

Status of the Catalan chub *Squalius laietanus* (Actinopterygii, Cyprinidae) in France: input from morphological and molecular data

G.P.J. Denys^{(1),*}, A. Dettai⁽²⁾, H. Persat⁽³⁾, I. Doadrio⁽⁴⁾, C. Cruaud⁽⁵⁾, P. Keith⁽¹⁾

Received September 7, 2012

Revised December 21, 2012

Accepted January 24, 2013

ABSTRACT

Key-words:
Squalius laietanus,
Squalius cephalus,
France,
DNA taxonomy,
hybridization

Two species of chubs in French drainages: the common chub *S. cephalus* and the Catalan chub *S. laietanus* restricted to Oriental Pyrenees (South of France). Morphological and molecular characters distinguishing the two species have been described on Spanish specimens, but not tested on French samples. 32 sites were sampled throughout the French water system, yielding 155 specimens of which 60 were sequenced for two molecular markers COI and rhodopsin. Two divergent haplotype clusters for COI are found, and indicate the presence of *S. laietanus* in France (from Massane to Agly basins). In some sites, both species are sympatric. In some cases, molecular results partially disagree with morphological data, suggesting possible hybridizations between the two. This might threaten Catalan chub in France, and its red list status should be re-evaluated in order to undertake conservation measures.

RÉSUMÉ

Le statut du chevesne catalan *Squalius laietanus* (Actinopterygii, Cyprinidae) en France : apport des données morphologiques et moléculaires

Mots-clés :
Squalius laietanus,
Squalius cephalus,
France,
taxonomie
moléculaire,
hybridation

Il y a deux espèces de chevesnes dans les bassins versants français, le chevesne commun *S. cephalus* et le chevesne catalan *S. laietanus* restreint aux Pyrénées Orientales. Les spécimens espagnols des deux espèces sont clairement distinguables aussi bien sur le plan morphologique que sur le plan moléculaire, mais aucun spécimen français n'avait été testé. Trente-deux stations sur tout le réseau hydrographique français ont permis de collecter 155 spécimens dont 60 ont été utilisés pour les analyses moléculaires sur les marqueurs du COI et de la rhodopsine. Deux clusters d'haplotypes sont présents pour le marqueur du COI, indiquant la présence du *S. laietanus* en France du bassin de la Massane à l'Agly. Les deux espèces sont sympatriques dans certaines stations. Il n'y a pas toujours de congruence entre les résultats moléculaires et les données morphologiques, suggérant d'éventuelles hybridations entre les deux espèces. *S. laietanus* pourrait être menacé en France et son statut de la Liste Rouge doit être réévalué afin d'entreprendre des mesures de conservation.

(1) Muséum national d'Histoire naturelle, UMR BOREA MNHN - CNRS 7208 - IRD 207 - UPMC, CP26, 57 rue Cuvier, 75231 Paris Cedex 05, France

(2) Muséum national d'Histoire naturelle, UMR SAE 7138, CP26, 57 rue Cuvier, 75231 Paris Cedex 05, France

(3) Écologie des Hydrosystèmes Naturels et Anthropisés, LEHNA UMR 5023, Bat. Forel, Université Claude Bernard Lyon 1, 69622 Villeurbanne Cedex, France

(4) National Museum of Natural Sciences, Spanish Research Council, Dpt of Biodiversity and Evolutionary Biology, José Gutiérrez Abascal 2 Str., 28006 Madrid, Spain

(5) Genoscope, Centre National de Séquençage, 2 rue Gaston Crémieux, CP5706, 91057 Évry Cedex, France

* Corresponding author: gael.denys@mnhn.fr

During two centuries, only one species of chub (*Squalius*, Actinopterygii, Cyprinidae) was known in France. It was however separated into two subspecies (Spillmann, 1961): a common form *Leuciscus cephalus cephalus* (Linnaeus, 1758) currently valid as *Squalius cephalus* (Linnaeus, 1758), and a meridional form *L. cephalus cabeda* (Risso, 1827) currently valid as *S. squalus* (Bonaparte, 1837). Kottelat and Freyhof (2007, pp. 275, 623), based on data cited as “Kottelat 2007b in press” considered the two French subspecies as synonyms. Doadrio *et al.* (2007) described a new species of chub, *S. laietanus* from Northeast Spain, from Ebre (Spain) to Agly (France) drainages on the basis of both morphological and cytochrome b sequence data. *S. laietanus* mainly differs from *S. cephalus* by having a mouth slightly subinferior (vs. terminal with well marked chin), lots of brown pigments on the upper and lower tips of the exposed part of the scales forming faint longitudinal stripes between scale rows (vs. no or few pigments on scales, forming clearly contrasted reticulate pattern), and 17 to 20 branched rays at pectoral fin (vs. 14 to 17). Genetic studies on *S. laietanus* were performed on Spanish specimens only (e.g. Doadrio *et al.*, 2007; Perea *et al.*, 2010), and never included French samples. However Doadrio (unpublished data) noticed that French *S. laietanus* populations differ morphologically from Spanish *S. laietanus* specimens. We therefore aim to (i) study the morphology and two DNA markers on a large and representative sampling from the French drainages: the mitochondrial gene coding for cytochrome C oxidase subunit 1 (COI) and the nuclear rhodopsin retrogene, (ii) compare the morphological and molecular characters and evaluate the presence of *S. laietanus* in France.

Sampling operations were performed in 2010 and 2011 with the collaboration of the French National Agency for Water and Aquatic Environments (Onema), the Fédération Nationale de la Pêche en France (FNPF), and Associations agréées de pêche et de protection des milieux aquatiques (AAPPMA). 155 specimens were caught by electrofishing in 32 stations, in the major drainages (Seine, Loire, Garonne, Rhine, Rhône, Adour), with a higher collection effort on the Mediterranean basins as Agly, Massane and Tech drainages (8 locations) (Figure 1). Fin clips were collected for molecular study in EtOH, and specimens were fixed in formalin. Specimens smaller than 15 cm were fixed by steps in 95% ethanol. All the specimens were identified morphologically using diagnostic characters given by Doadrio *et al.* (2007). 60 specimens were used for DNA analyses. Tissue samples from well identified Spanish *S. laietanus* were also included (see Appendix I). For DNA extraction, PCR, sequencing and quality control, we followed Dettai *et al.* (2011) except for the use of CodonCode Aligner 3.9 (Codon Code Corporation) for sequence cleaning and control. This yielded two datasets: partial COI gene (567 pb) and partial rhodopsin retrogene (755 pb), in which we also included some sequences from other *Squalius* species available in BOLD and GenBank, as well as *Alburnoides*, *Chondrostoma*, *Leuciscus* and *Rutilus* sequences as outgroups (Perea *et al.* 2010) (Appendix I). All new sequences were deposited in the Barcode of Life database (Ratnasingham and Hebert, 2007) with their voucher information. BOLD and GenBank Accession numbers are listed in appendix. Alignment was performed manually, as neither marker includes indels. Heterozygous sequences of rhodopsin were separated into the two alleles using as guides homozygote sequences (e.g. Sousa-Santos *et al.*, 2005) from the same drainage with SEQPHASE (Flot, 2010) and PHASE (Stephens *et al.*, 2001). Phylogenetic analyses were performed with Bayesian inference (MrBayes 3.2, Ronquist *et al.*, 2012). For COI, a model computed by JModelTest 2.1.1 (Darriba *et al.*, 2012), and partitioned by codon position was specified, on four analyses with 10 million generations and sampling every 200 generations. For rhodopsin, the dataset was not partitioned because of the low sequence variability. 10% of trees were eliminated as burnin after checking convergence. Intra- and inter-specific distances (p-distances) were calculated with the software MEGA 5 (Tamura *et al.*, 2011).

For the COI analysis (Figure 2), there are two distinct haplotype clusters, with almost no within cluster variability: only one specimen differing by 2 bases (FFFTag10843) in the first cluster, and in the second cluster one specimen differs by a single base from the others (FFFTag4180). There are 22 diagnostic sites differentiating the two clusters. The first haplotype includes specimens from all French drainages including Mediterranean basins. It clusters with low support (0.58 ppv) with other *S. cephalus* from Elbe and Danube drainages in Czech

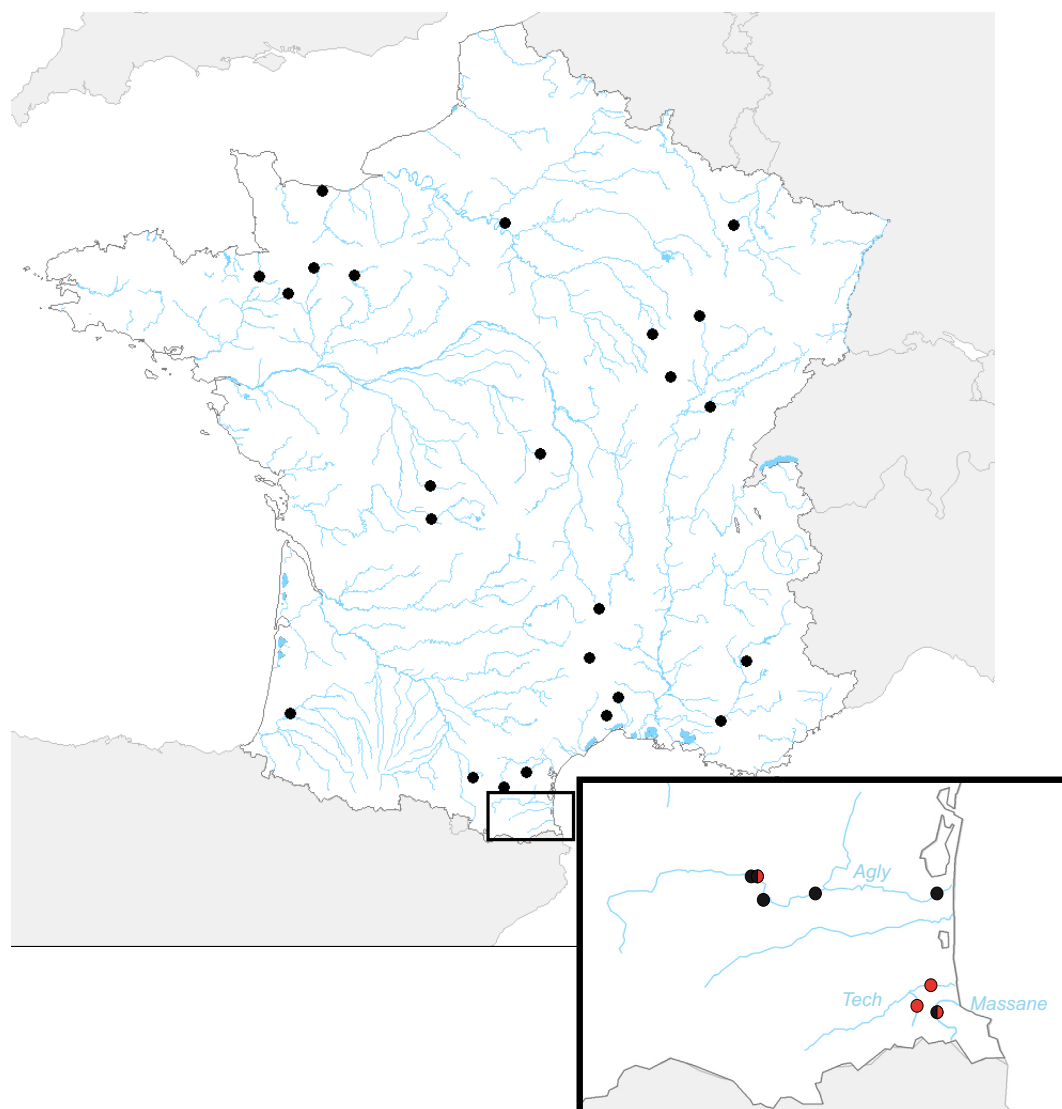


Figure 1

Location of the 32 sampling sites, showing the presence of *Squalius cephalus* haplotype (black dots), and *S. laietanus* (red dots).

Republic. This group also includes a distinct Central European group with *S. vardarensis* and *S. cephalus* (0.91 ppv) from Danube basin. This branch and the *S. cephalus* sequences differ by 5 positions. The second haplotype cluster includes Spanish *S. laietanus*, and samples from Agly, Tech and Massane drainages (0.69 ppv). It is included into a North Mediterranean group as the sister group of a Greek chub *S. orpheus* (1 ppv), and is distant from the Mediterranean group *sensu* Perea *et al.* (2010). Two locations, Agly at Torreilles and the Massane stream, yielded specimens belonging to both clusters. Central European and North Mediterranean groups constitute the Euroasiatic group *sensu* Perea *et al.* (2010).

Morphological identification of chubs from the principal drainages was done without any difficulty using the diagnostic characters (Doadrio *et al.*, 2007). However, the specimens from Agly and Tech basins were more problematic, for both species. Some (morphological characters placed on the COI topology, Figure 2) presented morphological data characteristic of both species (*i.e.* subterminal/terminal mouth, presence/absence of faint longitudinal stripes on flanks). All chubs caught in these Mediterranean basins have 17 pectoral fin rays, the maximum number for *S. cephalus* and the minimum for *S. laietanus*.

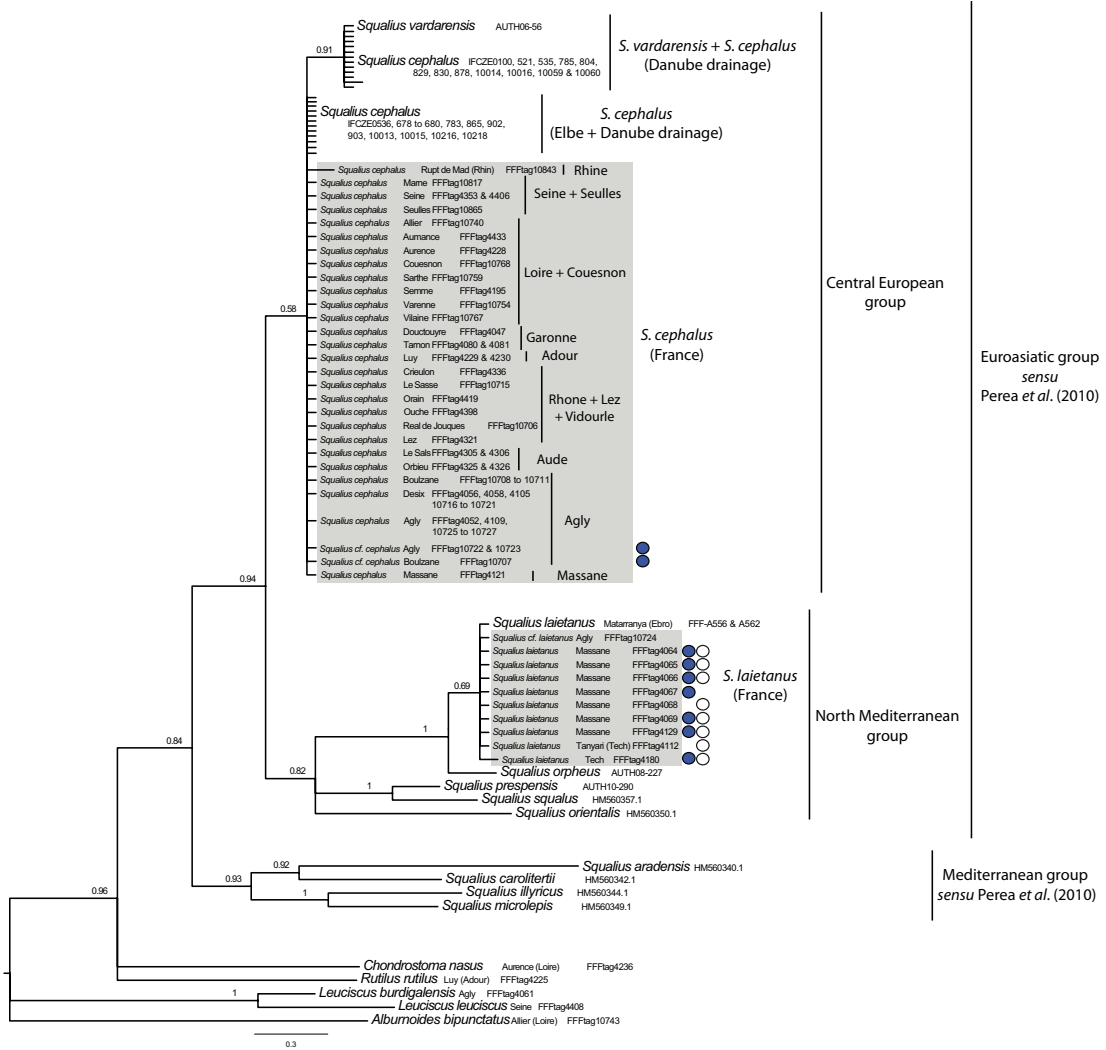


Figure 2

Bayesian tree of the cytochrome c oxidase subunit I (COI). Numbers on the nodes represent posterior probabilities. The mean a posteriori values of the parameters are (respectively for first, second and third codon position): TL = 19.515126; alpha = 94.564172, 99.421936, 76.659005; pinvar = 0.841327, 0.994746, 0.089359. French samples (grey boxes) have drainage indicated. Character states supposedly specific to *S. laietanus* are indicated by dots: subterminal mouth (blue), presence of brown pigments on scales on upper and lower tips of exposed part forming faint longitudinal stripes between scale rows (white).

There are 9 haplotypes for the rhodopsin dataset (Appendices 2 and 3), mostly represented by a single specimen, only one (H6) is present in both species and drainages. They correspond neither to taxonomy nor to geography, and some specimens are heterozygous. This pattern might be due to incomplete lineage sorting (Edwards, 2009) among other processes, but this needs further study. Thus, rhodopsin is not a good marker for DNA identification of *Squalius* genus.

The COI dataset, like the *cyt b* dataset (Perea et al., 2010), clearly separates the two species. More variable nuclear sequences would be needed for further analyses of these species.

Our results corroborate the presence of the Catalan chub in France from the Tech to Agly drainages (Doadrio et al., 2007), and add a new occurrence in the Massane basin. The two species are sympatric in Agly and Massane basins, at least at two stations, but currently only *S. laietanus* has been identified in the Tech basin. Moreover, some specimens from these

basins have a mix of diagnostic characters from both species: this might be evidence of hybridization or introgression between *S. cephalus* and *S. laietanus*. Doadrio *et al.* (2007) suspected exchange of genetic material based on two specimens identified as *S. laietanus* from the Agly river with the lower jaw somewhat more developed. Common chub has been largely transported and introduced since Middle Ages (Keith *et al.*, 2011), and is considered invasive in Ireland, where it is not native (Caffrey *et al.*, 2008). In Spain, *S. laietanus* is sympatric with *S. pyrenaicus* (Günther, 1868) in the south of Ebro basin (see Doadrio and Carmona, 2006), where introgression is known (Doadrio, pers.com.). In France, introgression has been described in many cyprinid species (see Keith *et al.*, 2011). Hybridization and introgression following the introduction of a non-native species are one of the major threats to endemic freshwater fishes (e.g. Ribeiro and Leunda, 2012). In France, until further data about its presence in the neighbour basins to the Agly and Tech (Têt and Baillauray drainages), Catalan chub has only been recorded in three small drainages, where it cohabits with a species with which it can probably hybridise. The use of appropriate nuclear markers would allow to verify this hypothesis. To our knowledge, only the Tech basin might shelter *S. laietanus* and no sympatric common chub. It would deserve further investigation and must be preserved from fish dumping. *S. laietanus* might therefore deserve a place in the Red List of threatened freshwater fish in France (Keith and Marion, 2002; UICN France *et al.*, 2010), where it would join the 70% of extant endemic freshwater fishes in the Mediterranean basin recognized as threatened by extinction (Ribeiro and Leunda, 2012).

ACKNOWLEDGEMENTS

This work was supported by the Onema, and we are particularly grateful to N. Poulet and S. Dembski. We thank all the Onema and FNPF agents, and the AAPPMA members for fish samplings. The ichthyology curators of MNHN and MNCN allowed access to the specimens. A. Amir helped with extractions and PCRs. This work was supported by the ‘Service de Systématique Moléculaire’ (project @ SPEED-ID “Accurate SPECiEs Delimitation and IDentification of eukaryotic biodiversity using DNA markers” proposed by F-BoL, the French Barcode of life initiative).

REFERENCES

- Caffrey J.M., Avededo S., Gallagher K. and Britton R., 2008. Chub (*Leuciscus cephalus*: a new potentially invasive fish species in Ireland. *Aquatic Invasions*, 3, 201–209.
- Darriba D., Taboada G.L., Doallo R. and Posada D., 2012. jModelTest 2: more models, new heuristics and parallel computing. *Nat. Methods*, 9, 772.
- Dettai A., Lautredou A.-C., Bonillo C., Goimbault E., Busson F., Causse R., Couloux A., Cruaud C., Duhamel G., Denys G., Hautecoeur M., Iglesias S., Koubbi P., Lecointre G., Moteki M., Pruvost P., Terceire S. and Ozouf C., 2011. The actinopterygian diversity of the CEAMARC cruises: Barcoding and molecular taxonomy as a multi-level tool for new findings. *Deep-Sea Research II*, 58, 250–263.
- Doadrio I. and Carmona J.A., 2006. Phylogenetic overview of the genus *Squalius* (Actinopterygii, Cyprinidae) in the Iberian Peninsula, with description of two new species. *Cybium*, 30, 199–214.
- Doadrio I., Kottelat M. and de Sostoa A., 2007. *Squalius laietanus*, a new species of cyprinid fish from north-eastern Spain and southern France (Teleostei: Cyprinidae). *Ichthyol. Explor. Fres.*, 18, 247–256.
- Edwards S.V., 2009. Is a new and general theory of molecular systematics emerging? *Evolution*, 63, 783–791.
- Flot J.-F., 2010. SEQPHASE: a web tool for interconverting PHASE input/output files and FASTA sequence alignments. *Mol. Ecol. Res.*, 10, 162–166.
- Keith P. and Marion L., 2002. Methodology for drawing up a Red List of threatened freshwater fish in France. *Aquat. Conserv.: Mar. Freshw. Ecosyst.*, 12, 169–179.

- Keith P., Persat H., Feuteun E. and Allardi J., 2011. Les Poissons d'eau douce de France, collection Inventaires & biodiversité, Biotope Editions, Publications scientifiques du Muséum, 552 p.
- Kottelat M. and Freyhof J., 2007. Handbook of European freshwater fishes, Publications Kottelat, Cornol, 646 p.
- Perea S., Böhme M., Zupancic P., Freyhof J., Sanda R., Özulug M., Abdoli A. and Doadrio I., 2010. Phylogenetic relationships and biogeographical patterns in Circum-Mediterranean subfamily Leuciscinae (Teleostei, Cyprinidae) inferred from both mitochondrial and nuclear data. *BMC Evol. Biol.*, 10, 1–27.
- Ratnasingham S. and Hebert P.D.N., 2007. BOLD: The Barcode of Life Data System (www.barcodinglife.org). *Mol. Ecol. Notes*, 7, 355–364.
- Ribeiro F. and Leunda P.M., 2012. Non-native fish impacts on Mediterranean freshwater ecosystems: current knowledge and research needs. *Fish. Manag. Ecol.*, 19, 142–156.
- Ronquist F., Teslenko M., van der Mark P., Ayres D.L., Darling A., Höhna S., Larget B., Liu L., Suchard M.A. and Huelsenbeck J.P., 2012. MrBayes 3.2: Efficient Bayesian Phylogenetic Inference and Model Choice across a Large Model Space. *Sys. Biol.*, 61, 1–4.
- Sousa-Santos C, Robalo J.I., Collares-Pereira M.-J. And Almada V.C., 2005. Heterozygous indels as useful tools in the reconstruction of DNA sequences and in the assessment of ploidy level and genomic constitution of hybrid organisms. *DNA Seq.*, 16, 462–467.
- Spillmann C.J., 1961. Poissons d'eau douce de France, Faune de France, P. Lechevalier, Paris, 303 p.
- Stephens M., Smith N.J. and Donnelly P., 2001. A new statistical method for haplotype reconstruction from population data. *Am. J. Hum. Genet.*, 68, 978–989.
- Tamura K., Peterson D., Peterson N., Stecher G., Nei M. and Kumar S., 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Mol. Biol. Evol.*, 28, 2731–2739.
- UICN France, MNHN, SFI and ONEMA, 2010. La liste rouge des espèces menacées en France, Chapitre Poissons d'eau douce de France métropolitaine. Paris, 12 p.

Appendix 1.

Species	Catalog number	BOLD	GenBank Accession Number		Stream (Basin)	Town	Country
			COI	Rhodopsin			
<i>S. cephalus</i>	MNHN 2010-1025	FFFtag4229	KC355008	KC355062	Luy (Adour)	Saunac et Cambrian	France
<i>S. cephalus</i>	MNHN 2010-1025	FFFtag4230	KC354977	KC355043	Luy (Adour)	Saunac et Cambrian	France
<i>S. cephalus</i>	MNHN 2011-827	FFFtag10725	KC354991	KC355050	Agly	Torreilles	France
<i>S. cephalus</i>	MNHN 2011-827	FFFtag10726	KC355001	KC355057	Agly	Torreilles	France
<i>S. cephalus</i>	MNHN 2011-827	FFFtag10727	KC355014	KC355065	Agly	Torreilles	France
<i>S. cephalus</i>	MNHN 2010-1836	FFFtag4052	KC355006	KC355060	Agly	La Tournère	France
<i>S. cephalus</i>	MNHN 2010-497	FFFtag4109	KC354982	KC355052	Agly	Latour-de-France	France
<i>S. cephalus</i>	MNHN 2011-825	FFFtag10708	KC354996	KC355052	Boulzane (Agly)	Saint-Paul-de-Fenouillet	France
<i>S. cephalus</i>	MNHN 2011-825	FFFtag10709	KC354973	KC355040	Boulzane (Agly)	Saint-Paul-de-Fenouillet	France
<i>S. cephalus</i>	MNHN 2011-825	FFFtag10710	KC355018	KC355069	Boulzane (Agly)	Saint-Paul-de-Fenouillet	France
<i>S. cephalus</i>	MNHN 2011-825	FFFtag10711	KC354986	KC355048	Boulzane (Agly)	Saint-Paul-de-Fenouillet	France
<i>S. cephalus</i>	MNHN 2011-825	FFFtag10712	KC355054	KC355054	Boulzane (Agly)	Saint-Paul-de-Fenouillet	France
<i>S. cephalus</i>	MNHN 2011-823	FFFtag10716	KC355000	KC355056	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-823	FFFtag10717	KC354972	KC355039	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-823	FFFtag10718	KC355016	KC355067	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-823	FFFtag10719	KC354976	KC355042	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-823	FFFtag10720	KC354998	KC355053	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-823	FFFtag10721	KC354987	KC355049	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2010-1838	FFFtag4056	KC355012	KC355046	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2010-1838	FFFtag4058	KC354984	KC355046	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2010-493	FFFtag4105	KC354983	KC355045	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-254	FFFtag4325	KC355009	KC355045	Desix (Agly)	Ansignan	France
<i>S. cephalus</i>	MNHN 2011-254	FFFtag4326	KC354975	KC355045	Orbieu (Aude)	Ribaute	France
<i>S. cephalus</i>	MNHN 2010-1834	FFFtag4047	KC355017	KC355068	Orbieu (Aude)	Ribaute	France
<i>S. cephalus</i>	MNHN 2010-1840	FFFtag4080	KC355005	KC355059	Douctouyre (Garonne)	Dun	France
<i>S. cephalus</i>	MNHN 2010-1840	FFFtag4081	KC355011	KC355064	Tarnon (Garonne)	Florac	France
<i>S. cephalus</i>	MNHN 2011-253	FFFtag4321	KC354990	KC355064	Tarnon (Garonne)	Florac	France
<i>S. cephalus</i>	MNHN 2011-818	FFFtag10740	KC355004	KC355058	Lez	Prades-le-Lez	France
<i>S. cephalus</i>	MNHN 2011-1271	FFFtag4433	KC354999	KC355004	Allier (Loire)	Saint-Haon	France
<i>S. cephalus</i>	MNHN 2010-1038	FFFtag4228	KC354993	KC355051	Aumance (Loire)	Cosne d'Allier	France
<i>S. cephalus</i>	MNHN 2011-914	FFFtag10768	KC354992	KC355051	Aurence (Loire)	Aixe-sur-Vienne	France
<i>S. cephalus</i>	MNHN 2011-922	FFFtag10759	KC354997	KC355051	Couesnon	Romazy	France
<i>S. cephalus</i>	MNHN 2010-1014	FFFtag4195	KC355002	KC355051	Sarthe (Loire)	Moulin-le-Carbonel	France
<i>S. cephalus</i>	MNHN 2011-912	FFFtag10754	KC354988	KC355051	Semme (Loire)	Droux	France
<i>S. cephalus</i>	MNHN 2011-912	FFFtag10754	KC354988	KC355051	Varenne (Loire)	Soucé	France

Appendix 1. Continued.

Species	Catalog number	BOLD	GenBank Accession Number		Stream (Basin)	Town	Country
			COI	Rhodopsin			
<i>S. cephalus</i>	MNHN 2011-918	FFFtag10767	KC355013		Vilaine (Loire)	Bourgon	France
<i>S. cephalus</i>	MNHN 2010-509	FFFtag4121	KC354981		Massane	Argelès	France
<i>S. cephalus</i>	MNHN 2011-898	FFFtag10843	KC355003		Rupt de Mad (Rhin)	Essey-et-maizerais	France
<i>S. cephalus</i>	MNHN 2011-248	FFFtag4336	KC354979		Crieulon (Vidourle)	Orthoux-Serignac-Quilhan	France
<i>S. cephalus</i>	MNHN 2011-273	FFFtag4305	KC354980		Le Sals (Aude)	Coustaussa	France
<i>S. cephalus</i>	MNHN 2011-273	FFFtag4306	KC354989		Le Sals (Aude)	Coustaussa	France
<i>S. cephalus</i>	MNHN 2011-824	FFFtag10715	KC355007		Le Sasse (Rhône)	Nibles	France
<i>S. cephalus</i>	MNHN 2011-391	FFFtag4419	KC355015		Orain (Rhône)	Saint-Baraing	France
<i>S. cephalus</i>	MNHN 2011-410	FFFtag4398	KC355010		Ouche (Rhône)	Fleurey-sur-ouche	France
<i>S. cephalus</i>	MNHN 2011-830	FFFtag10706	KC354974		Real de Jouques (Rhône)	Jouques	France
<i>S. cephalus</i>	MNHN 2011-863	FFFtag10817	KC354978		Marne (Seine)	Rolampont	France
<i>S. cephalus</i>	MNHN 2011-235	FFFtag4353	KC354994		Seine	Epinay-sur-Seine	France
<i>S. cephalus</i>	MNHN 2011-394	FFFtag4406	KC354985		Seine	Nod-Sur-Seine	France
<i>S. cephalus</i>	MNHN 2011-1135	FFFtag10865	KC354995		Seulles	Tierceville	France
<i>S. cf. cephalus</i>	MNHN 2011-826	FFFtag10722	KC354969		Agly	Torreilles	France
<i>S. cf. cephalus</i>	MNHN 2011-826	FFFtag10723	KC354968		Agly	Torreilles	France
<i>S. cf. cephalus</i>	MNHN 2011-828	FFFtag10707	KC354970		Boulzane (Agly)	Saint-paul-de-fenouillet	France
<i>S. laietanus</i>	MNHN 2010-1831	FFFtag4064	KC355019		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-1831	FFFtag4065	KC355021		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-1831	FFFtag4066	KC355022		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-1831	FFFtag4067	KC355029		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-1831	FFFtag4068	KC355023		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-1831	FFFtag4069	KC355027		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-517	FFFtag4129	KC355026		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-500	FFFtag4112	KC355024		Massane	Argelès	France
<i>S. laietanus</i>	MNHN 2010-994	FFFtag4180	KC355025		Tanyari (Tech)	St-Génis-des-Fontaines	France
<i>S. cf. laietanus</i>	MNHN 2011-827	FFFtag10724	KC354971		Tech	Eine	France
<i>S. cephalus</i>		IFCZE0783	HQ960908		Agly	Torreilles	France
<i>S. cephalus</i>		IFCZE10014			Becva		Czech republic
<i>S. cephalus</i>		IFCZE10015			Dyje		Czech republic
<i>S. cephalus</i>		IFCZE10016			Dyje		Czech republic
<i>S. cephalus</i>		IFCZE0521			Dyje		Czech republic
<i>S. cephalus</i>		IFCZE0829			dam (Hamry)		Czech republic
<i>S. cephalus</i>					Liboc		Czech republic

Appendix 1. Continued.

Species	Catalog number	BOLD	GenBank Accession Number		Stream (Basin)	Town	Country
			COI	Rhodopsin			
<i>S. cephalus</i>		IFCZE0830	HQ960949		Liboc		Czech republic
<i>S. cephalus</i>		IFCZE0679	HQ960810		Lubina		Czech republic
<i>S. cephalus</i>		IFCZE0680	HQ960811		Lubina		Czech republic
<i>S. cephalus</i>		IFCZE10218			Luznice		Czech republic
<i>S. cephalus</i>		IFCZE0878	HQ960995		Morava		Czech republic
<i>S. cephalus</i>		IFCZE10013			Morava		Czech republic
<i>S. cephalus</i>		IFCZE10059			Morava		Czech republic
<i>S. cephalus</i>		IFCZE10060			Morava		Czech republic
<i>S. cephalus</i>		IFCZE0903	HQ961020		Odrava		Czech republic
<i>S. cephalus</i>		IFCZE0865	HQ960983		Ohre		Czech republic
<i>S. cephalus</i>		IFCZE0902	HQ961019		OhrexRolava_confluence		Czech republic
<i>S. cephalus</i>		IFCZE0804	HQ960926		Olsava		Czech republic
<i>S. cephalus</i>		IFCZE0535	HQ960687		Orlice		Czech republic
<i>S. cephalus</i>		IFCZE0678	HQ960809		Ostravice		Czech republic
<i>S. cephalus</i>		IFCZE10216			sand pit outflow		Czech republic
<i>S. cephalus</i>		IFCZE0536	HQ960688		Sazava		Czech republic
<i>S. cephalus</i>		IFCZE0100	HQ960463		Svatka		Czech republic
<i>S. cephalus</i>		IFCZE0785	HQ960909		Becva (Vsetinska)		Czech republic
<i>S. alburnoides</i>				EU199441_1	Tejo		Portugal
<i>S. aradensis</i>	MNCNAT1272		HM560340.1		Algibre (Arade)		Portugal
<i>S. carolifertii</i>	MNCNAT2739		HM560342.1		Limia		Spain
<i>S. illyricus</i>	MNCNCEITINA1		HM560344.1				Adriatic
<i>S. laietanus</i>	MNCNA556	FFF-A556	KC355020	KC355070	Matarranya (Ebro)	Nonaspe	Spain
<i>S. laietanus</i>	MNCNA560	FFF-A560		KC355076	Matarranya (Ebro)	Nonaspe	Spain
<i>S. laietanus</i>	MNCNA562	FFF-A562		KC355082	Matarranya (Ebro)	Nonaspe	Spain
<i>S. laietanus</i>	MNCNA569	FFF-A569		KC355071	Matarranya (Ebro)	Nonaspe	Spain
<i>S. laietanus</i>	MNCNA202	FFF-A202		KC355079	El Goia	Querol	Spain
<i>S. laietanus</i>	MNCNA395	FFF-A395		KC355080	Corb (Segre)	Belvis	Spain
<i>S. microlepis</i>	MNCNBOS14		HM560349.1				Adriatic
<i>S. orientalis</i>	MNCNCEPHALUS2		HM560350.1				Minor Asia
<i>S. pyrenaicus</i>				EU199442_1	Tejo		Portugal
<i>S. squalus</i>	MNCNAT3959	HM560357.1					Italy

Appendix 1. Continued.

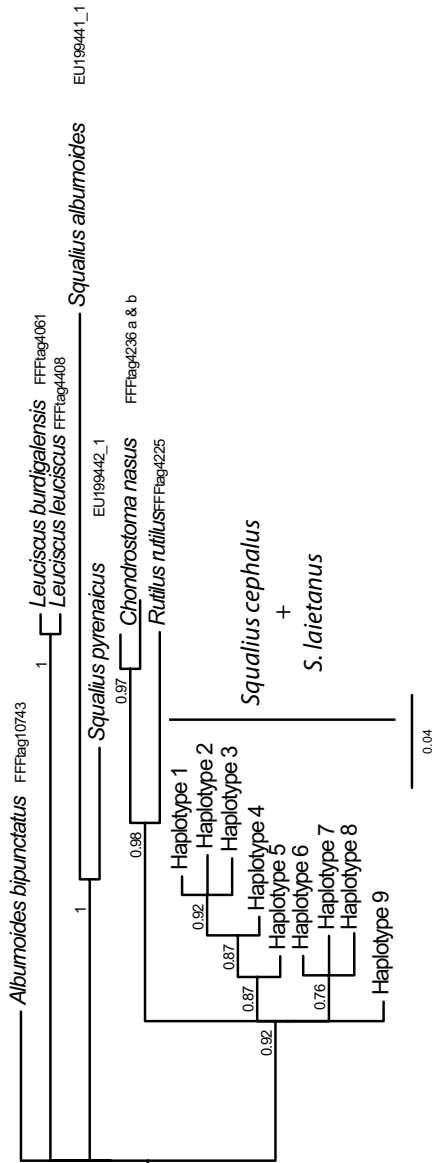
Species	Catalog number	BOLD	GenBank Accession Number		Stream (Basin)	Town	Country
			COI	Rhodopsin			
<i>S. orpheus</i>		AUTH08-227	HQ600791		Kerkini lake		Greece
<i>S. prespensis</i>		AUTH10-290	HQ600792		Prespa (Mikri)		Greece
<i>S. vardarensis</i>		AUTH06-56	HQ600797		Megalo rema (Doirani lake)		Greece
<i>Alburnoides bipunctatus</i>	MNHN 2011-821	FFFtag10743	KC354963	KC355030	Allier (Loire)	Saint-Haon	France
<i>Chondrostoma nasus</i>	MNHN 2010-1054	FFFtag4236	KC354964	KC355031	Aurence (Loire)	Aixe-sur-Vienne	France
<i>Leuciscus burdigalensis</i>	MNHN 2010-1830	FFFtag4061	KC354965	KC355032	Agly	Latour-de-France	France
<i>Leuciscus leuciscus</i>	MNHN 2011-396	FFFtag4408	KC354966	KC355033	Seine	Nod-Sur-Seine	France
<i>Rutilus rutilus</i>	MNHN 2010-1028	FFFtag4225	KC354967	KC355034	Luy (Adour)	Saunac et Cambran	France

Appendix 2. Bayesian tree of the nuclear rhodopsin retrogene haplotypes of french *Squalius cephalus* + *S. laietanus*. The tree is a majority consensus of 4 independent analyses run on 10 million generations with sampling every 500 generations, with a GTR + I model as selected by jModeltest 2.1.1 (Darriba et al., 2012). 10% of the trees were discarded as burnin after checking convergence had been reached. Numbers on the nodes represent posterior probabilities. a posteriori values of the parameters are: $TL = 2.200311$

$r(A \leftrightarrow C) = 0.002389$ $r(A \leftrightarrow G) = 0.896940$ $r(A \leftrightarrow T) = 0.023308$

$r(C \leftrightarrow G) = 0.006064$ $r(C \leftrightarrow T) = 0.064756$ $r(G \leftrightarrow T) = 0.006543$

$pi(A) = 0.198809$ $pi(C) = 0.284553$ $pi(G) = 0.294480$ $pi(T) = 0.222158$ $pinvar = 0.743371$.



Appendix 3.

Taxon	BOLD identifier	GenBank Accession number	Stream (Basin)	Number of heterozygotic positions	Squalius cephalus + S. laietanus haplotypes														
					H1	H2	H3	H4	H5	H6	H7	H8	H9						
<i>S. cephalus</i>	FFFtag4229*	KC355062	Luy (Adour)	0n															
<i>S. cephalus</i>	FFFtag4230	KC355043	Luy (Adour)	0n	2														
<i>S. cephalus</i>	FFFtag10724	KC355038	Agly	0n															
<i>S. cephalus</i>	FFFtag10725	KC355050	Agly	0n															
<i>S. cephalus</i>	FFFtag10726	KC355057	Agly	0n															
<i>S. cephalus</i>	FFFtag10727	KC355065	Agly	0n															
<i>S. cephalus</i>	FFFtag4052	KC355060	Agly	0n															
<i>S. cephalus</i>	FFFtag10707	KC355037	Bouizane (Agly)	0n															
<i>S. cephalus</i>	FFFtag10708	KC355052	Bouizane (Agly)	0n															
<i>S. cephalus</i>	FFFtag10709	KC355040	Bouizane (Agly)	3n				1											
<i>S. cephalus</i>	FFFtag10710	KC355069	Bouizane (Agly)	3n				1											
<i>S. cephalus</i>	FFFtag10711	KC355048	Bouizane (Agly)	0n															
<i>S. cephalus</i>	FFFtag10712	KC355054	Bouizane (Agly)	3n				1											
<i>S. cephalus</i>	FFFtag10716	KC355056	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag10717	KC355039	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag10718	KC355067	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag10719	KC355042	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag10720	KC355053	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag10721	KC355049	Desix (Agly)	2n						1									
<i>S. cephalus</i>	FFFtag4058	KC355046	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag4105	KC355045	Desix (Agly)	0n															
<i>S. cephalus</i>	FFFtag4047	KC355068	Douctouyre (Garonne)	4n	1														
<i>S. cephalus</i>	FFFtag4080	KC355059	Tarnon (Garonne)	2n		1													1
<i>S. cephalus</i>	FFFtag4081*	KC355064	Tarnon (Garonne)	0n	2														
<i>S. cephalus</i>	FFFtag10740	KC355058	Allier (Loire)	5n			1												
<i>S. cephalus</i>	FFFtag4228*	KC355051	Aurence (Loire)	0n															
<i>S. cephalus</i>	FFFtag4121*	KC355044	Massane	0n															
<i>S. cephalus</i>	FFFtag10715*	KC355061	Le Sasse (Rhône)	0n															

