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Integrative taxonomy does not support the occurrence of two species of the *Squalius squalus* complex (Actinopterygii, Cypriniformes, Cyprinidae) in Italy

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ABSTRACT

The systematic is still unresolved for the genus *Squalius* (Cyprinidae, Leuciscinae), a rich group of small to large fishes widely distributed throughout Europe. The distinction of one of the Italian narrowly endemic species, *Squalius albus* (Bonaparte, 1838), described for the area surrounding lake Trasimeno, from the more common and widespread *Squalius squalus* (Bonaparte, 1837) is doubtful. The application of integrative taxonomy, with DNA taxonomy and quantitative morphometric, using both living and preserved individuals collected from lake Trasimeno before *Squalius* sp. restocking, allowed us to explicitly test for the identity of the two species in the complex. *COI* barcoding data, used for phylogenetic reconstructions, underlined that two clades may exist in the complex; nevertheless, DNA taxonomy (ABGD and GMYC) and morphometrics show no statistical support for their identity as separate species. Moreover, during our survey of the genetic diversity of the Italian *Squalius*, we provided further support for the species status of *Squalius lucumonis*, and found evidence of the occurrence in Southern Italy of another chub species, *Squalius vardarensis* (Karaman, 1928), previously known only from the Southern part of the Balkan Peninsula.

1. Introduction

Chubs, small to large fishes of the genus *Squalius* (Family Cyprinidae), are widely distributed throughout Europe; about 40 species are currently recognized in this genus, showing a particularly high diversity in the Mediterranean area, where some species are extremely localised and endemic to single basins (Kottelat and Freyhof, 2007; Bogutskaya and Zupancic, 2010). As underlined by Kottelat and Freyhof (2007) "the systematics of *Squalius* is still not resolved. Several species of southern Europe have quite similar appearances and earlier authors had very contradictory views on species definitions".

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One of the taxa of unclear validity is the Trasimeno chub, *Squalius albus* (Bonaparte, 1838), living in lake Trasimeno and in the surrounding waters of the river Tevere basin in Central Italy (Kottelat and Freyhof, 2007). This species has been the subject of several taxonomic revisions: for Bonaparte (1838) and Pietravalle (1908) it had the status of a species, whereas Tortonese (1970) and Bianco and Recchia (1983) suggested it as a subspecies of *Squalius squalus* (Bonaparte, 1837). Gandolfi et al. (1991) questioned the validity of the separation of the two taxa, suggesting that the characteristics showed by *S. albus* were included within the morphological variability of the more widespread *S. squalus.* Recently, Kottelat and Freyhof (2007) proposed this taxon again on the basis of morphological traits such as body depth of 19–23% of standard length, a lower proportion than in *S. squalus*, between 22 and 27%. Furthermore, faint black pigments along the free margin of flank scales above the lateral line should be present in *S. albus*, whereas they are markedly black in *S. squalus*. However, none of these values are absolutely divergent between the two species (Kottelat and Freyhof, 2007). Apparently, there is no evidence that morphometric features could allow for an unambiguous distinction of the chubs sampled in lake Trasimeno nowadays, potentially belonging to *S. albus*, from *S. squalus* (Lorenzoni et al., 1994). Nevertheless, chubs have been introduced for fishing activities in several localities of the river Tevere basin, including lake Trasimeno (Lorenzoni et al., 1994). Such activities, culminating in a highly altered fish community in the area (Lorenzoni et al., 2006), could make it difficult to discriminate between the two entities on morphology, basing their identity on geographic origin only. The situation could be complicated by the presence of a third species of the genus in the area, *Squalius lucumonis* (Bianco, 1983). Its separation from *S. squalus* has been question by Gandolfi et al. (1991) and Zerunian (2002), even if Tancioni et al. (2013) have shown that its genetic, morphological and ecological characters are unambiguous for species identification (Kottelat and Freyhof, 2007; Giannetto et al., 2013).

Testing for the separation between the species of the *S. albus/squalus* complex using animals from lake Trasimeno and attributing them to the putative taxon *S. albus* (whose type locality is lake Trasimeno) cannot be considered as a feasible or supported choice. The ambiguous situation created by the translocation of fish in Central Italy prevents any conclusive consideration about the taxonomic identity of *S. albus* based on geographic origin of the samples and morphological analyses. Nevertheless, the presence in the Natural History Gallery of the University of Perugia (Casalina – Deruta, PG) of samples belonging to lake Trasimeno and surrounding areas collected before the restocking practices became a habit in Italy (Sommani, 1967; Lorenzoni et al., 1994) may represent the key to understand (i) whether a distinct taxon, *S. albus*, actually exists, and (ii) whether the population currently present in lake Trasimeno is still the same or it has been effectively mixed with allochthonous chubs.

The aim of the present study is to apply an integrative taxonomy approach to the problem, first by DNA taxonomy to assess the distinctiveness of *S. albus* taking advantage of historical and living samples, and then by testing for differences in morphological characters between the clades identified by DNA taxonomy.

2. Materials and methods

2.1. Sampling

We sampled 203 adult individuals of the *S. albus/squalus* complex from several populations in Italy: 34 individuals from 2 populations in Northern Italy (rivers Boesio and San Giovanni, river Po basin); 138 individuals from 15 present-day populations in Central Italy (rivers Aggia, Assino, Carpina, Chiani, Lama, Paglia, Soara, Tevere, Topino and lake Trasimeno, from river Tevere basin; Chiassa and Corsalone from river Arno basin; Burano from river Metauro basin) and 9 individuals from the Natural History Gallery of the University of Perugia (Casalina – Deruta, PG), sampled in lake Trasimeno and rivers Clitunno and Rio di Bosco (river Tevere basin) in the period 1889–1900, before the restocking campaigns in the area) and 31 samples from 4 populations in Southern Italy (rivers Basento, Bradano, Noce, and Ofanto, each with its own independent basin). Additionally, we sampled 53 adult individuals of the other Italian species of the genus, *S. lucumonis* from 11 populations: rivers Aggia, Assino,Fersinone, Lama, Niccone, Paglia, Passano, Soara and Ventia, (river Tevere basin) and rivers Chiassa and Corsalone (river Arno basin). The full list of the analysed individuals is reported in Supplementary File S1.

All living specimens were sampled by electrofishing and immediately released in the environment of origin. A small piece of caudal fin (about 10 mg) or few scales were collected to extract genetic material (Lucentini et al., 2006). In order to minimize the damage of the sampling procedures on the historical samples and to allow their display in the Gallery, a small portion of muscle was picked up with a pair of tweezers through the anus.

For a subsample of the specimens standard length and body-depth were also measured to calculate the BD/SL ratio (see later under '2.5 Morphometrics').

2.2. DNA extraction, amplification and sequencing

Genomic DNA was extracted as previously reported for conservative samples (Lucentini et al., 2006) and stored at −20 °C. DNA concentration was estimated in a 1.0% agarose gel electrophoresis in presence of Mass Ruler DNA Ladder Mix (Fermentas) and by spectrophotometric assay (GeneQuant, GE).

Initially, PCR amplifications of a cytochrome oxidase c subunit 1 (COI) fragment were performed by means of the primers already tested in fishes (Ivanova et al., 2007). Obtained amplicons clearly showed, apart from the expected fragment, a short fragment of 100 bp, probably due to NUMTs (nuclear copies of mtDNA). Thus, species-specific primers (Leu–COI–F 5'-GGCCGAACTAAGCCAAC-3'; Leu–COI–R 5'-ATGACTAGACTTCTGGGTGG-3') were newly designed and used to perform PCR

amplifications of the longer fragment. Amplifications were performed through Ready-to-go DNA PCR Beads (GE Healthcare) in a total volume of 25 μl, using 25 ng of total DNA, 20 μl of dsH_2O and 1 μl of 10 mM of each primer (MWG – Biotech AG) through the PCR program: 95 °C for 6 min; 30 cycles: 94 °C for 45 s, 60 °C for 1 min, 72 °C for 1 min followed by a final extension of 20 min at 72 °C. Amplicons were run in 1% agarose gel and controlled for the expected fragment sizes (592 bp) (Lucentini et al., 2011a).

PCR products were purified using ExoSAP-IT® For PCR Product Clean-Up (usb) following the manufacturer's instructions. They were sequenced in forward and reverse directions following a modified protocol (Lucentini et al., 2011a). Sequence identities were evaluated by GenBank BLAST procedure (http://www.ncbi.nlm.nih.gov/BLAST/). DNA sequences were aligned and edited using MacClade 4.08 (http://macclade.org/). No sequences displayed frame-shifts, stop codons, unexpected insertions/deletions, ambiguities or excessive differences from other *Squalius*. Haplotypes were identified from the aligned sequences; haplotype identity of all the sequenced specimens is reported in Supplementary File S1.

2.3. Phylogenetic reconstructions

Phylogenetic relationships were estimated through Maximum Likelihood (ML) reconstructions. A ML tree was obtained using PHYML 3.0 (Guindon and Gascuel, 2003), with the GTR + G model of evolution, selected by ModelGenerator v0.85 (Keane et al., 2006). Node support was assessed by 1000 bootstrap replicates. The dataset for the phylogenetic reconstruction included all the samples sequenced for this study (*S. albus/squalus* and *S. lucumonis*), together with *COI* sequences of the genus *Squalius* available in GenBank by September 2011. As a way to control for the quality of the sequences we downloaded from GenBank, we used only those that did not contain any ambiguity (Supplementary File S1). The phylogenetic reconstruction included 13 additional species of the genus, *Squalius aphipsi*, *Squalius aradensis*, *Squalius castellanus*, *Squalius illyricus*, *Squalius malacitanus*, *Squalius orientalis*, *Squalius orpheus*, *Squalius prespensis*, *S.* sp., *Squalius svallize*, *Squalius torgalensis*, *Squalius valentinus*, and *Squalius vardarensis*. As outgroup, we used two cyprinid species, *Abramis brama* and *Leuciscus leuciscus* (Supplementary File S1).

2.4. DNA taxonomy

To assess whether there is genetic evidence of distinct species in the complex *S. albus/squalus* in Italy and to further support the identity of *S. lucumonis*, we applied two different DNA taxonomy approaches: the Generalised Mixed Yule Coalescent model (GMYC: Fujisawa and Barraclough, 2013), and the Automatic Barcode Gap Discovery (ABGD: Puillandre et al., 2012). These methods use single locus genetic information and have been shown to perform well on a wide selection of animals (Tang et al., 2012).

The GMYC model is a maximum likelihood method based on the topology of phylogenetic trees (Pons et al., 2006; Fujisawa and Barraclough, 2013): it identifies the most likely threshold dividing speciation events (Yule) by within-species diversification (Coalescent), using a maximum likelihood approach to optimize the shift in the branching patterns of the gene tree. The result is the identification of clusters of sequences corresponding to independently evolving entities, akin to species. We first tested that the sample of sequences does not belong to a single population obeying a single coalescent process. Then, a threshold age, T, was optimised, such that nodes before the threshold are considered to be diversification events with branching rate and scaling parameter estimated from the tree. Branches crossing the threshold define clusters each obeying a separate coalescent process. Models were fitted in R 2.15.1 (R Development Core Team, 2012) with the package *splits* 1.0–14 (http://splits.r-forge.r-project.org). An ultrametric tree, needed as input for the GMYC model, was obtained with the function *chronopl* of the package *ape* 3.0–4 (Paradis et al., 2004), after pruning the ML tree and keeping only the haplotypes of the *S. albus/squalus* complex.

The ABGD uses a maximum likely approach that identifies the barcoding gap between species by iteratively searching for the most likely threshold in the dataset (Puillandre et al., 2012). We performed the ABGD using the online web tool: http://wwwabi.snv.jussieu.fr/public/abgd/abgdweb.html, with the default settings starting from the alignment of all the sequenced individuals of the *S. albus/squalus* complex.

We calculated genetic distances between and within taxa as uncorrected pairwise distances using the package *ape* in R. We calculated such distances for the species we sequenced and for all the other species in the genus *Squalius* for which we had at least three individuals. In order to have a direct comparison with what is known about genetic diversity in fish (Ward et al., 2009), we also calculated genetic distances as Kimura's 2-parameter model (K2P: Kimura, 1980).

2.5. Morphometrics

We performed morphometric analyses on the same individuals of the *S. albus/squalus* complex that have been used for the DNA taxonomy. In this way we would be able to evaluate whether the proportions in body size used for the original description of *S. albus* and *S. squalus* and then reported by Kottelat and Freyhof (2007) can be consistently used for the identification of the two species or not. We measured body depth (BD) and standard length (SL) for each of the following 103 fish: 12 specimens of *S. albus/squalus* complex from lake Trasimeno and surrounding areas from the historical collection of Casalina, 21 specimens from present-day lake Trasimeno, 36 specimens from other eight populations in Central Italy, and all

the 23 sequenced specimens from Northern Italy. Moreover, other additional 11 specimens from the same populations of Northern Italy (where only *S. squalus s.s.* was found) were measured in order to increase sample size.

We performed linear models (LM) to test whether the proportion BD/SL was significantly different between the clades identified by DNA taxonomy, controlling for potential differences due to size (SL). We used arcsin − square root transformation for the proportion BD/SL, as such data are bound to both extremes (Crawley, 2007). Analyses were performed on all specimens (103 with genetic identification + other 11 from Northern Italy); we then repeated the analyses using only those individuals with standard length of at least 100 mm, in order to avoid the confounding effect of immature organisms, and thus disregarding the 19 smallest individuals. In order to control for the possibility of introducing a geographical bias by including also animals from Northern Italy and animals that have not been sequenced, we repeated the analyses excluding the 23 + 11 animals from Northern Italy (Supplementary File S2).

3. Results

The 592 base pair (bp) region of the *COI* gene was successfully sequenced for all the individuals and deposited in GenBank (Accession Numbers JF317935, JF317936, GU985093 − GU985101, GU985104 − GU985108; KF836095-KF836105). The sequences could be aligned unambiguously with no insertions/deletions or missing data for the entire sample set, and 27 haplotypes were identified. The reading frame and translation of the nucleotide sequences were successfully carried out, with no sequence identified as a potential nuclear copy; no stop codons, insertions/deletions and high amino acid substitutions were found.

3.1. Phylogenetic reconstruction

Seven haplotypes were identified for *S. lucumonis*, 19 for the complex *S. albus/squalus*, and 1 that clustered with *S. vardarensis* (Fig. 1).

All the historical specimens, coming from lake Trasimeno and from two tributaries of the river Tevere (rivers Clitunno and Rio del Bosco), had the same haplotype, s01 (Supplementary File S1) and could potentially represent *S. albus*; individuals with such haplotype are still present in lake Trasimeno (27 of the 32 individuals sampled in the present day population of the lake). Moreover, the same haplotype was found in other individuals sampled in the river Chiassa (Arno basin), in rivers Aggia, Assino, Carpina, Chiani, Lama, Paglia, Soara, Tevere and Topino (Tevere basin), and in river Burano (Metauro basin). Haplotype s01 clustered with other three haplotypes, s02 from river Soara, s03 from lake Trasimeno, and s24 from lake Trasimeno and rivers Bradano (Southern Italy) and Lama (Fig. 1, clade A). The other cluster, clade B, included 15 haplotypes of the *S. albus/squalus* complex from individuals sampled in rivers Assino, Arno, Burano, Carpina, Chiani, Corsalone, Paglia, and Tevere and in one individual from lake Trasimeno from Central Italy; in all the chubs from Northern Italy; in all but three individuals from Southern Italy; in Slovenia (Fig. 1, Supplementary File S1).

Clade A and clade B are sister clades, closely related to *S. prespensis*. The other Italian chub, *S. lucumonis*, was sister to *S. illyricus*, in another part of the tree. Thus, *S. lucumonis* will not be considered any further in the following analyses on the *S. albus/squalus* complex, given that its genetic separation from *S. albus/squalus* is completely unambiguous. Two individuals from Southern Italy, misidentified as belonging to the *S. albus/squalus* complex during the sampling activities, suggest the potential presence of a new species for Italy, *S. vardarensis*. The two individuals from rivers Basento and Bradano had the same *COI* haplotype and clustered with the sequence of *S. vardarensis* from GenBank (Fig. 1).

3.2. DNA taxonomy

Uncorrected genetic distances ranged up to 0.3% within clade A, and up to 1.2% within clade B; distances between the two clades ranged from 0.8% to 1.6%. The same numbers were found by calculating genetic distances as K2P distances. Genetic distances within the other species were 1.2% in *S. aphipsi*, 0.2%–0.9% in *S. lucumonis*, 0.2%–0.3% in *S. orpheus*, and 0.2% in *S. prespensis*. Uncorrected genetic distances between all the other species in the phylogeny ranged from 1.1% to 10.1%.

Even if the two clades could be visually identified in the phylogenetic reconstruction, with bootstrap support above 0.91 (clades A and B in Fig. 1), the GMYC model, performed on the rooted ultrametric tree with the 19 haplotypes of the *S. albus/squalus* complex, did not support the existence of two independently evolving entities (likelihood of null model = 34.9, maximum likelihood of GMYC model = 37.0, likelihood ratio = 4.19, $p = 0.24$). The ABGD provided the same result, with only one entity and no evidence of a clear barcoding gap between clade A and clade B.

3.3. Morphometrics

The proportion between body depth (BD) and standard length (SL) was not significantly different between clade A and clade B (Table 1A). No significant difference could be found even when analysing only animals longer than 10 cm (Table 1B). The same result is supported when including only animals from the geographical area where clade A and clade B co-occur, Central and Southern Italy (Supplementary File S2).

There is a clear overlap in the BD/SL ratio between the two clades, even if we could not find very long animals for clade A (Fig. 2).

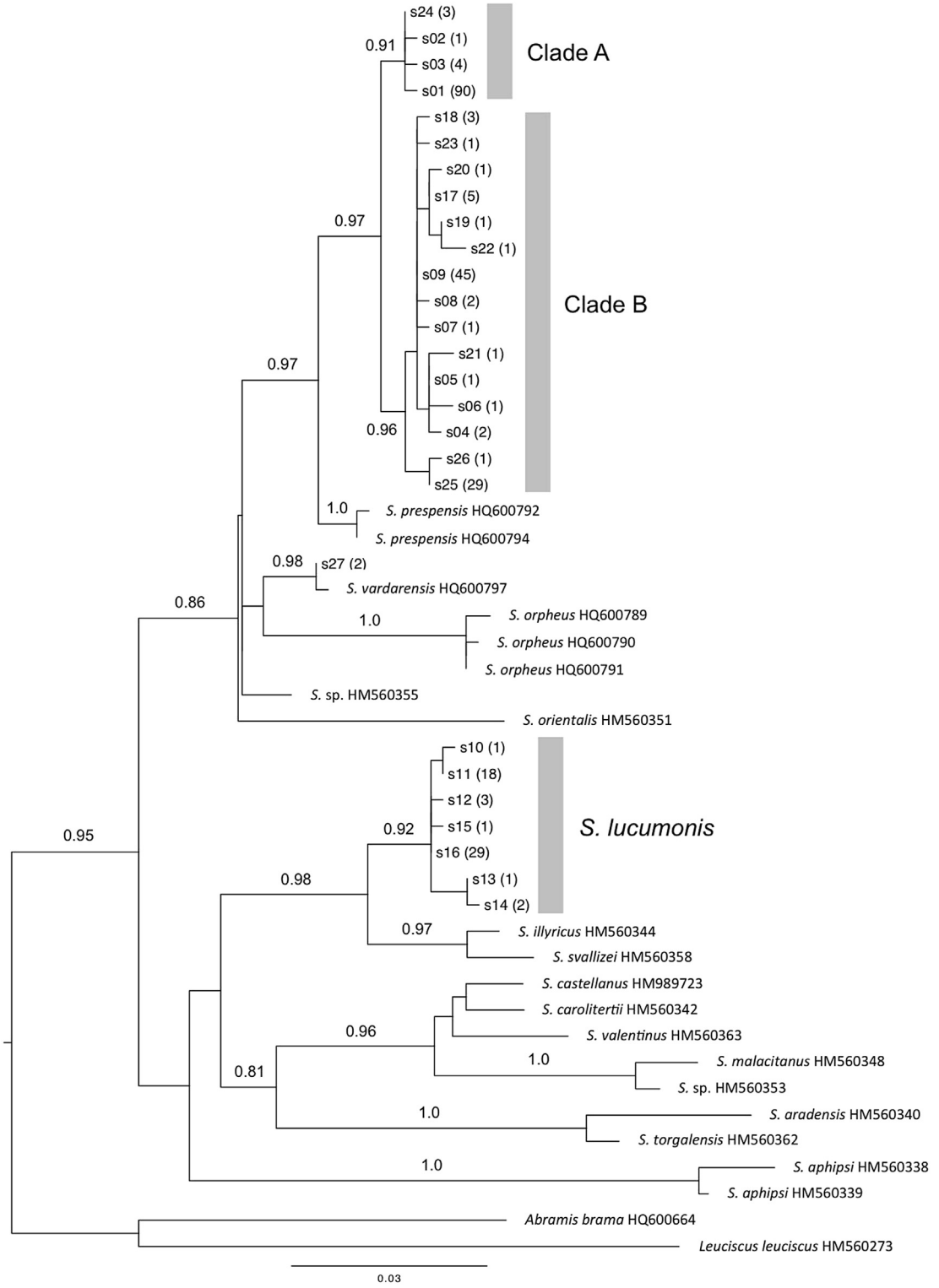

Fig. 1. Maximum likelihood phylogenetic reconstruction on the *COI* dataset ML phylogenetic tree of *COI* dataset for the genus *Squalius*, including the complex *S. albus/squalus* (haplotypes s01-s09 and s17-s26), *S. lucumonis* (haplotypes s10-s16), together with additional sequences from GenBank and the potential *S. vardarensis* (haplotype s27). Bootstrap support values are expressed as percentages on 1000 replicates and reported on branches; values below 80 and for short terminal branches are not reported.

Table 1
Results of the linear model to assess the importance of the explanatory variables in explaining the differences in the proportion between Body Length (BD) and Standard Length (SL) (Fig. 1). Estimates, *t*-values (*t*) and probabilities (*p*) are reported. A, whole dataset; B, only individuals longer than 100 mm.

	Estimate	*t*	*p*
A			
(intercept)	0.46 ± 0.007	59.79	<0.0001
Taxon (clade A *vs* clade B)	−0.02 ± 0.011	−1.91	0.0584
Body size (SL)	0.00 ± 0.000	0.37	0.7072
Taxon * Body size	0.00 ± 0.000	3.84	0.0002
B			
(intercept)	0.46 ± 0.013	34.63	<0.0001
Taxon (clade A *vs* clade B)	−0.01 ± 0.0116	−0.71	0.4814
Body size (SL)	0.00 ± 0.000	1.11	0.2695
Taxon * Body size	0.00 ± 0.000	2.12	0.0375

Such differences in the presence of longer animals (and with higher BD/SL ratio) only for one clade may create the significant interaction between the explanatory variables (Table 1). The interaction disappears when excluding the animals from Northern Italy, mostly longer than the ones from Central and Southern Italy (Supplementary File S2).

4. Discussion

We found unambiguous evidence that all the historical individuals and most of the present-day individuals from lake Trasimeno and the surrounding areas in the river Tevere basin belong to one clade (clade A), whereas all the individuals from Northern Italy, some from Central Italy and all but one of those from Southern Italy belong to a different clade (clade B). The geographic origin of clade A would suggest that it can be considered as *S. albus* with clade B being *S. squalus sensu stricto*, but none of the approaches from DNA taxonomy (ABGD and GMYC) supports the idea that the two clades could be considered separate species. Moreover, individuals of clade A are morphologically undistinguishable from those of clade B in the morphometric ratio Body Depth/Standard Length (BD/SL); such morphological difference corresponds to the one proposed by Bonaparte in the original description of *S. albus* (Kottelat and Freyhof, 2007) with BD/SL smaller than 23%, and ranging from 22 to 27% in *S. squalus*. Our analyses of the two clades did not support such difference (Fig. 2).

Not even a DNA barcoding approach supports the distinctiveness of clade A and clade B. Genetic distances between clade A and clade B, on average around 1.4%, are lower than those found in other organisms, usually around 3% (e.g. Hebert et al., 2003; Tang et al., 2012). Fish are already known to have a low barcoding threshold (Ward et al., 2009; Lucentini et al., 2011b; Mat Jaafar et al., 2012), but none of the tests we performed on DNA taxonomy (ABGD and GMYC) supported their existence as separate entities. Overall, notwithstanding the potential original geographical subdivision between clade A and clade B, we cannot support the distinction of two species in the *S. albus*/*squalus* species complex. The tests to look for evidence of independent evolution of the clades (Pons et al., 2006; Puillandre et al., 2012) suggest only one and not two entities. The fact that there is indeed genetic and morphological variability is not surprising, because the existence of genetic and phenotypic variability, even more structured in space than in the analysed case, is a common feature in animals (e.g. Bentz et al., 2011; Lind et al., 2011; Kieneke et al., 2012). Thus, we support the conclusion by Gandolfi et al. (1991) that the variability within the *S. albus/squalus* complex is not enough to identify independently evolving entities. We cannot exclude that the system we analysed represents a case of potential incipient speciation (Bilton et al., 1998). Incipient speciation with ongoing gene flow could produce an unclear scenario, similar to the one we observed for the *S. albus/squalus* species complex.

If the two clades are indeed geographical variants, the presence in lake Trasimeno of one individual of clade B, haplotype s09, could be an indication of the persistence in the lake of haplotypes introduced during restocking campaigns (Lorenzoni et al., 1994). Still, only one individual out of the 32 we analysed from the present day population of lake Trasimeno resulted in the clade B of the *S. albus/squalus* complex. Thus, the system seems to be resilient to invasion of allochthonous haplotypes.

The Italian Peninsula is known to have two distinct biogeographical districts for its fish fauna, one in Northern Italy, centred around the river Po, and one along the rest of the peninsula (Bianco, 1995), where often pairs of vicariant species exist, one in each district, e.g. *Barbus plebejus* (Bonaparte, 1839)/*B. tyberinus* (Bonaparte, 1839), *Rutilus aula* (Bonaparte, 1841)/*R. rubilio* (Bonaparte, 1837), *Padogobius bonelli* (Bonaparte, 1846)/*P. nigricans* (Canestrini, 1867), *Alburnus arborella* (De Filippi, 1844)/*A. albidus* (Costa, 1838) in the Northern and peninsula district respectively. Apparently, this is not the case for the *S. albus/squalus* complex, where clade B is present across all the districts, whereas clade A is present in the central part of the peninsular district. Such distribution in the analysed chubs could easily be due to restocking. In Italy, the restocking programs are considered one of the main sources of the introduction of exotic species since most often specimens from North Italy are translocated in Central Italy (Lorenzoni et al., 2006), leading to an alteration of the original distribution and compromising the genetic integrity of the native populations.

A task of major concern in biological conservation is the maintenance of intraspecific genetic diversity in target species (Nelson and Soulé, 1987; Ryman et al., 1995). Also, the IUCN has recognized genetic resources as one level of diversity requiring protection (McNeely et al., 1990). Up to now *S. squalus* does not represent an issue of concern for conservation

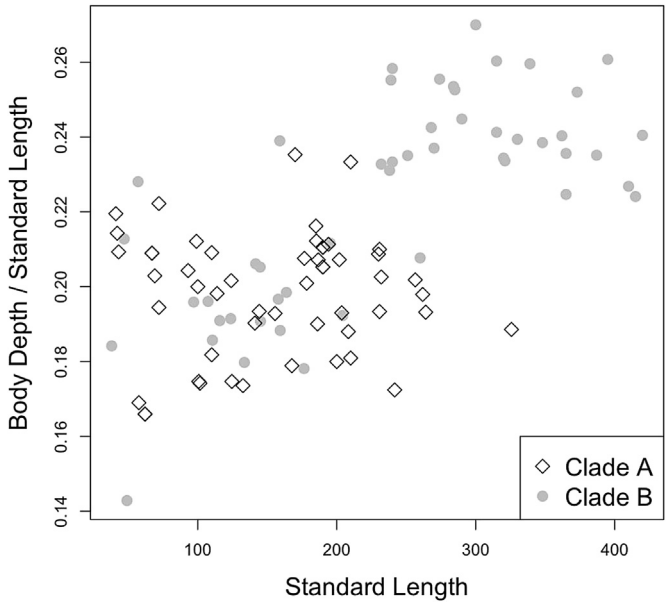

Fig. 2. Scatterplot of the morphometric parameters. Scatterplot proportion between Body Depth (BD) and Standard Length (SL), as a function of the Standard Length (SL, in millimeters) for the 103 specimens measured in the two clades evidenced in Fig. 1.

purpose and is listed as 'Least Concern' both by IUCN Red List of Threatened Species (IUCN, 2013) and Red List of Italian Vertebrates (Rondinini et al., 2013). However, the findings of this research raise some questions about the adequacy of management practices of most of the Italian populations of *S. squalus*. If clade B represents native animals from the Northern district and clade A animals from the peninsular district (or at least endemic to lake Trasimeno), the two groups of populations should be managed independently. Even if they do not represent different species, they belong to different genetic clades. As a general suggestion for fishery management, the translocations of specimens between watershed should be avoided when there are genetic differences as the ones between clade A and clade B, in order to prevent any possible mixture between different clades, even within the same species.

An interesting additional result of our analysis is that a new species for the Italian peninsula may be present, *S. vardarensis*. Specimens of this species were found at the same latitudes of the known distribution of the species in Greece and Macedonia (Kottelat and Freyhof, 2007). An interesting hypothesis is that this species may persist in the Southern parts of the Italian and Balkan Peninsulas as a disjunct remnant of past larger distribution. More plausibly it represents another case of translocation; nevertheless, there are previous finding of Balkan species found in Italy without any information or support for their translocation, as in the case of *Pachychilon pictum* Heckel and Kner, 1858 in fish (Delmastro and Balma 1990) and *Carabus cavernosus* in beetles (Casale et al., 1982). With the currently available information, in particular without any data about *S. vardarensis* acclimation, presence, consistence and distribution we cannot state which of the two hypotheses (autochthonous or allochthonous origin) is more reliable. In conclusion, even if no support has been found for the existence of two distinct species in the S. albus/squalus species complex in Italy, two clades were found (A and B) and, from the conservation point of view, they should be managed as separate units as a cautionary approach.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bse.2014.07.005.

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