

CURRENT KNOWLEDGE ABOUT EUROPEAN EEL *ANGUILLA* *ANGUILLA* (L.) mtDNA D-LOOP REGION HAPLOTYPIC VARIETY

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Abstract. Today, there are only two information sources about *Anguilla anguilla* mtDNA D-loop region haplotypic variety and both are quite old. Our research group updated the ten year-old mtDNA D-loop region database of 80 sequences by adding the homological sequences of 45 European eels caught in Lithuanian territorial waters, i.e., in the Baltic Sea, the Curonian Lagoon, and in inland Lakes Dringis and Siesartis. Even 37 unique haplotypes of European eel were found. Based on all available mtDNA D-loop sequence data, 115 polymorphic sites (S), 132 mutations (η), 0.9965 (\pm 0.002) overall haplotype diversity (H) and 0.02523 nucleotide diversity (π) were determined. No significant clustering related to the sampling location was identified after the evaluation of the distribution patterns of different haplotypes in the phylogenetic tree constructed using 125 European eel mtDNA D-loop sequences. However, limited genetic differentiation based on geographic distribution of some haplogroups of *A. anguilla* became evident after the refinement of haplotype relationships using a network approach. The ambiguous clustering of specimens representing geographically related sampling locations in the phylogenetic tree derived using the neighbor-joining method and a non-random distribution of some haplotypes in the median joining network are discussed.

Key words: *A. anguilla*, European eel, mtDNA, D-loop, haplotype, panmixia, IBD, IBT

INTRODUCTION

Variation within and between populations and stock discrimination within exploited species are important issues not only for fisheries management, but also for conservation programmes (Okumuş & Çiftci 2003). Molecular genetic studies of natural populations are dependent on polymorphic neutral markers and offer the possibility to investigate the population structure, to provide scientific data for the regulation of harvest in order to protect weaker populations and, finally, to enable the long-term management of fisheries resources (Çiftci & Okumuş 2002). However, many commercially exploited marine fish species exhibit no or a low degree of genetic differentiation in neutral marker genes. The lack of genetic differentiation, typically attributed to a high degree of gene flow in the marine environments, sometimes supports the thinking that genetically indistinguishable stocks can be managed as one panmictic population (Cano *et al.* 2008).

European eel (*Anguilla anguilla* (L.)) is a facultatively catadromous (Lin *et al.* 2007) commercially exploited fish (Huertas & Cerdà 2006; Trautner 2006), the catches of which started to decline in Europe a few decades ago (Palstra *et al.* 2006; Maes & Volckaert 2007). At the moment, the stock is outside safe biological limits

(Åström & Dekker 2007), therefore this species has been recently added to Appendix II of the CITES Red List of Endangered Species (Maes & Volckaert 2007). Due to the complex life cycle of European eel (Van Ginneken *et al.* 2007; Bonhommeau *et al.* 2008), the conservation and management of this species is a difficult task, therefore many 30 year-long efforts to produce economically profitable quantities of eels in aquaculture failed (Maes & Volckaert 2007). Thus, future studies on the genetics of *A. anguilla* should focus on the conservation issues and evolution history of this fish with the aim of integrating genetics into management (Maes & Volckaert 2007). While the panmixia status of this species is on debate (Dannewitz *et al.* 2005; Maes & Volckaert 2007; Wysujack 2007), the restocking strategy for this fish should be viewed with precaution (Sruoga *et al.* 2007).

In most species, mtDNA is highly variable and therefore is a good marker for detecting possible genetic differentiations (Okumuş & Çiftci 2003), however, few attempts were made to study *A. anguilla* mtDNA using the direct sequencing of cytochromes or D-loop sequences (Lintas *et al.* 1998; Daemen *et al.* 2001). To our knowledge, only Daemen *et al.* 2001 reported the results about European eel mtDNA cytochrome *b* and four microsatellite loci, and only Lintas *et al.* 1998

published the article with the description of 55 *A. anguilla* mtDNA D-loop sequences. Data on the combined European eel mtDNA D-loop and cytochrome *b* (*cyt b*) (Murgia *et al.* 1998) are still unpublished (sequence data are deposited in GenBank). Moreover, due to unclear reasons, *A. anguilla* mtDNA D-loop investigations were terminated 10 years ago. Therefore, a new investigation into D-loop haplotypic diversity of *A. anguilla* with utilisation of DNA sequencing data deposited in GenBank under accession numbers AJ225953-AJ226007 and AJ246983-AJ247007 was initiated by our research group in order to get a better understanding of intraspecific genetic variability of this species.

The main goals of the investigation are to: 1) update the European eel mtDNA D-loop region sequence database; 2) test the median joining network method for the recovery of the evolution history of this species; 3) verify the panmictic status of European eel; 4) increase the knowledge about *A. anguilla* mtDNA D-loop haplotypic diversity; 5) find out if the haplotype variety of predominantly naturally recruited European eels caught in the Baltic Sea and in the Curonian Lagoon differs from that of introduced eels from Lakes Dringis and Siesartis; 6) provide new information for the conservation for this species.

MATERIAL AND METHODS

Sampling

During this research, a total of 45 European eels were investigated. Animals were caught during the period 2004–2006 in the Lithuanian territorial waters of the Baltic Sea, Curonian Lagoon, Lake Dringis and Lake Siesartis (Fig. 1).

DNA extraction and amplification

DNA was extracted using universal and rapid salt-extraction of genomic DNA for PCR-based analysis (Aljanabi & Martinez 1997). DNA was extracted from frozen or ethanol preserved muscle tissue. In some cases, DNA was extracted using blood samples.

Instead of a primer pair created for *A. japonica* and *A. marmorata* by Lintas *et al.* 1998, which enabled the amplification of a 493 bp fragment of mtDNA (part of the tRNA for threonine, the tRNA for proline and part of the D-loop), a new primer pair (Ang1-F and Ang1-R), specific for *A. anguilla*, was created using the Primer 3 program (Rozen & Skaletsky 2000) and data from GenBank, i.e., comparison of all available mtDNA D-loop region sequences in *Anguilla* species enabled to choose the most suitable primer annealing area. Oligonucleotide primer sequences were as follows: 5'-TCGGTTTTGTAATCCGAAGA-3' for Ang1-F and

5'-CCAAATGCCAGTAATAGTTCATTTTA-3' for Ang1-R. The chosen oligonucleotides were synthesized by biomers.net GmbH.

PCR was performed using the Eppendorf Mastercycler gradient PCR machine. PCR volume for each sample was 25 µl and consisted of: 5 µl of genomic DNA at a concentration of approximately 50 µg/µl, 0.75 µl Taq DNA Polymerase LC (MBI Fermentas), 2.5 µl Taq buffer + KCl, 2.5 µl MgCl₂, 2.5 µl 2 mM dNTP Mix, 1 µl of each oligonucleotide, i.e., Ang1-F and Ang1-R, and water.

Amplification started with an initial denaturation step for 5 min at 95°C, followed by 35 cycles of denaturation for 45 sec at 94°C, annealing for 45 sec at 54°C and elongation for 1 min at 72°C, ended with a final elongation step for 5 min at 72°C.

After amplification, 5 µl of each PCR product was mixed with 2 µl loading dye and then loaded in 1.5% agarose gel. AGE (Agarose Gel Electrophoresis) was carried out in for 1 hour at 100 V in the Pharmacia Gel GNA-100 apparatus. Agarose gel, after staining with ethidium bromide, was examined using the Biometra BioDocAnalyze apparatus. The amplified fragment was approximately 610 bp. Only well-amplified fragments were selected for sequencing after the incubation of 5 µl of amplicate mixed with 1 µl CIAP (MBI Fermentas) and 0.5 µl exonuclease I (MBI Fermentas) for 15 min at 37°C and 15 min at 85°C, respectively. Purified PCR products and the same primers Ang1-F and Ang1-R were used for DNA sequencing in the Sequencing Centre of the Institute of Biotechnology (Lithuania) using the Big-Dye[®] Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) and 3130xl Genetic Analyzer (Applied Biosystems, USA).

A. anguilla mtDNA sequence analysis

After sequencing, chromatogram files were displayed and transferred to other programs using the CHROMAS 2.33 program (free software from www.technelysium.com.au). Newly detected *A. anguilla* mtDNA D-loop region sequences were aligned together with 80 homological fragments determined by previous investigators (data on 55 European eel mtDNA sequences (AJ225953-AJ226007) (Lintas *et al.* 1998) and 25 European eel homological mtDNA sequences (AJ246983-AJ247007) (Murgia *et al.* 1998) deposited in GenBank). A total of 125 sequences were aligned together with one sequence of *A. rostrata* (AB030662) using the CLC Sequence Viewer 5.1.1 program (free software from www.clcbio.com).

The number of polymorphic sites (S), total number of mutations (η), haplotype diversity (H), nucleotide diversity (π) and θ were estimated using the DNASP 4.50.3 program (Rozas *et al.* 2003). θ values were estimated according to the infinite sites model (Ewens 1972) using



Figure 1. Sampling locations of European eel during this research (small dots) and two earlier investigations (large dots): Lintas *et al.* 1998 and Murgia *et al.* 1998 (article unpublished; data from GenBank). Sampling locations of earlier investigations are approximate (particularly the samples from France).

three different estimators: θ_s , θ_π and θ_n (Tajima 1996). The DNASP 4.50.3 program eliminated all positions containing gaps, except where otherwise stated. Most phylogenetic analyses were conducted in MEGA4 (Tamura *et al.* 2007), but all possible shortest and least complex phylogenetic trees were constructed using the median joining (MJ, Bandelt *et al.* 1999) option in the NETWORK 4.5.1.0 program (free software from www.fluxus-engineering.com/). The MEGA4 program eliminated from the dataset all positions containing gaps (complete deletion option), whereas the NETWORK 4.5.1.0 program calculated the positions

containing gaps. In MEGA4, the evolutionary history was inferred using the neighbour-joining method (NJ, Saitou & Nei 1987). The bootstrap consensus tree was inferred from 500 replicates (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site.

The rho statistic for time estimates was used first by Morral *et al.* 1994 under a different name and then explored by Forster *et al.* 1996. The calculation of standard errors for rho was presented by Saillard *et al.* 2000.

Therefore, in this investigation rho statistic was selected for time estimations. Both rho and standard errors for rho were calculated using the NETWORK 4.5.1.0 program. Aoyama and Tsukamoto 1997 suggest that molecular evolution in eels may be much slower than in other animals, therefore an approximately four times slower mutation rate than that for human mtDNA in the stretch between np 16,090 and np16,365 was selected. Thus, one mutation in 80000 years was selected instead of one mutation in 20180 years (default option).

Based on DNASP 4.50.3 program θ values, the effective population female size was estimated according to $\theta = 2 N_e \mu$ model, with N_e – the effective population female size and μ – the mutation rate. The assumed mutation rate of 10^{-6} mutations per locus per generation in cyt *b* in fishes (Canatore *et al.* 1994 cited in Daemen *et al.* 2001) allowed to presume that a mutation rate of 10^{-5} in the D-loop in *A. anguilla* should be sufficient for approximate analyses, since mutations in the D-loop region accumulate faster than in other mtDNA regions.

K_{XY} , the divergence between groups of sequences as measured by the uncorrected average number of nucleotide substitutions per site between populations (Nei 1987) and some other measures of the extent of DNA divergence among populations were calculated using the DNASP 4.50.3 program. Note that during these calculations gaps as the fifth state were considered. The obtained pairwise distances between populations were used for neighbour-joining analysis in MEGA4. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

RESULTS

Data on mtDNA sequence variation

Based on a 493 bp fragment of mtDNA, 110 haplotypes were observed (Fig. 2). Consequently, all current knowledge about *A. anguilla* mtDNA 493 bp fragment variability are presented in Table 1. Currently, 36 new haplotypes are found in Lithuania (the sequences are deposited in the GenBank database under accession numbers FJ707255-FJ707280), but none of them was detected twice. Thus, comparison of 125 European eel sequences revealed 14 newly detected D-loop region sequences previously identified by other authors as haplotypes GL3 (2), FR4 (1), LS4 (1), M1 (5), NG1 (2), NG3 (1), NL2 (1), NL4 (1) and RM3 (1).

Using the DNASP 4.50.3 program, the following calculations (gaps excluded) were made: polymorphic sites (S) = 115, total number of mutations (η) = 132, overall haplotype diversity (H) = 0.9965 (\pm 0.002) and nucleotide diversity (π) = 0.02523.

Table 1. Current knowledge about *A. anguilla* mtDNA (493 bp amplified fragment comprised of part of the tRNA for threonine, tRNA for proline and part of the D-loop noncoding region) haplotypes.

Haplotypes	This research only	Earlier investigations (Lintas <i>et al.</i> 1998 and Murgia <i>et al.</i> 1998)	Total
Different	36	74	110
Repetition	9	6	15
Total	45	80	125

Phylogenetic tree

The phylogenetic tree derived from 125 European eel sequences and one homological sequence of American eel is presented in Figure 3. Comparison of the results derived from our phylogenetic tree and those of phylogenetic analysis by Lintas *et al.* 1998 suggests the absence of any significant clustering related to sampling locations. However, in contrast to previous reports, one specific group of phylogenetically and geographically related haplotypes BJ3, BJ9 and KM6 was detected predominantly in the Lithuanian territorial waters of the Baltic Sea and the Curonian Lagoon (see arrow, Fig. 3), with one exceptional specimen NL1 detected in the Netherlands. Interestingly, NETWORK 4.5.1.0 MJ results (Fig. 4) also indicate that these four haplotypes belong to one phylogenetic group (D1).

Networks and haplogroups

More traditional methods developed to estimate inter-specific relationships, such as maximum likelihood, maximum parsimony and minimum evolution, cannot properly take account of the fact that, at the population level, several phenomena violate some of their assumptions (Posada & Crandall 2001). Interestingly, networks are appropriate representations of intraspecific genetic variation even if they also have some limitations (Posada & Crandall 2001). Consequently, one network programme was selected. All possible shortest and least complex phylogenetic trees were constructed using the NETWORK 4.5.1.0 program MJ option. The estimated number of mutations of the shortest tree in this intraspecific network was 319. According to the results presented in Figure 4, at least several haplogroups could be defined. In haplogroups A and B, smaller subgroups were not defined because it was impossible to determine correctly the number of additional subgroups. Haplogroup C was not divided into subgroups either, since it was not reasonable to split it into smaller units having only 1–2 representatives (in most cases). However, it is obvious that haplogroup C, like haplogroup B, can be separated into several subgroups. Since haplogroups F and G have only three representatives, only haplogroups D and E

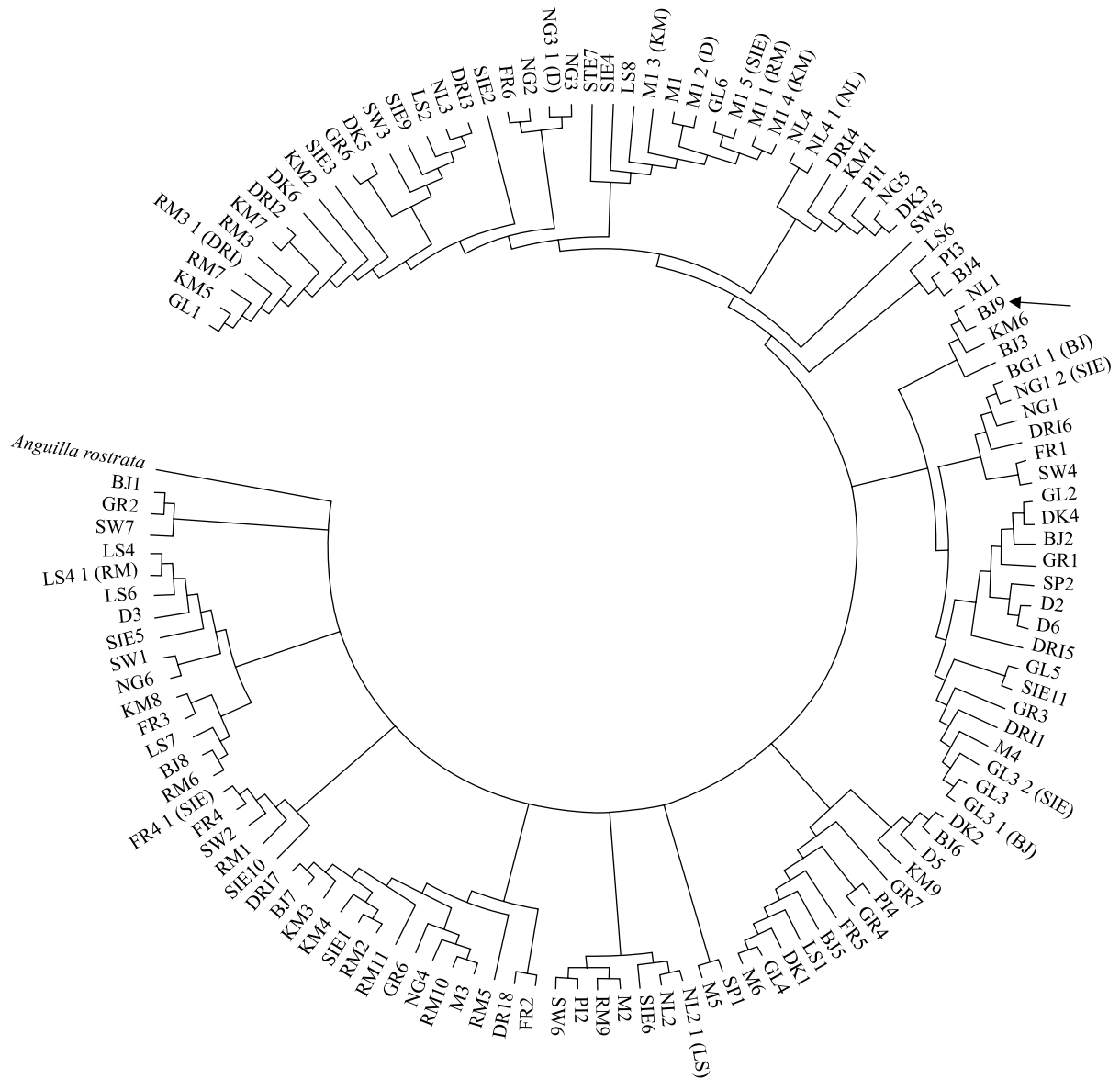


Figure 3. Evolutionary relationships of 126 taxa (*A. rostrata* sequence accession number in GenBank is AB030662). The evolutionary history was inferred using the neighbor-joining method. The bootstrap consensus tree inferred from 500 replicates. Specimens with same haplotypes have different names with additional numbers and sampling location indications.

were divided into smaller units. Haplogroup E was divided into two clearly separated subgroups. It should be noted that subgroups combined into haplogroup D could be distinguished as different branches, since NETWORK revealed a few possible ways for some branches. In order to find out if all haplogroups and subgroups are defined correctly, further investigations are necessary.

Time estimates

The application of the median joining network, constructed using only one available *A. rostrata* sequence and 125 *A. anguilla* sequences, showed that haplogroup B was the most similar to *A. rostrata* sequence. It took

at least 1320000 ± 226274 years for haplogroup B to evolve from the ancestor of these two species. It seems that at least 560000 ± 80000 years have passed since all other haplogroups evolved from ancestral haplogroup B. The above calculations suggest that the evolution of haplogroups A and C–G has lasted not less than 844138 ± 125846 years. Consequently, the estimated divergence times obtained by our research group appeared to be a bit shorter in comparison to those of earlier reports suggesting that *A. rostrata* and *A. anguilla* speciation occurred between 3 (Daemen *et al.* 2001) and 10 (Aoyama & Tsukamoto 1997) MA (million years ago).

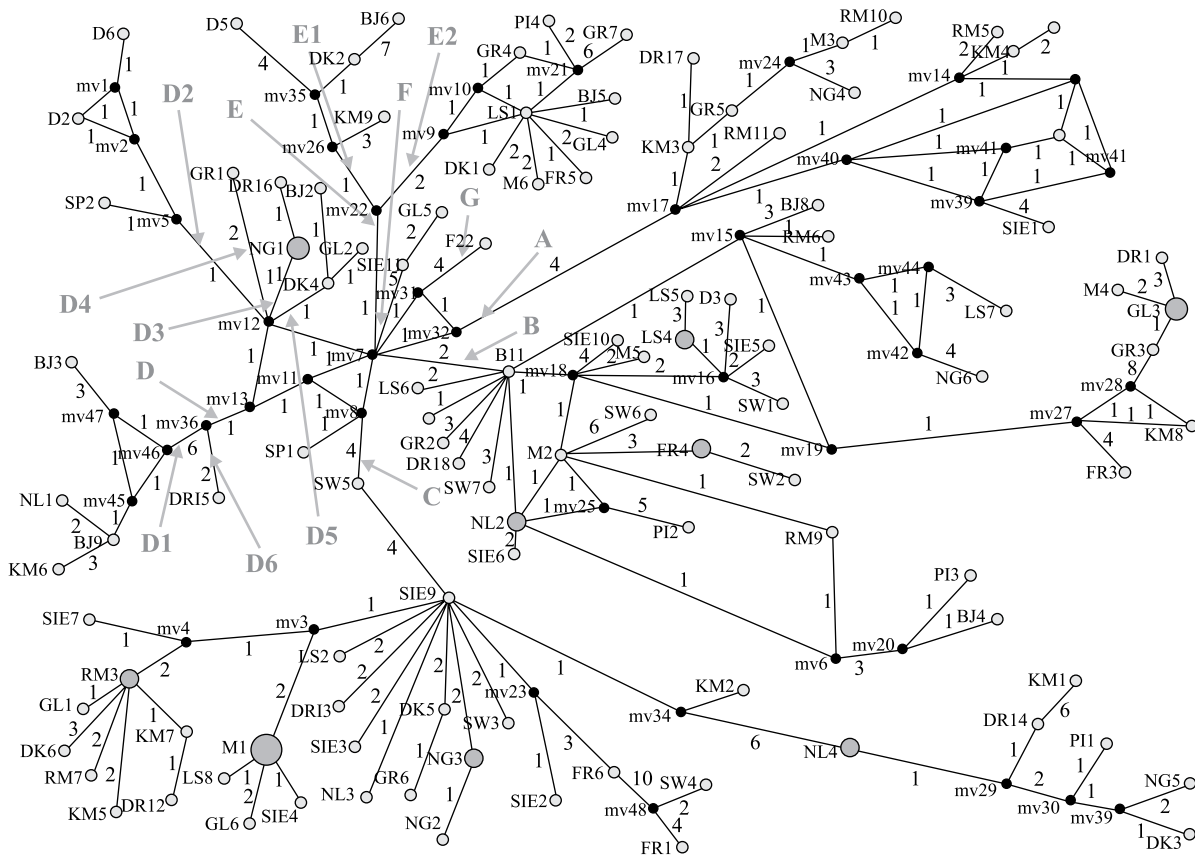


Figure 4. Network of 125 *A. anguilla* sequences. Numbers between nodes indicate mutational steps, node sizes are proportional to frequencies, mv (any number) is median vectors, i.e., hypothetical sequences which were not found.

Female effective population size

The female effective population size (N_e) calculated according to θ_π , θ_S and θ_η estimators are presented in Table 2. Based on the absence of significant differences between previous sequencing data (Murgia *et al.* 1998; Lintas *et al.* 1998) and the results obtained in this research, we suggest that there was no apparent bottleneck in *A. anguilla* population during the last decade.

Population structure

Characteristics of the *A. anguilla* population, which was divided into subpopulations depending on large scale and small scale geographic regions, were derived from

mtDNA D-loop region sequences (Table 3). The average number of nucleotide differences (K) was largest in the hypothetical northern *A. anguilla* subpopulation which included the samples from the Curonian Lagoon, the Baltic Sea and the North Sea. Despite complex evolutionary relationships among different haplogroups in the haplotypic network, we focused our attention on close evolutionary relationships of individuals attributed to haplogroup D1. Among four individuals from the northern subpopulation, three individuals with given haplotypes were detected only in the inshore area of the Baltic Sea and in the Curonian Lagoon (Lithuanian territorial waters), whereas the fourth one was caught in the Netherlands (haplotype NL1). So, it seems that part

Table 2. Comparison of female effective population size (N_e) values derived from θ_S , θ_π and θ_η estimators calculated using data obtained by three different research groups.

Investigation	Investigated sequences	Estimator				N_e	
		θ_π	θ_S	θ_η			
Our research group	45	0.02666	0.03771	0.04048	1333	1885	2024
Murgia <i>et al.</i> 1998	25	0.02659	0.03544	0.03642	1329	1772	1821
Lintas <i>et al.</i> 1998	55	0.02519	0.04323	0.04668	1259	2161	2334

Table 3. Characteristics of *A. anguilla* populations: 1) hypothetical population divided into Atlantic, Mediterranean and northern subpopulations, and introduced eels caught in Lithuanian inland waters; 2) Lithuanian samples of eels representing the Baltic Sea, Curonian Lagoon, Lake Dringis and Lake Siesartis (n – samples, S – polymorphic sites, H – haplotype diversity, K – average number of nucleotide differences).

Analysis	Population	n	Haplotypes	S	H	K
1)	Atlantic	18	18	55	1	12.90196
	Mediterranean	38	36	64	0.99716	11.57610
	North	46	43	90	0.99710	13.82029
	Introduced	23	23	51	1	12.71542
	Total	125	110	119	0.99652	12.88452
2)	Baltic Sea	11	11	47	1	13.94545
	Curonian Lagoon	11	10	39	0.98182	12.29091
	Lake Dringis	9	9	41	1	13.55556
	Lake Siesartis	14	14	39	1	12.47253
	Total	45	41	75	0.99495	13.19899

of European eels living in the North Sea and the Baltic Sea differs from the rest of the *A. anguilla* population, though the overall genetic differentiation between artificially composed subpopulations is not significant.

Comparison of eels representing low scale subpopulations, such as of the inshore zone of the Baltic Sea and the Curonian Lagoon, revealed that the average number of nucleotide differences (K) between these two low scale subpopulations was different. Higher K values were detected among the samples representing the Baltic Sea subpopulation. However, it is not surprising that the Baltic Sea eel subpopulation occupies the largest water body as compared to other small scale sampling areas (Curonian Lagoon and two inland lakes). According to the results obtained by Shiao *et al.* 2006 from otolith analysis, about 20% of European eels caught in the Curonian Lagoon originated from the stock of introduced eels. Thus, theoretically it is possible to detect the recurrence of the same haplotypes in the Curonian Lagoon, i.e., the K value for the lagoon should be lower than in the Baltic Sea sample. This assumption was partly confirmed by the detection of animals with haplotype M1 both in Curonian Lagoon and in Lake Siesartis samples.

Haplotype M1 is the most common *A. anguilla* haplotype, which, besides Lithuania, was also found in Ireland and Italy, therefore the possibility of natural recruitment of M1-haplotype eels found in the Curonian Lagoon cannot be excluded. Regardless of this, it is more likely that M1-haplotype individuals (attributed to haplogroup C) migrated to the Curonian Lagoon after they escaped from inland lakes. It should be noted that haplogroup C representatives have not yet been detected in the Baltic Sea near Lithuania, although the representatives of this haplogroup have been observed in the Baltic Sea near Sweden (Fig. 5).

Two NJ trees based on the mean number of pairwise nucleotide differences between populations (K_{XY}) are presented in Figure 6. With regard to overall genetic similarity, the Curonian Lagoon sample as compared to the Baltic Sea sample seems to be closer to the samples of introduced eels from Lake Dringis and Lake Siesartis, at least on a small geographic scale. The pooling of samples into subpopulations representing large scale geographic differentiation revealed differences between the hypothetical northern subpopulation comprised of Curonian Lagoon, Baltic Sea and North Sea samples and the other two hypothetical subpopulations representing the Atlantic and Mediterranean pools of haplotypes. As for the Lithuanian case, it seems that the sample representing introduced eels should not be attributed to any of the three hypothetical *A. anguilla* subpopulations. It is obvious that introduced eels could be recognised as possessing a haplotypic pool which is intermediate between the northern and the rest of the European population.

DISCUSSION

Haplotype variety

New results based on *A. anguilla* mtDNA D-loop region sequencing obtained in this study renewed investigations of haplotypic variability in European eel initiated by two research groups in 1998 (Lintas *et al.* 1998; Murgia *et al.* 1998, unpubl. data). It has become obvious that at least this particular species displays a wide mtDNA D-loop region variety: a total of 111 different haplotypes were found among the 125 animals investigated. Comparable data on haplotypic variability in the mtDNA cytochrome *b* partial sequence of *Anguilla* species were provided by another research group (Dae-

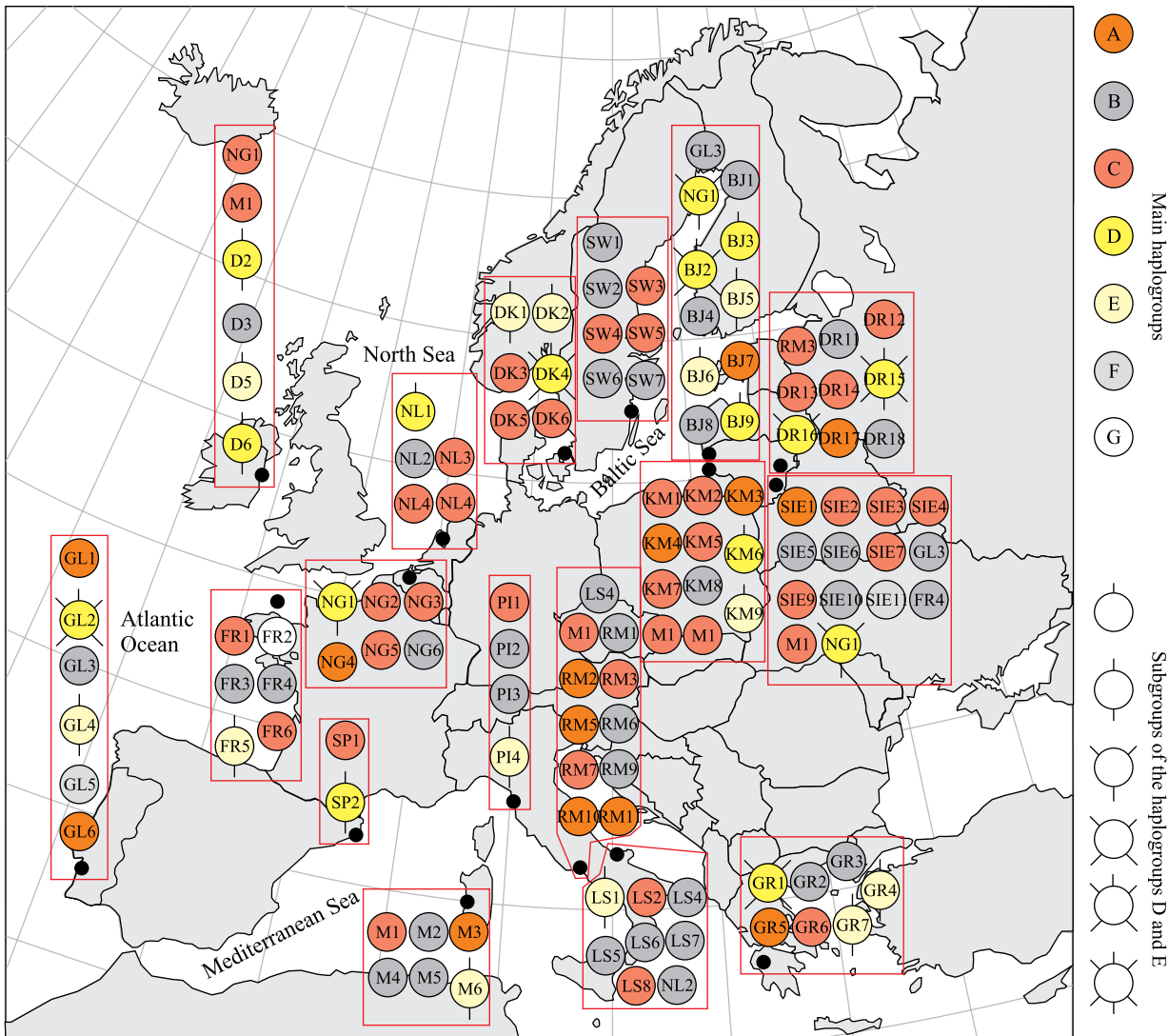


Figure 5. Distribution of haplotypes listed according to sampling locations (small dots represent our research areas, large dots indicate areas studied by other authors): GL (Portugal, Atlantic coast), SP (Spain, Barcelona), M (Italy, Cabras Lagoon), PI (Italy, Pisa), RM (Italy, Rome, Tiber River, LS (Italy, Lesina Lagoon), GR (Greece, Messolonghi Lagoon), D (Ireland, Dublin), FR (France, West Coast), NG (Netherlands, Rhine River), NL (Netherlands, Nieuw Koop Lagoon), DK (Denmark, Copenhagen, Lake Arresø), SW (Sweden), BJ (Lithuania, Baltic Sea), KM (Lithuania, Curonian Lagoon), DRI (Lithuania, Lake Dringis), SIE (Lithuania, Lake Siesartis). Spectrum of colours represents different haplogroups, D and E haplogroups divided into subgroups, different subgroups have different shape. Some haplotypes found in different sampling areas were named according to one sampling location. Sampling locations of earlier investigations are approximate.

men *et al.* 2001). The investigation of 107 *A. anguilla* mtDNA cytochrome *b* (392 bp) sequences revealed only 17 different haplotypes, 10 of which were singletons (Daemen *et al.* 2001). Thus, the mutation rates in the *A. anguilla* mtDNA cytochrome *b* sequence are more than six times lower than those in the mtDNA D-loop region.

Network and haplogroups

Studies on European eel were initiated more than 100

years ago and hypotheses concerning its population structure were tested using newly developed techniques every time they appeared (Maes & Volckaert 2007). Intraspecific gene evolution cannot always be represented by a bifurcating tree because traditional methods for estimating phylogenies are not designed and might not be adequate for within-species phylogeny (Posada & Crandall 2001). Unfortunately, only bifurcating trees have been used so far in the investigations of *A. anguilla* and *A. japonica* within-species phylogenies. Therefore,

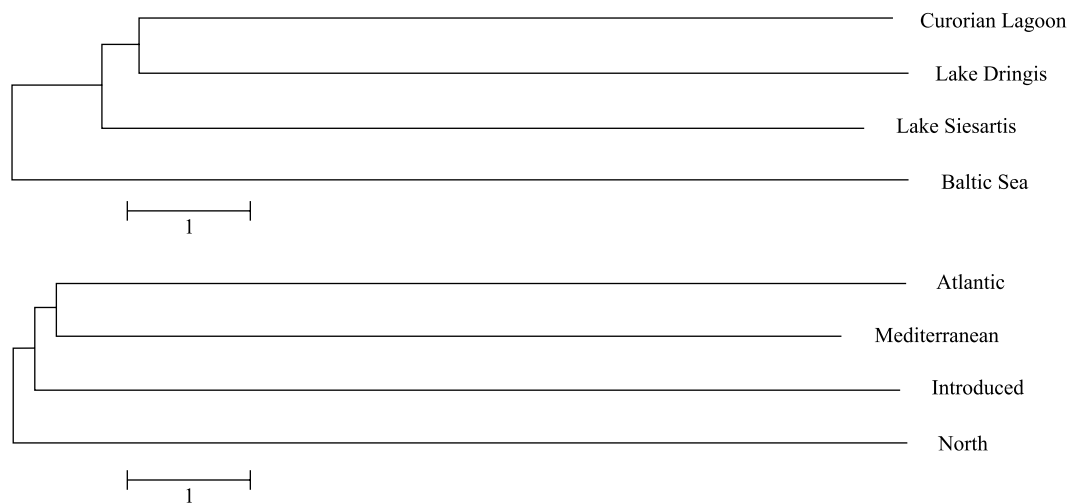


Figure 6. NJ trees of *A. anguilla* populations based on pairwise K_{XY} distances between mtDNA haplotypes. At the top: relationships among eel samples representing small scale geographic differences between some sampling locations in Lithuania. At the bottom: relationships between three hypothetical *A. anguilla* subpopulations and the sample of eels introduced into Lithuanian inland lakes.

a considerable amount of valuable information about these species has been missed. Consequently, previous investigations (Lintas *et al.* 1998; Sang *et al.* 1994 and Ishikawa *et al.* 2001) failed to reach full potential, i.e., valuable information which could have been obtained from *A. anguilla* and *A. japonica* D-loop region variability was omitted. Nevertheless, the accumulated data could be used as initial database for future investigations.

A wide mtDNA D-loop region haplotype variety allowed, instead of haplotypes, to use a haplogroup category as the most appropriate category to estimate the connection between the sampling location and genetic variability. Since a network based approach can incorporate population processes in the construction of the refinement of haplotype relationships and enable a more detailed display of population information than strictly bifurcating trees (Posada & Crandall 2001), haplogroup category was quite informative when used for evaluation of phylogenetic relationships among different *A. anguilla* samples. Several main haplogroups, i.e., A-G, and a few subgroups of haplogroups D and E, were defined after a detailed evaluation of the haplotypic network. It should be taken into account that if new haplotypes are detected during further investigations, some branches of the network might change, but the probability of dramatic changes in phylogenetic relationships between the defined haplogroups is very low. Actually, it is very important for future research to use historical material and/or new sampling regions, as well as all possible information about otolith investigation results, other genetic markers, etc.

***A. anguilla* and *A. rostrata* speciation and European eel within-species evolution**

A. anguilla and *A. rostrata* speciation dates from 3 (Daemen *et al.* 2001) to 10 (Aoyama & Tsukamoto 1997) MA. New *A. anguilla* and *A. rostrata* speciation time estimates are in accordance with the results of earlier studies. Time estimates obtained in this study also show that the time of divergence of these two species is near 3 MA. It could be assumed that after the speciation event haplogroup B was formed as one of the most ancestral haplogroup of *A. anguilla* that still has representatives even today. The pattern of haplotypic network structure (Fig. 4) suggests that after *A. anguilla* and *A. rostrata* speciation only one European eel evolution branch occurred, from which all presently found haplogroups evolved. Since the evolution of haplogroups A and C-G is estimated to be approximately 0.85 million years, we suggest that the within-population divergence in *A. anguilla* species lasted for at least ~2 MA. According to *cyt b* investigation results by Daemen *et al.* 2001, time estimates of within-population divergence were 1.9–3.8 million years before present. In order to get a better understanding of *A. anguilla* evolution, further more detailed investigations are necessary.

***A. anguilla* genetic differentiation complexity**

Panmixia in *A. anguilla* was widely accepted until three independent genetic studies (Daemen *et al.* 2001; Wirth & Bernatchez 2001; Maes & Volckaert 2002) reported evidence indicating a weak, but significant population structure (Dannewitz *et al.* 2005; Palm *et*

al. 2009). Genetic differentiation in *A. anguilla* populations could be explained not only by the isolation-by-distance (IBD) pattern (Wirth & Bernatchez 2001, 2003), but also by the role of temporal genetic variation (Maes & Volckaert 2002; Dannewitz *et al.* 2005). Temporal restriction on gene flow is called IBT to acknowledge its analogy with IBD. During the presence of IBT, variation in selection throughout the reproductive season may lead to adaptive temporal variation in phenotypic traits (adaptation-by-time) (Hendry & Day 2005). Actually, isolation-by-time (IBT) is even more important in shaping the genetic structure of *A. anguilla* than IBD (Maes & Volckaert 2007).

Unfortunately, during this investigation temporal samples were collected only from the Curonian Lagoon and Lake Dringis, thus discussions about IBT are not appropriate. Current knowledge about *A. anguilla* mtDNA D-loop region haplotype variety suggests the absence of IBD within this species. This view is supported by similar assumptions from earlier investigations of the mtDNA D-loop region in *A. anguilla* and *A. japonica* (Lintas *et al.* 1998; Sang *et al.* 1994; Ishikawa *et al.* 2001). For example, a wide haplotype variety, low nucleotide diversity (π), node bifurcations of the NJ phylogenetic tree were not strongly supported. However, analysis of 125 European eel mtDNA D-loop region sequences without any additional information, i.e., otolith results, DNA microsatellites, etc., does not allow the conclusion about the absence of genetic differentiation within this species. Furthermore, even genetic homogeneity does not necessarily imply a single panmictic population (Lintas *et al.* 1998). Consequently, even a low level of geographical genetic differentiation at neutral molecular markers may lead to serious underestimation of quantitative and adaptive differentiation between populations (Maes & Volckaert 2007).

Interestingly, the employment of haplogroup category, demonstrated limited evidence of genetic differentiation in *A. anguilla*. Analysis of the haplotypic network (Fig. 4) showed that special attention should be focused on the haplotypes of subgroup D1, attributed to haplogroup D, because of a limited geographic area where the representatives of this subgroup were detected: they were found only in Lithuanian territorial waters and the Netherlands (haplotype NL1). The possibility that haplotypes of subgroup D1 were grouped according to geographic location accidentally seems to be unlikely. First, the values of populational parameters (n – number of the investigated eels, S – number of polymorphic sites and K – average number of nucleotide differences) derived from mtDNA sequences (Table 3) were much higher in the hypothetical northern *A. anguilla* population than in other hypothetical *A. anguilla* populations.

Second, the haplotypes of subgroup D1 differ in very few mutational steps which suggest that they closely coevolved long enough to evolve into a haplotypic subgroup. Third, the paradigm that European eel constitutes a panmictic population is difficult to maintain because of recent evidence from the related studies (Daemen *et al.* 2001). Finally, the haplotypic network indicates that *A. anguilla* has never been a panmictic animal. Consequently, the definition of D1 as subgroup representing geographically and genetically connected individuals requires further investigations.

Diadromy in eels is more complex than simple separation into catadromous and non-catadromous individuals (Daverat *et al.* 2006). Actually, Daverat *et al.* (2006) identified more than six types of the main habitat use by eels. Interestingly, the types of habitat use were observed in different proportions in different geographic regions (Daverat *et al.* 2006). However, it is still unknown whether this is due to a remnant genetic trait that determines if an individual enters freshwater or not, or if it is simply due to behavioural plasticity that enables each species to use the maximum range of habitats (Tsukamoto & Arai 2001). Evidence exists that semi-catadromous European eels live in the Baltic Sea (Tzeng *et al.* 2000), therefore further investigations of the mtDNA D-loop region, combined with otolith analysis, might yield valuable information about the discussed subject.

Naturally recruited and introduced European eels in Lithuania

According to Shiao *et al.* (2006), most of European eels which live in the Baltic Sea and in the Curonian Lagoon are naturally recruited, whereas all European eels that inhabit Lithuanian lakes are introduced from west European countries. The fact that the representatives of haplogroup E and the haplotypes of subgroup D1, though absent in inland lakes, were detected in the Baltic Sea, leads to conclusion that the haplotypic spectrum of naturally recruited eels differs from that of introduced eels. Furthermore, the representatives of haplogroup C were not detected in the Baltic Sea, whereas the haplotypes of this haplogroup were common in the Curonian Lagoon and inland lakes. Such differences in the haplotypic spectrum between the Baltic Sea and the Curonian Lagoon are observed due to a significant percentage of introduced eels inhabiting the Curonian Lagoon. Our results, based on otolith analysis are in agreement with those reported by Shiao *et al.* 2006 indicating that at least 20% of eels migrate from inland lakes to the Curonian Lagoon. In the Baltic Sea, near Lithuania, introduced European eels account only for 2% of all eels living there (Shiao *et al.* 2006).

***A. anguilla* conservation**

Challenges for genetic research on European eel management were reviewed by Maes and Volckaert (2007). Accurate estimation of the effective population size is one of the most important aims in developing an appropriate conservation strategy for eels (Maes & Volckaert 2007). Our investigation results suggest that during the period of approximately 10 years no bottleneck in the *A. anguilla* population probably occurred and that female effective population size was low.

Today, investigations of genetic differentiation of European eel populations based on the current mtDNA D-loop region sequence data are not statistically significant to confirm genetic differentiation between European eels living in the North Sea and Lithuanian territorial waters and the rest of the *A. anguilla* population. Nevertheless, limited evidence of genetic differentiation was found among European eel subpopulations based on the distribution patterns of evolutionary close haplotypes. Interestingly, limited evidence of genetic differentiation observed in the Baltic Sea was reported by Daemen *et al.* 2001. Recent work based on molecular markers has highlighted the possibility of the existence of local population structure within eel populations of Europe and North Africa (Kettle *et al.* 2008). Non-random distribution of individuals with evolutionary close haplotypes of subgroup D1 detected in the Baltic Sea and in the Curonian Lagoon during this investigation could be interpreted as evidence of natal homing and self-replenishment of local populations recently described by Cano *et al.* 2008. Thus, we support the assumption that such phenomenon may be more common in marine fish than previously anticipated.

In order to make appropriate conclusions, a more representative *A. anguilla* mtDNA D-loop region database should be created. Therefore, combined investigations of the mtDNA D-loop region, other genetic markers and otoliths are urgent. Additional attempts should be made to connect haplotypic information with data on other genetic or ecological markers useful for the development of a long-term management strategy. Finally, the detection of negative changes and reductions in biological diversity will be impossible unless the amount and distribution of this diversity are systematically studied (Laikre *et al.* 2008).

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- UPINIO UNGURIO *ANGUILLA ANGUILLA* (L.) MTDNR D-KILPOS REGIONO HAPLOTIPŲ ĮVAIROVĖ**
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- SANTRAUKA**
- Iki šiol buvo tik du informacijos šaltiniai apie upinių ungurių *Anguilla anguilla* mtDNR D-kilpos regiono haplotipų įvairovę. Mes papildėme prieš dešimt metų sukurtą mtDNR D-kilpos regiono duomenų bazę 45 homologinėmis sekomis, kurios reprezentuoja Baltijos jūroje, Kuršių mariose ir Dringio bei Siesarčio ežeruose neseniai pagautus upinius ungurius. Iš šių 45 sekų net 36 – iki šiol neidentifikuoti haplotipai. Remiantis *A. anguilla* DNR sekų duomenimis iš Genų banko, buvo nustatyti šie parametrai: $S = 115$, $\eta = 132$, $H = 0.9965 (\pm 0.002)$ ir $\pi = 0.02523$. Panaudojus 125 upinių ungurių duomenis, pagal mtDNR D-kilpos sekas sukonstruotame NJ filogenetiniame medyje akivaizdus haplotipų grupavimosi pagal žuvų pagavimo vietas neaptikta. Genetinės diferenciacijos įrodymai gauti kitu, haplotipų tinklo konstravimo metodu. Šiame darbe aptariama *A. anguilla* vidurūšinė evoliucija, populiacinė struktūra, rūšies apsaugos aspektai ir NJ filogenetinio medžio bei haplotipų tinklo metodikų tinkamumas *A. anguilla* vidurūšinės evoliucijos tyrimams.

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