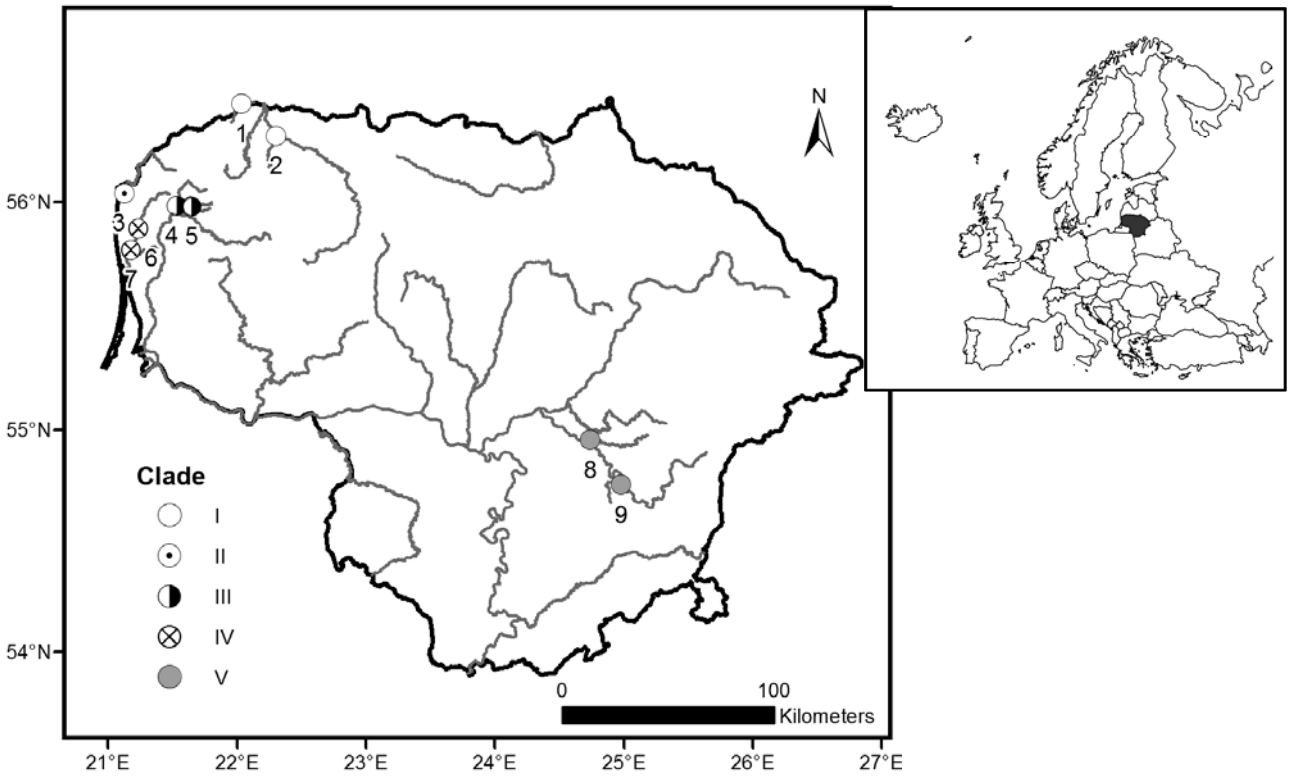

Fig. 1. **Lamprey sampling sites in Lithuania (circles). Clades organized according to the catchment region.** Site locations: 1, the Lūšis; 2, the Viešetė; 3, the Šventoji; 4, the Blendžiava; 5, the Mišupė; 6, the Danė, 7, the Eketė, 8, the Musė, 9, the Bražuolė.

Materials and methods. Adult lampreys were captured during the pre-spawning or spawning period from April to June in three consecutive years, 2011, 2012 and 2013. Pre-spawners were collected by electric fishing and spawning individuals were caught mainly manually by a dip net. Placement of the individuals into different clades was inferred from their sampling site location in different catchments. The Viešetė and Lūšis Rivers represent clade I (the Venta river basin), the Šventoji River corresponds to clade II (discharges directly into the Baltic Sea), the Blendžiava River and the Mišupė River represent clade III (the Minija river basin), the Danė River and the Eketė River form clade IV (the Danė-Akmena river basin), both rivers Musė and Bražuolė clade correspond to the Neris River basin and form clade V (Fig. 1).

A total of 60 *L. fluviatilis* and *L. planeri specimen* (57 mature individuals and three ammocoetes) were investigated for mtDNA D-loop region polymorphism (partial sequences including non-coding region I according to Almada et al., 2008). Adults were classified according to the specimen's size and morphology (Gardiner, 2003). Three lamprey ammocoetes collected in the Bražuolė River were assigned to the migratory form as there were no records of the *L. planeri* population. DNA was extracted from frozen at -20 °C or ethanol preserved muscle tissues and fin clips using Salting Out protocol (Aljanabi & Martinez, 1997) with slight variations. DNA fragments of the control region of total 611 base pairs (bp) were amplified using primers Lamp-1F 5'-ACACCCAGAAACAGCAACAAA-3' and Lamp-1R 5'-GCTGGTTTACAAGACCAGTGC-3' (Almada et al., 2008). The PCR volume for each sample was 25 µ l and consisted of: 1 × Taq buffer (with 50 mM KCl), 0.2 mM dNTP, 0.2 µM of each primer, 2.5 mM $MgCl_2$, 0.75 U Taq DNA polymerase), and 0.05 µg of template DNA. PCR conditions followed Almada et al. (2008). Agarose Gel Electrophoresis was carried out in the Pharmacia Gel GNA-100 ® equipment for 40 minutes at 100–120 V. Purified PCR products were sequenced in DNA Sequencing Centre of the Institute of Biotechnology, Vilnius University (http://www.ibt.lt/) using the Big-Dye® Terminator v3.1 Cycle Sequencing Kit and 3130xl Genetic Analyzer (Applied Biosystems; www.appliedbiosystems.com). The obtained sequences were aligned with MEGA 6.05 (Tamura et al., 2013) using CluctalW algorithm. In the control region, an indel of 39 bp was widespread, which is homologous to a part of the repetitive motif expressed in both *L. fluviatilis* and *L. planeri* in Europe and *Lampetra aepyptera* in North America (White and Martin, 2009; Pereira et al., 2010). The insertion had a conserved sequence and did not vary in the number of repeats, and most populations were polymorphic for the indel. In order to avoid misleading results, the indel was excluded from subsequent analyzes. The distance between the groups of sequences was calculated in line with Kimura 2-parameter (K2P) plus gamma (K80+G) method in MEGA 6.05 (Tamura et al., 2013). The gamma shape (α=0.0661) parameter was calculated directly from the newly obtained data and the

data available in GenBank (accession numbers EU595965 – EU596199 and GQ340523 – GQ340554; Pereira et al. 2010). The substitution pattern and rates were estimated under the Tamura and Nei (1993) model. Phylogenetic relationships among the haplotypes were also examined using maximum likelihood (ML) analyses with 1000 bootstrap replicates. K2P distances were visualized by constructing a neighbour-joining (NJ) tree again using MEGA 6.05 (Tamura et al., 2013). Both the number of polymorphic sites (S), haplotype diversity (H), the average number of nucleotide differences (K as measured by the uncorrected average number of nucleotide substitutions per site between the populations (K_{XY}) (Nei, 1987) and the fixation index (Φ_{ST}) with the significance level (P) were estimated using the DNASP 5.10.01 program (Librado and Rozas, 2009). During these calculations, all positions with gaps were included into the analysis.

Results. A total of 60 sequences were obtained. Sequencing mtDNR revealed 21 haplotypes defined by 35 variable sites (Table 1). The amplified fragment was approximately 611 bp, but the alignment of DNA sequences was made with 562–572 bp (indel of 39 bp was excluded, see Materials and Methods for further details) homologous fragments. These fragments of mtDNA comprised of partial NADH dehydrogenase subunit 6 gene and partial sequence of control region. Overall haplotype diversity was 0.575 ± 0,076 (±SD) and nucleotide diversity was 0.0067 ± 0.0019 (±SE). The majority (85.7%) of the haplotypes were only found in one or two clades (Table 2). The most common haplotypes (H1, H8, and H9) were shared among 38 lampreys belonging to five clades. These haplotypes were also the only ones which were shared by both the *Lampetra fluviatilis* and the *Lampetra planeri* populations.

Table 1. ***Lampetra*** **genus mtDNA D-loop region haplotypes found in Lithuania**

No.	7	32	57	110	119	121	122	124	125	131	132	134	138	143	144	145	145	148	149	153	154	155	156	158	160	162	173	296	328	334	336	360	410	486	498	Clade				
																																					I	II	III	IV	V
H1	C	-	G	C	C	A	T	A	C	C	G	C	G	C	A	A	T	C	C	-	-	A	A	C	C	C	-	A	A	T	G	C	T	A	C	1	4	2	4	4
H2	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	G	.	.	.	.	.	.	.		1			
H3	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.	1				
H4	.	-	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.		1			
H5	.	-	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.				1	
H6	.	-	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	-	.	.	.	-	.	.	.	.	.	.	.	.				1	
H7	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	.	.	.	.	-	.	.	.	.	.	.	.	.		2			
H8	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.	2	2	3	2	4
H9	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	.	.	.	.	.	-	.	.	.	.	.	.	.	.	4	1	2	1	2
H10	.	-	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.					1
H11	.	-	.	.	.	T	.	T	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.				2	
H12	.	-	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	C	-	.	.	.	.	.	-	.	.	.	.	.	.	.	.		1			
H13	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	-	.	.	.	-	.	.	.	A	.	.	.	.		1			
H14	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	-	-	.	.	.	.	.	-	.	.	.	.	.	C	.	.					1
H15	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	-	.	.	.	-	.	.	.	.	.	.	.	.		1			
H16	T	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	.	-	.	-	.	.	.	.	.	.	.	.		1	1		
H17	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	.	.	.	-	.	.	.	A	.	.	.	.					1
H18	.	-	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	.	.	.	-	.	.	.	A	.	.	.	-					1
H19	.	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	C	C	.	-	.	**i**	.	.	.	.	.	.	.	.		1			
H20	.	-	T	.	.	G	.	.	T	T	.	T	A	T	C	T	A	T	T	C	-	.	.	A	.	A	-	G	G	C	.	T	.	C	.	1	1			
H21	T	-	T	.	.	G	.	.	T	T	.	T	A	T	C	T	A	T	T	C	-	.	.	A	.	A	-	G	G	C	.	T	.	C	.		1			

All sequences are given with reference to H1 haplotype and the identical sequence from GenBank (accession number EU596094; Pereira et al. 2010). Due to 11 bp insertion (i-TTCCTCACCTA) in haplotype H19, other sequences are shown as 572 bp fragments instead of 561bp.

The haplotypes were highly shared between the clades and private haplotypes accounted for 25% - 50 % of the detected haplotypes. Lowest values of haplotype diversity were found in clade I (the Lūšis and Viešetė Rivers), highest in clades II (the Šventoji River) and clade III (the Blendžiava and Mišupė rivers). All other genetic parameters such as nucleotide diversity (π), the number of polymorphic sites (S), and the average number of nucleotide differences within a population (K) was also higher among those two clades.

N is the number of individuals studied; *h* is haplotype diversity, π is nucleotide diversity, S is polymorphic sites and K is the average number of nucleotide differences within a population.

Table 2. **Parameters of mtDNA D-loop region genetic diversity in different *Lampetra* sp. populations in Lithuania**

	N	Haplotypes	Private haplotypes	*h*	Π±SE	S	K
Clade I	8	4	25%	0.750	0	3	1.071
Clade II	16	11	45.5%	0.933	0.009±0.003	28	5.083
Clade III	11	7	28.6%	0.909	0.019±0.006	26	8.145
Clade IV	11	6	50%	0.855	0.002±0.00	6	2.036
Clade V	14	7	42.9%	0.857	0.002±0.001	9	1.220

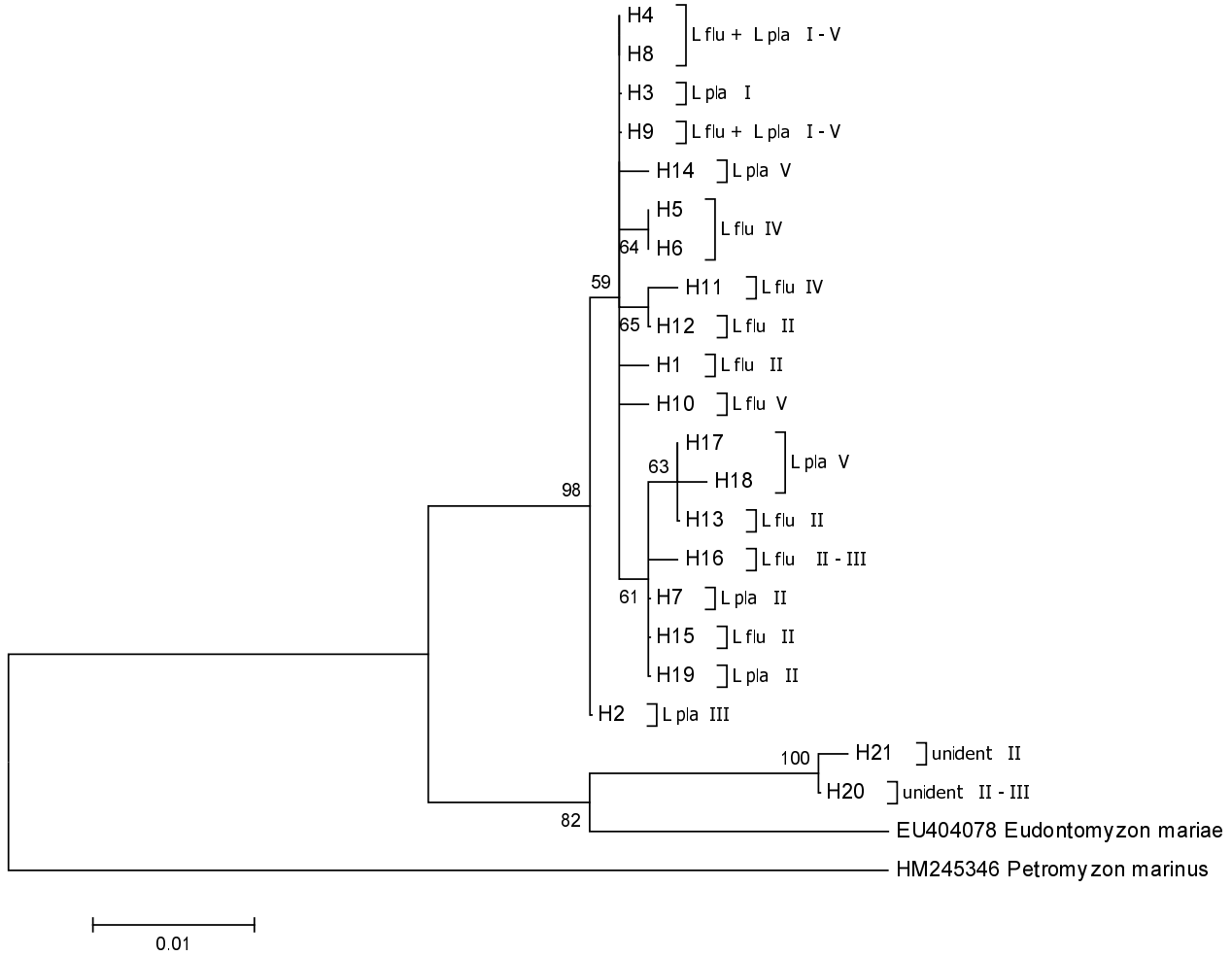

Fig. 2. **Molecular Phylogenetic analysis of 21 lamprey haplotypes of mitochondrial non-coding region I by using the Maximum Likelihood method based on the Kimura's (1980) two-parameter distance model; numbers at nodes are bootstrap values for 1,000 replications.** The abbreviations are as follows: L pla = *Lampetra planeri*, L flu = *Lampetra fluviatilis*, unident = unidentified species. Sequence data were obtained from GenBank: EU404078 (Blank et al., 2008), HM245346 (Pereira et al. 2012).

The phylogenetic analysis carried out using ML methods identified two distinct lineages in the lamprey populations (see Fig. 2), one lineage was widespread in all the rivers (clades) studied, and the other one was present exclusively in clades II and III with a limited distribution in the majority of north-western rivers of Lithuania. The bootstrap values supporting the *L. planeri*/*L. fluviatilis* and the newly emerged lineage were high (82–98%). This lineage was clustered together with *E. mariae*. It is likely that these lampreys were erroneously identified and described as *L. planeri*. Further on, we describe these individuals as unidentified species.

Average genetic distances attained from the hypervariable domain of the mtDNA control region between the evolutionary lineages indicate the levels of nucleotide diversity between *L. fluviatilis* and *L. planeri* to be low 0.2% ± 0.1% (±SE) (p=0.33). On the contrary, the distance between *L. fluviatilis* and *E. mariae* 5.1% ±

1.7% (±SE) ($p<<0.01$) and between *L. planeri* and *E. mariae* 5% ± 1.7% (±SE) ($p<<0.01$) is substantial and clearly indicates different species. The distance between the unidentified lampreys and both *L. fluviatilis* and *L. planeri* was 5.7% ±1.9% (±SE) ($p<<0.01$) following by similar distance calculated between unidentified individuals and *E. mariae* 5.2% ± 1.7% (±SE) ($p=0.17$).

The analysis of genetic differentiation between the two nominal species *L. fluviatilis* and *L. planeri* showed no substantial subdivision between the species ($\Phi_{ST}=0.074$). Pairwise Φ_{ST} values for five clades ranged from weak to moderate (Φ_{ST} 0 – 0.1) although none of the values was statistically significant.

Discussion. The genetic diversity studies of lamprids based on D-loop sequences in Europe are scarce. Prior to the current study, the Iberian Peninsula, particularly the rivers of Portugal were studied most extensively (Pereira et al. 2010). The results of the current study suggest that the diversity of the *L. fluviatilis* and *L. planeri* populations in Lithuanian river catchments (clades I to V) is higher compared to that previously discovered in the North Sea, the Baltic Sea and the Iberian Peninsula. Making use of the same DNA fragment, we found 18 haplotypes in 57 specimens in contrast to the 58 haplotypes identified among 267 specimens by Pereira et al. (2010).

The current study brought out major differences in genetic diversity between the river catchments in western and eastern Lithuania. It seems that the greatest haplotype diversity could be found in the Šventoji, the Blendžiava and the Mišupė Rivers representing clades II and III. The results of the phylogenetic analysis suggested the existence of two major evolutionary lineages: the *Lampetra* genus and the unidentified group of lampreys. Differences in genetic parameters between the localities emerged mainly due to the presence of unidentified lampreys.

The estimated level of nucleotide diversity in the *Lampetra* sp. population in Lithuanian watersheds was low ($\pi=0.0016$). It is very similar to what was shown in *Lampetra* sp. based on the cyt *b* gene in the populations of central and northern Europe ($\pi=0.00185$) (Espanhol et al., 2007). The variability of both markers is likely to be of similar magnitude and differences given by both markers can be compared to some extent. Johns and Avise (1998) stated that based on the cyt *b* gene 90% of cryptic species of vertebrates show sequence divergences exceeding 2%. Reid et al. (2011) calculated from 2.85 to 3.20% sequence divergence between the *L. pacifica* and the *L. richardsoni,* which proves these findings to be correct. The divergence between the *L. planeri* and the *L. fluviatilis* in the current study accounted for 0.2%, which indicates a lack of separation between the nominal species. On the other hand, divergence of the unidentified group of lampreys and the *Lampetra* sp. accounted for 5.7%, which could imply the co-existence of the undescribed cryptic species and well defined species of the *Lampetra* genus.

The lineage of the cryptic species found in a few river basins in western Lithuania demonstrates an affinity to *E. mariae*. Thus far, there have been no confirmed reports of *E. mariae* in the Lithuanian waters although, according to the IUCN redlist data and Kottelat and Freyhof (2007), the Nemunas River basin is referred to as the natural range for this species. Nevertheless, there is still an ongoing discussion among ichthyologists about the taxonomic status of *E. mariae*. According to Renaud (2011), this species needs a complete re-evaluation, and it is possible that this name harbour a complex of species. The idea is strongly supported by recent findings. Docker (2009) regarded *E. mariae* and *Eudontomyzon danfordi* as closely related species or, moreover, it represents a pair of satellite species. The analysis of the multiple mitochondrial DNA markers also suggests reinclusion of *E. mariae* into the *Lampetra* genus (Blank et al. 2008). It is very likely to be correct as three adult hybrids between *L. planeri* and *E. mariae* in the Jeziorka River, Poland have been reported (Rembiszewski 1968), and communal spawning during the current study was also observed between the *L. fluviatilis*, *L. planeri* and the unidentified lampreys.

The divergence between the cryptic species and *E. mariae* was of the same extent as that between the *Lampetra* sp. and *E. mariae* 5.2% ($p=0.17$). It could be supposed that these few specimens of an unidentified lineage described in our study could be representatives of an isolated peripatric species, which is most closely related to *E. mariae* and possibly originated from the Vistula River watershed, Baltic Sea basin or the Dniepr River watershed, Black Sea basins. In general, *E. mariae* life cycle is similar to that of *L. planeri* and adults are non-parasitic. However, it is strongly suggested that feeding type plasticity within the lamprey species (Hubbs 1971) occurs and cases of *E.mariae* ectoparasitic behaviour in the Jelesná Brook, Slovakia, and the Prut River, the Ukraine (FishBase 2013), have been recorded. These findings could correspond to the new fish parasite species found in inland waters. Hence, in order to clear the evidence on this cryptic species additional genetic and morphological markers have to be thoroughly investigated.

Conclusion. The analyses of mtDNA D-loop sequences diversity proved that the river and brook lamprey populations in Lithuanian water bodies originated within one distinct evolutionary lineage. The current study also revealed co-existence of the cryptic species affined to the Ukrainian brook lamprey within the river and brook lamprey populations.

Acknowledgements

The authors express sincere gratitude to N. Nika and K. Bagdonas from the Coastal Research and Planning Institute of Klaipėda University for field assistance in collecting samples.

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Received 27 January 2014
Accepted 1 July 2014