Molecular and Phenotypic Evidence of a New Species of Genus *Esox* (Esocidae, Esociformes, Actinopterygii): The Southern Pike, *Esox flaviae*  

Livia Lucentini1*, Maria Elena Puletti1, Claudia Ricciolini1, Lilia Gigliarelli1, Diego Fontaneto2, Luisa Lanfalone1, Fabiana Bilò3, Mauro Natali4, Fausto Panara1  

1 Dipartimento di Biologia Cellulare e Ambientale, Università degli Studi di Perugia, Perugia, Italy, 2 Division of Biology, Imperial College London, Silwood Park Campus, Berkshire, United Kingdom, 3 Veneto Agricoltura Viale dell’Università, Legnaro, Italy, 4 Provincia di Perugia, Via Angelucci, Perugia, Italy  

Abstract  
We address the taxonomic position of the southern European individuals of pike, performing a series of tests and comparisons from morphology, DNA taxonomy and population genetics parameters, in order to support the hypothesis that two species of pike, and not only one, exist in Europe. A strong relationship emerged between a northern genotype supported by COI, Cytb, AFLP and specific fragments, and a phenotype with round spot skin colour pattern and a large number of scales in the lateral line, clearly separated from a southern genotype with other skin colour pattern and a low number of scales in the lateral line. DNA taxonomy, based on a coalescent approach (GMYC) from phylogenetic reconstructions on COI and Cytb together with AFLP admixture analysis, supported the existence of two independently evolving entities. Such differences are not simply due to geographic distances, as northern European samples are more similar to Canadian and Chinese samples than the southern Europe ones. Thus, given that the differences between the two groups of European pike are significant at the phenotypic, genotypic and geographical levels, we propose the identification of two pike species: the already known northern pike (*Esox lucius*) and the southern pike (*E. flaviae* n.sp.). The correct identification of these two lineages as independent species should give rise to a ban on the introduction of northern pikes in southern Europe for recreational fishing, due to potential problems of hybridisation.

Citation: Lucentini L, Puletti ME, Ricciolini C, Gigliarelli L, Fontaneto D, et al. (2011) Molecular and Phenotypic Evidence of a New Species of Genus *Esox* (Esocidae, Esociformes, Actinopterygii): The Southern Pike, *Esox flaviae*. PLoS ONE 6(12): e25218. doi:10.1371/journal.pone.0025218  

Editor: Sharyn Jane Goldstien, University of Canterbury, New Zealand  
Received July 14, 2011; Accepted August 29, 2011; Published December 2, 2011  
Copyright: © 2011 Lucentini et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors have no support or funding to report.  
Competing Interests: The authors have declared that no competing interests exist.

* E-mail: livia@unipg.it

Introduction

Freshwater habitats support most ecosystem services, but their integrity is continuously affected by anthropogenic threats [1–3], as the introduction of non-native species and individuals for recreational fishing [4,5]. These introductions may have a major impact on local diversity, as they are carried out by organised fishing associations, which re-stock local populations of target species using non-native animals. The impoverishment of phenotypic and genetic diversity is a known consequence of these activities in European salmonids [6,7], where local populations can represent divergent lineages, potential endemic taxa [8,9]. Actions should be taken to counteract extinction of endemic taxa and populations because of stocking with non-native individuals. Concerns on threatened fish other than salmonids are indeed present, and identification of divergent, endemic lineages should be considered urgently, especially for widespread species targeted by recreational fishing. Among those, the northern pike, *Esox lucius* Linnaeus, 1758, is extensively managed in Europe.

The genus *Esox* is the only living genus in the family Esocidae, with five currently known species inhabiting North America, Europe and Eurasia. The phylogenetic relationships and biogeography of these species have been already studied [10–12], and the northern pike is the only native esocid in Europe (Text S1). Recent studies indicated a decline of southern populations [13–15], potentially due to its negative sensitivity to increasing water temperature [16], suggesting that this decline might be partly due to climate changes, as demonstrated for other species [17]. The negative effects of the contraction of these populations in Mediterranean countries have been managed through stocking programs with fries from northern Europe. This practice brought, as a consequence, the appearance of a phenotype, described as a yellow spot on a dark-coloured base (Figure 1), that was never seen earlier than 15 years ago in southern Europe [5;6] and that is the one described by Fickling [18] as with round/oval spots. To date, the question of whether this morphological difference between southern and northern European pikes suggests the existence of putative genetic separation of the two areas has not been investigated, even though the presence of distinct evolutionary entities in the European pike concerns biodiversity preservation, fish aquaculture and freshwater ecosystem management.

Here we explicitly test the hypothesis that the different phenotypes of the pike, geographically isolated in Europe, may represent two different evolutionary entities, in order to have reliable information for the management of wild populations of pikes in Europe. We analysed phenotypic and genetic differences sampling several individuals from different populations of European pike: as for the phenotype, we tested statistical
We also included information from populations outside Europe, differences from amplified fragment-length polymorphism (AFLP). In order to support this signature with nuclear genetic data we applied a coalescent based approach to mtDNA phylogeny; in order to identify the genetic signature of cryptic speciation we meristic characters as the number of scales in the lateral line. In differences in the general appearance of the skin pattern and in meristic characters as the number of scales in the lateral line. In order to identify the genetic signature of cryptic speciation we applied a coalescent based approach to mtDNA phylogeny; in order to support this signature with nuclear genetic data we evaluated the degree of historical admixture, testing overall genetic differences from amplified fragment-length polymorphism (AFLP). We also included information from populations outside Europe, present in the literature.

Our results strongly support the existence of two distinct lineages: thus, we here describe a new species of pike and provide phenotypic and genetic features to identify it unambiguously. As a consequence, we recommend stopping the stocking of pike in southern Europe using northern pike from other European countries, as this could greatly impact the survival of this newly discovered species in its native range.

Materials and Methods

The work performed during the analyses carried out for this manuscript is consistent with National regulations and indications of the Ethics Committee of the University of Perugia. Approval by Ethics Committee was not necessary given the nature of the data collected (counts of lateral line scales, photos and conservative specimens) and the method of data recovery, without any animal suffering. In fact, all the animals were anaesthetized, samples for DNA analyses were collected with non invasive techniques, and animals were all released in the same sampling site, after recovery in freshwater tanks. In such cases the Ethics Committee waives the need for consent. The only dead individual is the holotype, one of the spawners used in supportive breeding, accidentally killed during artificial spawning activity.

Sample collection

Sampling was not invasive: pictures of the right side of animals were collected for the morphological analyses, and 10 mg of caudal fin or a few scales were collected for the genetic analyses. The right side of 1306 mature individuals, longer than 30 cm was photographed by means of a digital photo camera. These samples were collected in three areas in Europe: continental Europe (Holland, Switzerland, Czech Republic, Hungary and Sweden), Northern Italy (Po basin, 11 populations) and Central Italy (Tevere basin, 4 populations) (Text S2).

Phenotypic analyses

The number of scales in the lateral line is a taxonomically informative meristic character that can provide clear-cut separation between populations of the northern and southern pike. The number of scales was counted for each individual. Counts were performed twice in different days, in order to check for consistency between measurements and no disagreements were found between repeated counts. Differences in the number of scales between the two potential groups of pike were tested by generalised linear models (GLM), implementing a quasi-Poisson distribution for count data, accounting for overdispersion [19]. GLM were performed in R 2.12.0 (R Development Core Team 2011).

The skin colour pattern is a qualitative character that can provide another useful tool for the discrimination of the two groups of pike in Europe. Presence/absence of such patterns can be analysed quantitatively to provide statistical support for the hypothesis of two separate entities. Five basic skin colour patterns were identified and noted, according to Fickling [18] with some modifications: we considered round spot (RS), stellate spot (SS), diagonal bars (DB), longitudinal bars (LB) and vertical bars (VB) (Figure 1). Differences in the presence of the five skin colour patterns in the two groups were tested by GLM in R, implementing a quasi-Poisson distribution.

Genetic analyses

All individuals were sampled non-invasively and were immediately reintroduced in the environment of origin. Genomic DNA was extracted and quantified as previously reported [20,21] on 374 samples belonging to the four identified areas.

Independent lineages of mtDNA. DNA taxonomy, providing species delimitation based on the topology of gene trees, is becoming a powerful tool to help taxonomists support their decisions [20,22]. We used two mtDNA loci, Cytochrome Oxidase Subunit I (COI) and Cytochrome b (Cytb), to reconstruct phylogenetic relationships and test for statistical evidence of two independently evolving entities using a coalescent-based approach. COI and Cytb are known to be useful in barcoding and DNA taxonomy in fishes [23–31]; moreover, these proteins might be related to oxygen availability and, thus, to water temperature, which is one of the main physical characters distinguishing northern and southern European freshwaters.

No nuclear copies of the COI and Cytb region have been reported in fishes; nevertheless, because preliminary PCR amplifications with Ivanova et al primers [32] displayed more than one band in electrophoretic visualization, we performed PCRs with more specific primers. COI amplicons of 651 bp were thus obtained using specifically designed primers F-COI-El GTGGCAATTCACCGCTG and R-COI-El CCGGGTGTTCCGGAAGATC. For Cytb, amplicons of 1079 bp were obtained using specifically designed primers cytbf TCGGACTCTACGAAGAATC and cytbr GTGCAACGGGTATTCCTCCA. PCR reactions were performed with Ready-to-go-DNA-PCR-Beads (GE) with the protocol described by Lucentini et al [14]. PCR products were purified and sequenced in forward and reverse directions as reported by Lucentini and colleagues [33].
Sequence identities were evaluated by blasting procedure (http://www.ncbi.nlm.nih.gov/BLAST/). Sequences were aligned and edited using MEGA 4.0. Moreover, we included in our dataset all COI and Cytb sequences of the genus *Esox* available from GenBank on May 20th, 2011 (Table 1). These sequences were used to widen the geographic coverage of the sample of the northern pike (including areas as Canada and China, geographically distant from our European samples), and to have outgroup information to root the phylogenetic trees.

Phylogenetic analyses were performed separately for each locus. The COI dataset comprised 30 haplotypes (20 from the present study and 10 from GenBank) with 651 bp. The Cytb dataset comprised 46 haplotypes (30 from the present study and 16 from GenBank) with 998 bp (Table 1). ModelGenerator 0.85 [34] was used to determine the best model of evolution for each dataset.

### Table 1. COI and Cytb datasets.

| COI | GenBank | Species | Haplotype Code | GenBank | Species       | Haplotype Code |
|-----|---------|---------|---------------|---------|---------------|---------------|
| EU524612 | *Esox niger* | - | | AY497428-30 | *Esox americanus* | - |
| EU524602 | *Esox masquinongy* | - | | AY497433-36 | *Esox americanus* | - |
| EU524598 | *Esox masquinongy* | - | | JN190460 | *Esox flavae* | Cythbap3 |
| EU524577 | *Esox americanus* | - | | JN190463 | *Esox flavae* | Cythbap6 |
| EU524573 | *Esox americanus* | - | | JN190464 | *Esox flavae* | Cythbap7 |
| HM563707 | *Esox flaviae* | COI hap 20 | | JN190465 | *Esox flavae* | Cythbap8 |
| HM563706 | *Esox flaviae* | COI hap 19 | | JN190471 | *Esox flavae* | Cythbap14 |
| HM563705 | *Esox flaviae* | COI hap 18 | | JN190472 | *Esox flavae* | Cythbap15 |
| HM563704 | *Esox flaviae* | COI hap 17 | | JN190476 | *Esox flavae* | Cythbap19 |
| HM563703 | *Esox flaviae* | COI hap 16 | | JN190477 | *Esox flavae* | Cythbap20 |
| HM563700 | *Esox flaviae* | COI hap 13 | | JN190481 | *Esox flavae* | Cythbap24 |
| HM563698 | *Esox flaviae* | COI hap 11 | | JN190482 | *Esox flavae* | Cythbap25 |
| HM563697 | *Esox flaviae* | COI hap 10 | | JN190484 | *Esox flavae* | Cythbap27 |
| HM563696 | *Esox flaviae* | COI hap 9 | | JN190485 | *Esox flavae* | Cythbap28 |
| HM563695 | *Esox flaviae* | COI hap 8 | | JN190486 | *Esox flavae* | Cythbap29 |
| HM563694 | *Esox flaviae* | COI hap 7 | | JN190487 | *Esox flavae* | Cythbap30 |
| HM563692 | *Esox flaviae* | COI hap 5 | | FJ425091-95 | *Esox lucius* | - |
| HM563691 | *Esox flaviae* | COI hap 4 | | FJ425097 | *Esox lucius* | - |
| HM563688 | *Esox flaviae* | COI hap 1 | | HM177469-70 | *Esox lucius* | - |
| EU524592 | *Esox lucius* | | | HM592193 | *Esox lucius* | - |
| EU524586 | *Esox lucius* | | | JN190458 | *Esox lucius* | Cythbap 1 |
| EU524578 | *Esox lucius* | | | JN190459 | *Esox lucius* | Cythbap 2 |
| HQ600729 | *Esox lucius* | | | JN190461 | *Esox lucius* | Cythbap 4 |
| FJ896108 | *Esox lucius* | | | JN190462 | *Esox lucius* | Cythbap 5 |
| HM563702 | *Esox lucius* | COI hap 15 | | JN190466 | *Esox lucius* | Cythbap 9 |
| HM563701 | *Esox lucius* | COI hap 14 | | JN190467 | *Esox lucius* | Cythbap 10 |
| HM563699 | *Esox lucius* | COI hap 12 | | JN190468 | *Esox lucius* | Cythbap 11 |
| HM563693 | *Esox lucius* | COI hap 6 | | JN190469 | *Esox lucius* | Cythbap 12 |
| HM563690 | *Esox lucius* | COI hap 3 | | JN190470 | *Esox lucius* | Cythbap 13 |
| HM563689 | *Esox lucius* | COI hap 2 | | JN190473 | *Esox lucius* | Cythbap 16 |
|  |  |  |  | JN190474 | *Esox lucius* | Cythbap 17 |
|  |  |  |  | JN190475 | *Esox lucius* | Cythbap 18 |
|  |  |  |  | JN190478 | *Esox lucius* | Cythbap 21 |
|  |  |  |  | JN190479 | *Esox lucius* | Cythbap 22 |
|  |  |  |  | JN190480 | *Esox lucius* | Cythbap 23 |
|  |  |  |  | JN190483 | *Esox lucius* | Cythbap 26 |
|  |  |  |  | AY497437 | *Esox niger* | - |
|  |  |  |  | AY497442 | *Esox reicherti* | - |
|  |  |  |  | AY497444 | *Esox reicherti* | - |

Haplotype codes are given for the individuals newly sequenced for this study, whereas no code has been given to sequences downloaded from GenBank. doi:10.1371/journal.pone.0025218.t001
used to select the best evolutionary models for the phylogenetic reconstructions, which resulted HKY+G for both datasets, according to AIC and BIC. Bayesian inference analyses were run in MrBayes 3.1.2 [35] for 3 million generations with two parallel searches. Maximum Likelihood (ML) reconstructions were performed using PhyML 3.0 [36], with 100 bootstrap replicates to provide support for the branching pattern.

The generalised mixed Yule coalescent (GMYC) model [37,38] was used to detect independent evolutionary lineages, evidence of distinct species from the topology of the tree. We used the output of the Bayesian inference as a starting tree for both loci, including only the dataset from Esox lucius, rooted with the closest of the other Esox (all other Esox for COI and only E. reicherti for Cytb). We then converted the tree into an ultrametric tree testing for the most appropriate smoothing parameter using r8s 1.71 [39]. The GMYC protocol considers a null model that the sample of individuals derives from a single evolutionary entity following a single coalescent process. The alternative is that the sample represents several independently evolving entities: i.e. selection and drift operate independently in different entities. In this case, coalescence occurs separately in different entities, leading over time to the appearance of discrete genetic clusters, separated from each other by longer internal branches. The method uses a maximum likelihood approach to optimize the shift in the branching patterns of the gene tree from interspecific branches (Yule model) to intraspecific branches (neutral coalescent), and thereby identifies clusters of sequences corresponding to independently evolving entities. It does this by optimizing the maximum likelihood value of a threshold such that nodes before the threshold are identified as species diversification events, while branches beyond the threshold are clusters following coalescent processes. We used a Likelihood Ratio test to support the scenario that the result of this Maximum Likelihood threshold is a better explanation of the tree topology than the null model (i.e. the sample of sequences belongs to a single population obeying a single coalescent process). Models were fitted in R 2.12.0 with the package splits [http://splits.r-forge.r-project.org].

Uncorrected pairwise distances between haplotypes were calculated in R, package ape 2.6–1 [40]. To assess distinctiveness level of European populations and their contribution to the overall genetic species variability, differences between European pike and congeneric from other continents were analysed using indices of divergence from population genetics. We identified variable domains, parsimony informative sites, translation of nucleotide sequences, pairwise genetic distances, locus base composition, transition/transversion ratios and the Tajima’s Neutrality Test (TNT), using MEGA4.0. Moreover, we calculated FST values between taxa using ARLEQUIN3.5 [41].

**AFLP analysis.** AFLP analysis produces reliable multilocus fingerprints of complex genomes without any previous species-specific information [42] and it has been already used to distinguish morpho-species and phenotypes in fishes [43–44]. Thus, AFLPs can screen the entire genome to search for genotype-phenotype relationships [45]. As briefly reviewed by Papa and colleagues [46] several analytical improvements have been made in recent years, in particular the use of fluorescence labelled primers and the resolution of the band pattern by means of automatic capillary sequencers instead of polyacrylamide gel. This has greatly reduced the time and cost, while improving pattern reproducibility [46]. The quantitative information for single individuals is essentially dependent on the specific endonucleases to be used for the restriction step; EorRI/TaqI combinations in genomes of high complexity have greatly improved polymorphism and profile quality if compared with EorRI/MseI combinations [46–48].

The present research applies, for the first time, an extensive application of ten different EorRI/TaqI AFLPs in European pike. We analysed the overall patterns of nuclear genetic variation among northern pike populations using a fluorescent AFLP procedure [46] with just a few modifications: 500 ng of genomic DNA were restricted with TaqI for 1.5 hour at 65°C and, subsequently, for 2 hours with EorRI at 37°C. The selective nucleotides were AAG/ACG, AGC/AAC, AGC/AAG, AAC/AAC, AGT/ACG, AAC/AAG, AAG/AAG, AAC/ACG, ACT/ACG, AGT/ACG. One micro litre of a mixture made of FAM, PET, NED, or VIC 5’-labelled amplicons and 0.3 µl of GeneScan-500 LIZ Size Standard were added to 18.7 µl of Hi-Di Formamide, denatured and run on an ABIPRISM310. The reproducibility of AFLP markers was tested as suggested by Papa et al. [46] and by analysing different extractions per sample, by repeating the AFLP assay with twenty random samples and using the chosen selective primer pairs. Fragments were analysed through GeneMapper 5.0, eliminating bands under 150 bp to reduce the risk of homoplasy [44]. Statistical analyses were both band-based (BB) and allele frequency-based (FB) and conducted as already assessed (see Table 1 in Bonin et al. [49]).

FST values between groups of individuals defined by the five categories of skin colour pattern were computed with AFLP-SURV [50]. Statistical significance of the differences in FST values between northern (round spot) and southern skin colour pattern (stellate spot, vertical, diagonal and longitudinal bars) was estimated using a permutational multivariate analysis of variance using distance matrices with the adonis function in R, package vegan 1.17-4 [51].

In order to independently verify if the skin colour pattern assignment was related to AFLP-derived genotypes, STRUCTURE 2.3.3 [52] was used to implement the non-spatial Bayesian clustering methods using the AFLP data aggregated by skin colour pattern. The applied algorithm divides sampled individuals into a number of clusters (K) and tests for the most likely value of K, given the data, and maximises log Pr[X|K] given the data (X); we performed the test setting K from 1 to 20. Twenty independent runs for each K between 1 and 20 using the admixture model and correlated allele frequencies were made. Exploratory structure runs demonstrated that a burn-in period of 100,000 steps, followed by 500,000 steps of data collection, ensure the convergence of the MCMC. Moreover, a Mantel test was performed between the matrix of FST values between the analysed populations and the geographical distances between them, in order to identify geographic signatures of genetic diversification in Europe. Geographical distances were specified as the matrix of all paired kilometric distances, estimated from angular distances between paired coordinates.

**Identification of specific polymorphic AFLP fragments.** In order to identify a quick and reliable genetic marker for the identification of the two species, thirty-two AFLP polymorphic clearly visible and distinct bands were selected. Following the Bensch et al. [53] protocol, these thirty-two bands were processed and, finally, two bands were identified and positively sequenced: band 9 and 24. Thus, additional primers were designed (9FW: CAGTGTGTAAGGCAGGAAAG 9RV: GGAAATACGTCTTGAGAACCTGC; 24extFW: GATCTCTGGTACATGGGAC 24extRV: TGGCTGACATGCCAGCATCAG) and used to amplify these bands in additional 71 individuals. Results were controlled verifying the presence/absence of each AFLP band correlating with the individual phenotype.

**Nomenclature Acts**

The electronic version of this document does not represent a published work according to the International Code of Zoological
Nomenclature (ICZN), and hence the nomenclatural acts contained in the electronic version are not available under that Code from the electronic edition. Therefore, a separate edition of this document was produced by a method that assures numerous identical and durable copies, and those copies were simultaneously obtainable (from the publication date noted on the first page of this article) for the purpose of providing a public and permanent scientific record, in accordance with Article 8.1 of the Code. The separate print-only edition is available on request from PLoS by sending a request to PLoS ONE, 1160 Battery Street, Suite 100, San Francisco, CA 94111, USA along with a check for $10 (to cover printing and postage) payable to “Public Library of Science”.

In addition, this published work and the nomenclatural acts it contains have been registered in ZooBank, the proposed online registration system for the ICZN. The ZooBank LSIDs (Life Science Identifiers) can be resolved and the associated information viewed through any standard web browser by appending the LSID to the prefix “http://zoobank.org/”. The LSID for this publication is: urn:lsid:zoobank.org:pub:B826CDA6-01B6-4D68-B998-5EFB7D70A9A9.

Results and Discussion

Phenotypic differences

The analysis of the skin colour pattern of European pikes showed a recognizable distribution of phenotypes around Europe: in Central-West and North-East Europe the round-spot phenotype was dominant (Figure 2) whereas in Italy it seems confined just to some populations; on the other hand, the other four skin colour patterns (stellate spot, diagonal, longitudinal and vertical bars) are particularly diffused in this area. The distribution of the round spot skin pattern, together with the fact that this phenotype was never seen in Italy before the start of the re-stocking activities [Natali M., personal communication], strongly suggests that its appearance in Italy could be due to artificial transportation.

The number of lateral line scales in our European samples ranged from 101 to 148, and almost completely overlaps with the numbers reported in the literature across all the palearctic range of the northern pike: between 103 and 148 [54]. The number of scales did not show significant differences among the four southern phenotypes (diagonal, vertical and longitudinal bars, and stellate spot; GLM: all p<0.4), whereas the difference was highly significant between the round spots and the other phenotypes (GLM: p<0.01).

![Figure 2. Distribution of the five colour patterns shown by pike in Europe.](https://example.com/figure2.png)
significant between these four phenotypes and the round spot skin pattern (GLM: $t = 11.9$, $p < 0.0001$). Independently of the geographic area of origin, samples showing the four southern skin colour patterns have a significantly lower number (GLM: $t = 20.8$, $p < 0.0001$) of lateral line scales, ranging from 101 to 115, than those showing the round-spot phenotype, whose scales ranged generally from 125 to 148 (Text S3). The number of scales supports a clear distinction between the two groups, almost without any overlap. Interestingly, three individuals with a round-spot phenotype had a lower number of scales, 102, 104 and 109, typical of the other skin colour patterns. All these individuals came from one population in Lake Maggiore, where 3 different skin colour phenotypes and a number of scales from 102 to 147 were found out of only 9 analysed individuals (Figure 3). It is possible that northern pikes introduced in this area from northern Europe hybridised with the local populations, producing hybrids with northern skin colour pattern and southern number of scales. Hybridisation in fishes is a common event, both in closely related species and even between non co-generic species (see Epifanio and Nielsen [55] for a review). In the genus Esox, hybridisation between co-occurring species is possible and has been demonstrated in North America, where different species have overlapping ranges [56].

Discriminating lineages with mtDNA

The 651 bp COI-region and the 998 bp Cytb-sequence were successfully sequenced and could be aligned unambiguously for the entire sample set. From our European dataset, 20 haplotypes were identified for COI, whereas 30 were identified for Cytb (Table 1). Phylogenetic reconstructions for both loci, including additional haplotypes from GenBank, provided evidence of two well-supported groups (Figure 4). The two groups received significant support from the GMYC model, which provided evidence for two independently evolving entities, equivalent to two distinct species, in the northern pike (Table 2).

For COI, one potential species, here called COI-A, included 15 haplotypes, 11 from southern Europe and 4 from the Danube river; the other potential species, COI-B, included 9 haplotypes, 6 from southern, central, northern and eastern Europe, 1 from Greece and 3 from Canada. Thus, according to the two COI groups, some European pikes are more similar to Canadian pikes than to most Italian ones. We can argue that for COI, pikes of the COI-B clade are native to all the palearctic, except for Italy, where its occurrence can be due to introduction for recreational fishing, whereas pikes of the COI-A clade are native only in Italy with some individuals in the Danube river. The sample from Greece can be regarded as geographical dubious, because autoctony of the northern pike in this country is questionable [57,58]. COI-B is the nominal species Esox lucius, whereas COI-A is a different species, which will be described later. Percentages of uncorrected genetic differences within each group were less than 1%, and from 1.1% to 2.6% between them (Table 3). Both within-species and between-species differences in other species of Esox have generally higher values (Table 3). Nevertheless, these values are congruent with those already reported on other freshwater fishes at both intra-specific and inter-specific-level. In fact, in Canadian fishes values below 1% within species and between 0% and 19.33% between species within genus have been found for COI [59].
values we report for *Esox* are expected to be lower, as we used uncorrected distances, instead of the K2P model used for Canadian fishes; thus, comparable values with our more conservative approach in computing genetic distances provide even stronger support for the significant distances between the two species of pike in Europe. Moreover, COI is not able to resolve the species complex *E. niger* – *E. americanus*; *E. americanus* was paraphyletic, with *E. niger* nested within its genealogy [56,59], suggesting that a high degree of introgression may have happened for these two species.

Non-synonymous substitutions in the COI sequence occurred in 24 amino acids out of 217; 11 of them occurred only in COI-A, 5 only in COI-B, 5 in both groups, and only 3 were completely discriminating the two groups. The nucleotide sequences composition across all haplotypes in the *E. lucius* species complex was moderately A+T rich (54.9%), with overlapping values between the two COI groups (COI-A: range 55.30–55.91%, average 55.73%; COI-B: range 55.45–55.76%, average 55.63%). These values are comparable with those of other esocids (55.3% for *E. masquinongy*; 55.8% for *E. americanus*). Tajima's test for neutrality underlined a p s across *Esox* species of 0.23, between the *E. lucius* species complex and *E. americanus* of 0.17, whereas it was 0.15 between the *E. lucius* species complex and *E. masquinongy*. A value of 0.04 was found between the two COI groups within the *E. lucius* species.
Table 3. Summary of the sequence divergence, transition/transversion ratios (Ti/Tv), number of variable sites (Vs) and number of parsimony-informative sites (Ps) between and within haplogroups.

| Haplogroups               | Average Divergence (range) | Ti/Tv 1st | Ti/Tv 2nd | Ti/Tv 3rd | Ti/Tv all | Vs    | Ps    |
|---------------------------|-----------------------------|-----------|-----------|-----------|-----------|-------|-------|
| Between multiple haplogroups | 1.80% (1.08–2.61)           | 6.15      | 220.62    | 0.00      | 6.64      | 23    | 0.03  |
| COI-A vs COI-B             |                             |           |           |           |           |       |       |
| Within haplogroups          | 0.42% (0.15–0.92)           | 222.59    | 0.00      | 2.93      | 1.28      | 15    | 0.02  |
| COI-A                      | 0.47% (0.15–0.77)           | 0.51      | 0.58      | 135.35    | 366.79    | 7     | 0.01  |
| COI-B                      |                             |           |           |           |           |       |       |
| Between multiple haplogroups | 2.19% (1.51–2.72)           | 348.87    | 317.87    | 20.85     | 24.55     | 40    | 0.04  |
| Cytb-A vs Cytb-B            |                             |           |           |           |           |       |       |
| Within haplogroups          | 0.34% (0.10–0.71)           | 321.05    | 11.66     | 21.46     | 260.96    | 13    | 0.01  |
| Cytb-B                      | 0.64% (0.10–1.41)           | 455.84    | 0.56      | 5.93      | 6.95      | 20    | 0.02  |

The overall transition/transversion bias is R = [(A+G)k1+(T+C)k2]/[(A+G)(T+C)]. All positions containing gaps and missing data were removed from the dataset (Complete-deletion option). The Ti/Tv ratios are presented for 1st, 2nd and 3rd codon positions, and also summarized across all codon positions.

doi:10.1371/journal.pone.00025218.t003

species complex. Fst values between species ranged from 0.93 (E. lucius/E. americanus-E. masquinongy) to 0.99 (E. masquinongy/E. niger), whereas Fst value between COI-A and COI-B was lower, only 0.57. This value, though lower than those obtained between other Esox species, is still high and greater than 0.25, i.e. the value that, according to Hartl and Clark [60], denotes a very great differentiation between isolated populations, which again may suggest the existence of distinct species.

The two putative species had a clear scenario in their skin colour pattern: 90.5% of the individuals in COI-A had southern pattern (stellate spot, diagonal, longitudinal or vertical bars), and 96.5% of the individuals in COI-B had the round spot pattern (Figure 5A). The number of scales in the lateral line was significantly different in the two COI groups (GLM: t = 9.9, p < 0.0001), with COI-A mostly having less than 115 scales and COI-B more than 125. The three individuals of COI-A with more than 125 scales all came from the Danube river, whereas 4 of the individuals of COI-B with less than 115 scales came from Maggiore lake and 1 from Chiusi lake (Figure 5B).

For Cytb, one potential species, here called Cytb-A, included 15 haplotypes, all from southern Europe, except for one individual from the Danube river, which shared haplotype 3 with many other Italian individuals; the other potential species, Cytb-B, included 22 haplotypes, 16 from southern, central, northern and eastern Europe, and 6 from China. Thus, in a similar scenario to the one for COI, according to the two Cytb groups, some European pikes are more similar to Chinese pikes than to most Italian ones. We can argue that also for Cytb, pikes of the Cytb-B clade are native only to the palearctic, except for Italy, and are indeed the nominal species E. lucius, whereas pikes of the Cytb-A clade are native only in Italy, and, again, also present in the Danube river. Cytb-A represents a new species, described later. Percentages of uncorrected genetic differences within each group were less than 1.5%, and from 1.5% to 2.7% between them (Table 3). Both within-species and between-species differences in other species of Esox have generally higher values (Table 3); even the differences between the two subspecies of E. americanus are above 3%. On the other hand, also for Cytb, E. niger falls within E. americanus.

Non-synonymous substitutions in the Cytb sequence occurred in 9 amino acids out of 332; 2 of them occurred only in Cytb-A, 5 only in Cytb-B, 2 in both groups, and none was completely discriminating the two groups. The nucleotide sequences composition in Esox is moderately A+T rich, with very similar values for Cytb-A (55.85%) and Cytb-B (55.96%); slightly lower than in the other species (E. americanus: 58.47%, E. reichertii: 57.56%). Tajima’s test for neutrality underlined a p s across Esox species of 0.23, between the E. lucius species complex and E. americanus of 0.20, whereas it was 0.12 between the E. lucius species complex and E. reichertii. A value of 0.05 was found between the two Cytb groups within the E. lucius species complex. Fst values across Esox species of 0.90, between the E. lucius species complex and E. americanus of 0.94, whereas it was 0.95 between the E. lucius species complex and E. reichertii. A value of 0.79 was found between the two Cytb groups within the E. lucius species complex. This value, though lower than those obtained between other Esox species, is still high and greater than 0.25, i.e. the value that, according to Hartl and Clark [60], denotes a very great differentiation between isolated populations, which again may suggest the existence of distinct species.

The two putative species had a clear scenario in their skin colour pattern: 87.7% of the individuals in Cytb-A had southern pattern, and 97.5% of the individuals in Cytb-B had the round spot pattern (Figure 5C). The number of scales in the lateral line was significantly different in the two Cytb groups (GLM: t = 10.3, p < 0.0001), with Cytb-A mostly having less than 115 scales and Cytb-B more than 125. One individual of Cytb-A with more than 125 scales came from the Danube river, whereas the two individuals of Cytb-B with less than 115 scales came one from Maggiore lake and one from Chiusi lake (Figure 5D). This scenario is very similar to the one described for COI groups. Actually, COI and Cytb groups overlapped almost completely: all individuals belonging to COI-A group were in Cytb-A, whereas only two individuals had COI-B and Cytb-A, one from the Danube and one from the Baccighione river.

AFLP

The analysed markers provided high percentages of polymorphism (Table 4), Fst values for comparison between different skin colour patterns (Table 5) suggest a dual separation of samples, with very low Fst values between the four southern phenotypes (stellate spot, longitudinal, vertical and diagonal bars), and Fst values higher than 0.16 between round spot pattern and the other skin colour pattern. The differences between the two groups are strong (adonis: R² = 0.99, p = 0.05).
The estimation of the most likely number of groups, K, was two, which means that AFLPs were able to identify 2 distinct lineages, here called AFLP-A and AFLP-B. The bar plot re-arrangement based on skin colour pattern (Figure 6) suggested a unique relationship between round spot skin colour pattern and AFLP-B: the prediction of historical admixture within each population performed with two partitions indicated that all samples showing the round spot pattern were assigned to the AFLP-B group. In contrast, none of the round spot individuals were assigned to AFLP-A. Thus, AFLPs are completely linked to colour skin patterns. On the other hand, the spatial distribution of the two AFLP groups are not so clear. All the samples from continental Europe showed the same genotype, AFLP-B, with only one individual from the Danube river with genotype AFLP-A; populations from Italy had both AFLP-A and AFLP-B. AFLP-B genotype has been identified in 45 Italian individuals scattered in
Identification of specific polymorphic AFLP fragments

The sequences obtained for the two polymorphic bands (9, 24) did not show any similarity with other GenBank sequences; thus, we cannot suggest any specific location or function for them. A biunivocal relationship with skin colour pattern was found: all the individuals with round spot pattern showed the allele 24 whereas all the individuals with the other four skin patterns showed the allele 9 (Figure 7). Thus, these two highly informative nuclear SNPs represent an important and fast procedure for the rapid identification of the two groups of European pike.

Conclusions

The northern pike is the most widespread Esocidae species in the world, and the only one naturally present in Europe, occupying a wide range of different lotic and lentic habitats. It has the ability to tolerate very different prey typologies, salinity and temperatures, even though its life-cycle is strictly dependent on water vegetation. This plasticity was considered the biological factor supporting and facilitating the migration of individuals from the north to the south of the distribution area. Nevertheless, this research clearly demonstrated that behind such plasticity there might be the genetic, phenotypic and geographic distinction between two different taxonomic units.

The present analysis of the northern pike revealed a species complex, with one species widely distributed in Europe, Asia and North America and one in Italy only. The widely distributed species is more homogeneous in its skin colour pattern, whereas the Italian one has a large variability in colour pattern. There is evidence of potential past connections between the two species, with individuals that have mismatches between their mtDNA, AFLP and phenotypic characters. Nevertheless, these individuals are present in areas where previous contact between the two species is plausible. The population in the Danube belongs to the widely distributed species, but some individuals have mtDNA loci of the Italian one, and even one individual with COI-A and Cytb-B. This may be a signature of introgression of the Italian species in northern areas in the past, when the paleo-delta of the Po River make the shores of the Adriatic sea closer to the present position of the Danube river [61]. On the contrary, most of the occurrences of the widely distributed species in Italy can be ascribed to recent introductions for recreational fishing. Such introductions potentially produced hybridisation between the two pike species, and this could explain the very few mismatches between phenotypes and genotypes. The large proportion of mismatches between phenotypes and genotypes in Maggiore Lake may suggest that, in this area, northern individuals could have migrated south in the past and maybe the history of hybridisation is here longer than the one due to recent human translocation of individuals.

Table 4. Primer combination, combination code and selective nucleotides used, together with the total number of fragments and of polymorphic bands for each combination.

| Combination code | Selective nucleotides | Total number of fragments per primer combination | Number of polymorphic bands per primer combination | % of polymorphisms |
|------------------|-----------------------|-----------------------------------------------|-----------------------------------------------|-------------------|
| A                | AAG/AAC               | 47                                            | 26                                            | 55%               |
| B                | AGC/AAC               | 51                                            | 22                                            | 43%               |
| C                | AGC/AAG               | 50                                            | 26                                            | 52%               |
| D                | AAC/AAC               | 58                                            | 22                                            | 38%               |
| E                | AGT/ACG               | 43                                            | 31                                            | 72%               |
| F                | AAC/AAG               | 54                                            | 26                                            | 48%               |
| G                | AAG/AAG               | 55                                            | 28                                            | 51%               |
| H                | AAG/ACG               | 71                                            | 36                                            | 51%               |
| I                | AGC/ACG               | 76                                            | 37                                            | 49%               |
| L                | AGT/AAC               | 67                                            | 30                                            | 45%               |

Table 5. Comparison of $F_{st}$ values for each phenotype pair.

|         | DB | LB | VB | SS | RS |
|---------|----|----|----|----|----|
| DB      | 0.00 |    |    |    |    |
| LB      | 0.01 | 0.00 |    |    |    |
| VB      | 0.00 | 0.00 | 0.00 |    |    |
| SS      | 0.01 | 0.00 | 0.00 | 0.00 |    |
| RS      | 0.23 | 0.16 | 0.26 | 0.28 | 0.00 |

$F_{st}$ values between groups of individuals defined by the five categories of skin colour pattern were computed on the basis of AFLP data by means of AFLP-SURV [50]. The related $p$-values were always $<0.00$. DB diagonal bars; LB longitudinal bars; VB vertical bars; SS stellate spot; RS round spot.

doi:10.1371/journal.pone.0025218.t004

doi:10.1371/journal.pone.0025218.t005
In a previous evaluation of the stocking impact, a poor performance and a low level of introgression of stocked fries into a brackish northern pike population were reported [62]. In the case of Italian populations it is difficult to evaluate the real performance of stocked individuals, because no quantitative data on the repopulation with allochthonous specimens are available. This fact, together with the presence of allochthonous genotypes in several Italian populations here reported, suggests that the performance of stocked fish was sufficient to allow the persistence of allochtonous genotypes across generations. The findings here reported agree with the few introgressions registered for the Stege Nor population [62]. In fact, a strict association between the allochthonous genotype and the “new” round spot skin colour pattern is present in Italy, supporting the hypothesis that the stocked fish are at least in part reproductively separated by the autochthonous populations. This poor introgression is in contrast with data reported by Launey et al. [63] that found an extensive introgression between French populations and introduced stocks. The higher level of introgression in French populations compared to the Italian ones can be attributed to the fact that native French populations belong to the same widely distributed northern lineage used for introductions, whereas in Italy local populations belong to

Figure 6. Illustration of the estimated population subdivision and the prediction of historical admixture. Two partitions (K) were empirically determined by STRUCTURE (version 2.3.3) for AFLP data aggregated by skin colour pattern, using a burn-in period of 100,000 steps, followed by 500,000 steps of data collection to ensure the convergence of the MCMC. Yellow bars refer to the northern genotype, here represented by round spot (RS) individuals. Green bars refer to the southern genotype, here represented by individuals showing the southern phenotypes stellate spot (SS), diagonal bars (DB), longitudinal bars (LB) and vertical bars (VB).

doi:10.1371/journal.pone.0025218.g006

Figure 7. Sequences obtained for the specific polymorphic AFLP fragments. Sequences obtained for the band 9 (A) and the band 24 (B) for individuals showing the five phenotypes: stellate spot (SS), diagonal bars (DB), longitudinal bars (LB), vertical bars (VB) and round spot (RS). The original bands were obtained from a stellate spot individual for band 9 and from a round spot individual for band 24. 9P: band 9, present; 24P: band 24, present. 9A: band 9, absent; 24A: band 24, absent.

doi:10.1371/journal.pone.0025218.g007
a different lineage, a separate species, for which hybridisation events are much rarer than sexual exchange between populations within the same species. French populations analysed by Launey et al. [42] in rivers with an outlet in the Mediterranean were all stocked, and not completely wild. Moreover, the autochthony of pike in Mediterranean France is dubious [54]. An alternative explanation for the different levels of introgression between the Italian and the French populations might be found in water temperature. The role of water temperature in pike biology is well established in differentiating reproductive periods [16] and French freshwaters temperatures may be more similar to those of other European countries than in Italian freshwater habitats, that are generally warmer.

The data reported here on statistically significant differences in mtDNA, AFLP, SNPs, skin colour pattern and number of scales in the lateral line support a clear differentiation between a northern lineage of pike, widely distributed in the palearctic region, and a southern lineage, distributed in Italy, and potentially in other areas presently or in the past connected to the Mediterranean (as the Danube area). All the analyses we performed show high concordance between genotypic and phenotypic markers to identify the two lineages. Moreover, a coalescent based approach on the tree topology for two different mtDNA loci and AFLP clustering supported the existence of the two independently evolving entities, that is, the genetic signature of two different species. The differentiation values between genetic markers, too high to belong to differentiation between populations, are still lower than those concerning the other Esocidae species; this suggests that the separation between the two species is relatively recent.

The type locality of Esox lucius is not specified in the original description, and it is only reported as “in Europa” [64]. We may assume that Linnaeus described the species from individuals from continental Europe, if not even from Sweden; thus, we suggest 1) to maintain the name E. lucius for the widespread northern lineage we identified in this analysis, and 2) to describe a new species for the southern samples showing the four skin colour pattern non present in continental Europe and having a lower number of scales in the lateral line. Surprisingly, no name has ever been proposed to underline the “latin” origin of the species. Moreover, we propose the vernacular name of southern pike for the new species, to point out the different origin with respect to northern pike.

**Type material.** Holotype: a male specimen in ethanol in a jar deposited at the Natural History Gallery of Casalina (http://www.unipg.it/camsol/galleria/g0.htm), Perugia, Italy, accession number GSD1. Moreover, a 1.5 ml test tube containing the extracted DNA of this individual is present as accompanying material, with accession number GSDNA1.

**Holotype male.** Total Length 450 mm; Standard Length: 415 mm; Colour pattern: stellate spot.

**Etymology.** The specific name flaviae is the female genitive of Flavia, the name of the first women of the Flavian Dynasty, which included the emperor Titus Flavius (both his mother and his sister were named Flavia), to whom Pliny dedicated his Naturalis Historia. Four books of Naturalis Historia are devoted to zoology and to an attempt of systematics and they became a reference for subsequent naturalistic books. We dedicate the new species to the same person, to underline the “latin” origin of the species. Moreover, we propose the vernacular name of southern pike for the new species, to point out the different origin with respect to northern pike.

**Differential diagnosis.** As for northern pike, it is distinguishable from all other European freshwater fishes by large size, up to 1000 mm in total length; a long and flat duckbill-like snout; a large mouth with many sharp teeth on gill arches; rearward position of dorsal and anal fins with the dorsal one located far to the rear; pectoral and pelvic fins low on body, paired fins paddle-shaped [54].

It is possible to distinguish E. flaviae from the only congeneric species in Europe on the basis of several meristic characters (Table 6), which discriminate also between E. flaviae and all the other species of the genus. The strongest discrimination from E. lucius sensu stricto is possible on the basis of the number of scales in the lateral line: this number ranges from 101 to 115 in E. flaviae and from 125 to 148 in E. lucius. The two species differ also for the skin colour pattern. E. flaviae is very variable and shows four different colour pattern (stellate spot, diagonal bars, longitudinal bars and vertical bars, Fig. 1A–D), but never the colour pattern typical of E. lucius described as round spot (Fig. 1E).

**Measurements (values referred to the population of the type locality).**

- Total length: 96–1000 mm (average 475 mm).
- Standard length: 95% of total length circa.
- Weight: average 0.8 kg [14] and up to above 10 kg.
- Maximum age: 13 years
- Length at first maturity: 300 mm
- Dorsal spines (total): 1–2
- Dorsal soft rays (total): 13–16
- Anal spines: 1–2
- Anal soft rays: 11–13
- Caudal fin rays: 14–18
- Mandibular pores: 3–8
- Opercular pores: 3–8

**Biolog.** As for the northern pike [54], the southern pike occurs in clear vegetated water bodies as lakes and large rivers. It is solitary and territorial, voracious predator feeding mainly on fishes, but also on frogs, crayfish and often cannibalistic. Males reproduce for the first time when one-year-old, females when two-years-old. As for northern pike, reproduction is closely related to the presence of submerged vegetation. Spawns in late winter (February-March) in central Italy and in early spring (March-April) in northern Italy. It is a valuable game fish for recreational fishing and may be impacted by habitat alteration and by competition and/or hybridisation with the northern pike previously used for stocking local populations in Italy.

**Distribution.** Central and northern Italy. Potentially, it can be present in other European water bodies in the Mediterranean.
area as those on the north-eastern shores of the Adriatic and in Mediterranean France. The individuals of *E. lucus* from the Danube river with genetic signature of introgression from *E. flaviae* have the typical phenotype of *E. lucus* (high number of scales, round spot skin colour pattern), and thus it is not likely that *E. flaviae* could be found in the Danube area.

**DNA barcoding.** GenBank accession number for holotype COI: HM563688.1 (COIhap1), holotype cyt b: JN190460 (CytbIhap3). GenBank accession numbers of additional individuals considered in this analyses for COI: HM563688- HM563707 (previously named COI-A), and for Cyb: JN190458 - JN190487 (previously named Cytb-A).

**Supporting Information**

**Text S1 Distribution of *Esox lucius*.** In green: countries where *Esox lucius* is considered native; in red: countries where is considered as introduced fish; yellow: countries for which incongruent informations are reported by different authors.

**Table 6.** Meristic characters of esocids (Minimum-Maximum).

| Name          | Mandibular Pores | Pelvic rays | Pectoral rays | Scales in lateral line |
|---------------|------------------|-------------|---------------|------------------------|
| *E. a. americanus* | 4*               | 8–9*        | 14–15*        | 102–116*               |
| *E. niger*     | 4*               | 9–10*       | 12–15*        | 117–135*               |
| *E. masquinongy* | 6–9*             | 11–12       | 14–19*        | 132–167*               |
| *E. reicherti* | 5*               | -           | -             | 130–163*               |
| *E. lucus s.s.* | 5*               | 9–10*       | 13–16*        | 125–148*               |
| *E. flaviae*   | 3–9*             | 6–15*       | 4–19*         | 101–115*               |

*Raat, 1988; Our data. doi:10.1371/journal.pone.0025218.t006

**Text S2 Samples origin.**

**Text S3 Original data for the 374 analysed samples.** N/ A means data Not Available.

**Text S4 Permission to publish Figure 1–Monday, July 11th, 2011 17.08.**

**Acknowledgments**

The authors are grateful to colleagues for collecting specimens. The authors thank Veneto Agricoltura for the permission to reproduce Lorenzo Stafani's paintings (Text S4).

**Author Contributions**

Conceived and designed the experiments: L. Lucentini. Performed the experiments: L. Lucentini MEP CR LG. Analyzed the data: L. Lucentini DF. Contributed reagents/materials/analysis tools: FB MN L. Lanfaloni FP. Wrote the paper: L. Lucentini DF.

**References**

1. Jenkins M (2003) Prospects for biodiversity. Science 302: 1175–1177.
2. Fitzhugh TW, Richter BD (2004) Quenching urban thirst: Growing cities and their impacts on freshwater ecosystems. Bioscience 54: 741–754.
3. Dodson D, Arrington AH, Gesner MO, Kawabata Z, Knowler DJ, et al. (2006) Freshwater biodiversity: importance, threats, status and conservation challenges. Biological Reviews 81: 163–182.
4. Leprêtre F, Beauchard O, Blanchet S, Oberdorff T, Brosse S (2008) Fish invasions in the world’s river systems: When natural processes are blurred by human activities. PLoS Biology 6: 404–410.
5. Pysek P, Jarovsk V, Hulme PE, Kühn I, Wild J, et al. (2010) Disentangling the role of environmental and human pressures on biological invasions across Europe. Proceedings of the National Academy of Sciences of the United States of America 107: 12157–12162.
6. Susnik S, Snoj A, Wilson IJ, Mrdalj D, Weiss S (2007) Historical demography of brown trout (*Salmo trutta*) in the Adriatic drainage including the putative *S. secura* endemic to Lake Ohrid. Molecular Phylogenetics and Evolution 44: 63–76.
7. Meraner A, Baric S, Polter B, Dalla Via J (2010) Microsatellite DNA data point to extensive but incomplete admixture in a marble and brown trout hybridisation zone. Conservation Genetics 11: 903–906.
8. Melott S, Alessio G (1990) Biology of carpione, *Salmo virgins L.*, an endemic species of lake Garda (Italy). Journal of Fish Biology 37: 687–696.
9. A/EI O, Cavall D, Eletren P, Grollino MG, Colombari PT, et al. (1996) Nuclear DNA content in *Salmo fario* in Lake Posta Fibreno, Italy. Journal of Fish Biology 61: 1051–1056.
10. Zaragoza-Bagil R, Lavoie S, Tuller A, Rouillé C, Leconte G (2002) Assessment of osteophalan and protactopharyngian concepts in the light of multiple phylogenies. CR Biol 325: 1–17.
11. Ortí G, Meyer A (1996) Molecular evolution of eponymph and the phylogenetic resolution of early divergences among osteolepid fishes. Mol Biol Evol 13: 556–573.
12. Terry G, Lates H, Lo PZ YA (2004) Phylogenetic relationships of extant esocid species (*Teleostei: Salmoniformes*) based on morphological and molecular characters. Copeia 4: 743–757.
13. Lorenzoni M, Corboli M, Dorr AJM, Mearelli M, Giovinazzo G (2002) The growth of pike (*Esox lucius* Linnaeus, 1758) in Lake Trasimeno (Umbria, Italy). Fish Res 39: 239–246.
14. Lucentini L, Palomba A, Gigliarelle L, Sgaravizzi G, Lancioni H, et al. (2009a) Temporal changes and effective population size of an Italian isolated and supportive-breeding managed northern pike (*Esox lucius*) population. Fish Res 96: 139–147.
15. Lucentini L, Palomba P, Gigliarelle L, Sgaravizzi G, Ricciolini C, et al. (2009b) Northern Pike: A Species In Crisis?. In: Columbus AM, Kuznetsov L, eds. Endangered Species: New Research. ISBN: 978-1-60692-241-5.
16. Lucentini L, Gigliarelle L, Palomba A, Puletti ME, Panara F (2010) Temporal study of stress-induced effects caused by developmental temperature changes and water quality in an isolated northern pike (*Esox lucius*) population. JWARP 2: 167–180.
17. Thomas CD (2010) Climate, climate change and range boundaries. Diversity Distrib 16: 483–495.
18. Fielding NJ (1982) The identification of pike by means of characteristic marks. Aquacult Res 13: 79–82.
19. Crawley MJ (2007) The R book. Chichester: John Wiley.
20. Birky CW, Ricci C, Melone G, Fontaneto D (2011) Integrating DNA and morphological taxon ofy to describe diversity in poorly studied microscopic animals: new species of the genus *Microthrix* Bryce, 1910 (Rotifera: Biloculidea: Philodinidae). Zool J Linnean Soc 161: 725–734.
21. Lucentini L, Caporillo S, Palomba A, Lancioni H, Panara F (2006a) A comparison of conservative DNA extraction methods from fins and scales of freshwater fish: A useful tool for conservation genetics. Cons Genet 7(6): 1009–1012.
22. Goldstein PZ, DeSalle R (2011) Integrating DNA barcode data and taxonomic practice: determination, discovery, and description. Bioessays 33: 135–147.
23. Ardura A, Pala IG, Gomes V, Garcia-Vazquez E (2010) Application of barcoding to Amazonian commercial fish labelling. Food Res Int 43: 1549–1552.
24. Steinke D, Zemlak TS, Hebert PDN (2009) Barcoding Nemo: DNA-based identifications for the ornamental fish trade. PLoS One 4(7): e6300. doi: 10.1371/journal.pone.0006300.
25. Ward KD, Zemlak TS, Innes BH, Last PR, Hebert PDN (2005) DNA barcoding Australia’s fish species. Philos T Roy Soc B 360: 1847–1857.
26. Logan JA (1999) Extraction, polymerase chain reaction, and sequencing of a 440 base pair region of the mitochondrial cytochrome oxidase I gene from two species of acetyl-preserved Daniofis (Odonton: Cyprinidae). Aquaculture, 183: 143–147.
27. Sevilla RD, Diez A, Noron M, Mouchel O, Jerome M, et al. (2007) Primers and polymerase chain reaction conditions for DNA barcoding teleost fish based on the mitochondrial cytochrome b and nuclear rhodopsin genes. Mol Ecol Notes 7: 730–734.
28. Zardoya R, Meyer A (1996) Phylogenetic performance of mitochondrial protein-coding genes in resolving relationships among vertebrates. Mol Biol Evol 13: 933–942.
29. Chen WJ, Bonillo C, Lecointre G (2003) Repeatability of clades as a criterion of reliability: a case study for molecular phylogeny of Acanthomorpha (Teleostei) with larger number of taxa. Mol Phylogenet Evol 26: 262–288.
30. Dettila A, Lecointre G (2005) Further support for the clades obtained by multiple molecular phylogenies in the atherinomorph family. Compt Rend Biol 329: 674–689.
31. Kyle CJ, Wilson CC (2007) Mitochondrial DNA identification of game and harvested freshwater fish species. Forensic Sci Int 166: 68–76.
32. Ivanova NV, Zemlak TS, Hanner RH, Hebert PDN (2007) Universal primer cocktails for fish DNA barcoding. Mol Ecol Notes 7: 544–548.
33. Lucenina L, Rebora M, Paletti ME, Gigliarelli L, Fontaneto D, et al. (2011) Geographical and seasonal evidence of cryptic diversity in the Batini dusiani complex (Ephemeroperae, Baetidae) revealed by means of DNA taxonomy. Hydrobiologia. DOI 10.1007/s10750-011-0778-1.
34. Keane TM, Czerev CJ, Pienz NY, Naughton TJJ, McElrane MJO (2006) Assessment of methods for amino acid matrix and their use on empirical data shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 6: 29.
35. Ronquist F, Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.
36. Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Systematic Biology 52: 696–704.
37. Pons J, Barraclough TG, Gomez-Zurita J, Cardoso A, Duran DP, et al. (2006) Sequence-Based Species Delimitation for the DNA Taxonomy of Undescribed Insects. Syst Biol 55(4): 595–609.
38. Fontaneto D, HermouEA, BossiC, Caprioli C, Melone G, et al. (2007) Independently evolving species in asexual bdelloid rotifers. PLoS Biol 5: 914–921.
39. Sanderson MJ (2002) Estimating absolute rates of molecular evolution and divergence times: A penalized likelihood approach. Mol Biol Evol 19: 101–109.
40. Paradis E, Claude J, Strimmer K (2004) APE: analyses of phylogenetics and evolution in R language. Bioinformatics 20: 209–210.
41. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 9(5): 1322–1322.
42. Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, et al. (1995) AFLP: a new technique for DNA fingerprinting. Nucl Acids Res 23(21): 4407–4414.
43. Excoffier L, Lischer HEL (2010) Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Mol Ecol Res 9(5): 1322–1332.
44. Papa R, Troggio M, Ajmone-Marsan P, Nonni Marzano F (2005) An improved protocol for the production of AFLP markers in complex genomes by means of capillary electrophoresis. J Anin Breed Genet 122: 62–68. doi: 10.1111/j.1439-0303.2004.00476.x.
45. Ajmone-Marsan P, Valentin A, Cascandri M, Vecchiotti-Antaldi G, Bertoni G, et al. (1997) AFLP markers for DNA fingerprinting in cattle. Anim Genet 28: 418–426. doi: 10.1111/j.1365-2052.1997.00204.x.
46. David L, Rajasekaran P, Fang J, Hillel J, Lav I (2001) Polymorphism in ornamental and common carp strains (Cyprinus carpio L.) as revealed by AFLP analysis and a new set of microsatellite markers. Mol Genet Genomics 266: 353–362.
47. Bonin A, Ehric D, Manel S (2007) Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Mol Ecol 16: 3737–3758. doi: 10.1111/j.1365-294X.2007.03435.x.
48. Vekemans X, Beauvens T, Lemaire M, Roldan-Ruiz I (2002) Data from amplified fragment length polymorphism (AFLP) markers show indication of size homoplasies and of a relationship between degree of homoplasies and fragment size. Molecular Ecology 11: 139–151.
49. Oksanen J, Blanchet FG, Kindt R, Legendre P, O’Hara RB (2010) vegan: Community Ecology Package. R package version 1.17–4. http://CRAN.R-project.org/package=vegan.
50. Hubisz MJ, Falush D, Stephens M, Princhi JK (2009) Inferring weak population structure with the assistance of sample group information. Mol Ecol Res 9(3): 1322–1322.
51. Bensch S, Akesson S, Irwin DE (2002) The use of AFLP to find an informative SNP: genetic differences across a migratory divide in willow warblers. Mol Ecol 11: 2359–2366.
52. Kotetel M, Fryhof J (2007) Handbook of European freshwater fishes. Publications Kotetela, Cornhol (CH).
53. Sala M, Nielsen J (2000) The role of hybridization in the distribution, conservation and management of aquatic species. Reviews in Fish Biology and Fisheries 10: 245–251.
54. Grande T, Laren M, Lopez JA (2004) Phylogenetic relationships of extant Esox species (Teleostei: Salmoniformes) based on morphological and molecular characters. Copeia 4: 743–757.
55. Economidis PS, Dimitriou E, Pagoni R, Michaloudi E, Natsis L (2000) Introduction and translated fish species in the inland waters of Greece. Fisheries Management & Ecology 7(4): 239–290.
56. Welcomme RL (1988) International introductions of inland aquatic species. FAO Fish. Tech Pup 294: 798.
57. Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, et al. (2008) Identifying canadian freshwater fishes through DNA Barcodes. PLoS ONE 3(6): e2490. doi:10.1371/journal.pone.0002490.
58. Hubert N, Hanner R, Holm E, Maudrik NE, Taylor E, et al. (2008) Identifying canadian freshwater fishes through DNA Barcodes. PLoS ONE 3(6): e2490. doi:10.1371/journal.pone.0002490.
59. Roff J, Clark AG (1997) Principles of Population Genetics. Sinauer Associates, ed. Sunderland, Massachusetts.
60. Amorosi A, Centinorio MC, Colalongo ML, Pasini G, Sarti G, et al. (2003) Facies Architecture and Late Pleistocene–Holocene Depositional History of the Po Delta (Cnossacchio Area), Italy. The Journal of Geology 111: 39–56.
61. Larsen PF, Hansen MM, Nielsen EE, Jensen LF, Lepscheke V (2003) Stocking impact and temporal stability of genetic composition in a brackish northern pike population (Esox flaviae) revealed by microsatellite DNA analysis of historical and contemporary samples. Heredity 95: 136–143.
62. Launey S, Morin J, Minery S, Laroche J (2006) Microsatellite genetic variation shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 6: 29.
63. Launey S, Morin J, Minery S, Laroche J (2006) Microsatellite genetic variation shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 6: 29.
64. Launey S, Morin J, Minery S, Laroche J (2006) Microsatellite genetic variation shows that ad hoc assumptions for choice of matrix are not justified. BMC Evol Biol 6: 29.
65. Lucentini L, Palomba A, Lancioni H, Gigliarelli L, Natali M, et al. (2006b) Microsatellite polymorphism in Italian populations of northern pike (Esox lucius). Fish Res 89: 251–262.