ORIGINAL ARTICLE

WILEY

Genetic and morphological discrimination of three species of ninespined stickleback *Pungitius* spp. (Teleostei, Gasterosteidae) in France with the revalidation of *Pungitius vulgaris* (Mauduyt, 1848)

Gaël P. J. Denys[1] | Henri Persat[2] | Agnès Dettai[3] | Matthias F. Geiger[4] |
Jörg Freyhof[5] | Justine Fesquet[1] | Philippe Keith[1]

[1]Unité Biologie des organismes et écosystèmes aquatiques (BOREA, UMR 7208), Sorbonne Universités, Muséum national d'Histoire naturelle, Université Pierre et Marie Curie, Université de Caen Basse-Normandie, CNRS, IRD, Paris, France

[2]Écologie des Hydrosystèmes Naturels et Anthropisés, LEHNA UMR 5023, Bat. Forel, Université Claude Bernard Lyon 1, Villeurbanne Cedex, France

[3]Institut de Systématique, Evolution, Biodiversité, ISYEB – UMR 7205 – CNRS, MNHN, UPMC, EPHE, Muséum national d'Histoire naturelle, Sorbonne Universités, Paris, France

[4]Zoologisches Forschungsmuseum Alexander Koenig, Leibniz Institute for Animal Biodiversity, Bonn, Germany

[5]Leibniz Institute of Freshwater Ecology and Inland Fisheries, Berlin, Germany

Correspondence
Gaël Denys
Email: gael.denys@mnhn.fr

Contributing authors: Henri Persat (Henri. Persat@univ-lyon1.fr); Agnès Dettai (agnes. dettai@mnhn.fr); Matthias Geiger (m.geiger@zfmk.de); Jörg Freyhof (j.freyhof@igb-berlin.de); Justine Fesquet (fesquetjustine@gmail.com); Philippe Keith (philippe.keith@mnhn.fr)

Abstract

The taxonomy of French ninespined sticklebacks (*Pungitius* spp.) has long been controversial. To clarify the taxonomy in this group, we use mitochondrial (*COI*) and nuclear (*RNF213*) sequence markers, as well as morphological data. In France, both genetic markers discriminate three evolutionary lineages. Morphological analysis on fresh and type specimens supports the different lineages and the existence of three species in France. *Pungitius pungitius*, occurring in the North of France and Rhone basin, is characterized by specimens longer than 35 mm SL, by a flat head with a straight or slightly concave snout, typically 9–10 dorsal spines, 10–11 dorsal soft rays, 9–10 anal soft rays, 0–12 scutes on the caudal peduncle with a keel reaching the last anal-fin ray, longer pelvic fin, post-dorsal and caudal peduncle lengths, and a slim caudal peduncle (caudal peduncle depth/length 11.8%–21.9%). *Pungitius laevis*, occurring in France, in the English Channel basins and Loire drainage, differs from the other species by a head rounded with concave snout in specimens longer than 35 mm SL, accentuating the impression of fleshy lips, 0–4 scutes on the caudal peduncle and a higher caudal peduncle depth/length ratio (15.7%–34.5%). Finally, *Pungitius vulgaris*, endemic to the Vienne River and rivers of south-western France as far north as the Garonne estuary, is differentiated by a rounded head with a straight or slightly convex snout, the absence of scutes on the caudal peduncle and by having 11 pectoral-fin rays. Our data confirm the existence of a hybridization zone in the North of France between *P. pungitius* and *P. laevis*. As a result, *Pungitius lotharingus* is invalid, as it was described based on hybrid specimens. A lectotype for *P. laevis* was designated because the syntypes included hybrids. This revision provides new perspectives for evolutionary biology studies and will have consequences for *Pungitius* conservation in France.

KEYWORDS
France, integrative taxonomy, mitochondrial DNA *COI*, *Pungitius*, *RNF213*

1 | INTRODUCTION

The ninespined stickleback *Pungitius pungitius* (Linnaeus, 1758) (Actinopterygii, Gasterosteidae) is a model organism for evolutionary biology, genetics, and behavioral research, almost as much as the three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 (Merilä, 2013). It has an almost complete circumpolar distribution, and lives in freshwater streams and ponds, but can also tolerate marine salinity (Bănărescu & Paepke, 2002; Kottelat & Freyhof, 2007).

Historically, *P. pungitius* has been repeatedly described as a gasterosteid having 8–11 spines in front of the dorsal-fin, and a slim caudal peduncle adorned with modified scales forming a keel on each side (eg, Blanchard, 1866; Cuvier, 1829; Mauduyt, 1848). Several morphological species were described from France. First Cuvier (1829) distinguished specimens with no lateral keel on the caudal peduncle and described *Gasterosteus laevis*. Mauduyt (1848), in a publication little noticed for more than two centuries, also distinguished two forms of ninespined sticklebacks in the Vienne basin (West of France), based on the same character, and named the keelless form *Gasterosteus vulgaris*. Blanchard (1866) examined other criteria such as the pelvic girdle, the snout, and the opercles and described three other species: a species with a keel on the caudal peduncle from Burgundy, *Gasterosteus burgundianus*, and two keelless species, one from Normandy, *Gasterosteus breviceps*, and one from the Meuse basin, *Gasterosteus lotharingus*.

The taxonomy of this group has often been discussed (Table 1). The ninespined sticklebacks are not placed in the genus *Gasterosteus* anymore, but into *Pungitius* Coste, 1848. *Pygosteus* Gill, 1861, a third genus, was also used but is now a junior synonym of *Pungitius*. This taxonomical change of genus names is supported by morphological (Keivany & Nelson, 2004; Mattern & McLennan, 2004; McLennan & Mattern, 2001), molecular genetic (Kawahara, Miya, Mabuchi, Near, & Nishida, 2009; Mattern, 2004; Mattern & McLennan, 2004) and behavioral data (Coste, 1848; Mattern & McLennan, 2004). In any case, most of the debate is focused on the validity of Cuvier and Blanchard's taxa. Some authors recognized only one species in France, *P. pungitius* (eg, Bănărescu & Paepke, 2002; Spillmann, 1961), either with different ecotypes (Moreau, 1881) or sometimes separated into two subspecies *P. pungitius pungitius* and *P. pungitius laevis* (Gross, 1979; Hureau & Monod, 1973; Keivany & Nelson, 2000; Münzing, 1969; Reshetnikov et al., 1997; Wootton, 1984). Other authors accepted Cuvier and Blanchard's species as valid (Bertin, 1925; Sauvage, 1874) or considered the presence/absence of a keel on the caudal peduncle a valid taxonomic criterion to discriminate the two species *Pungitius pungitius* and *Pungitius laevis* (Berg, 1949; Kottelat, 1997; Kottelat & Freyhof, 2007). Münzing (1969) noticed a cline variation between these two taxa and identified a relation with geographical separation: Populations of *P. pungitius laevis* with no keel on the caudal peduncle are restricted to the South of France and the North of the United Kingdom, whereas populations with a keel (*P. pungitius pungitius*) are present in the North of Europe. Kottelat (1997) in his taxonomical review commented the study of Münzing (1969). He noticed an area in

TABLE 1 Evolution of the taxonomical points of view of the French nine-spined sticklebacks *Pungitius* spp. since Sauvage (1874)

	Gasterosteus pungitius Linnaeus 1758	*Gasterosteus burgundianus* Blanchard 1866	*Gasterosteus laevis* Cuvier 1829	*Gasterosteus breviceps* Blanchard 1866	*Gasterosteus lotharingus* Blanchard 1866	*Gasterosteus vulgaris* Mauduyt 1848
Sauvage (1874)	*Gasterosteus (Gasterostea) pungitia*	*Gasterosteus (Gasterostea) burgundiana*	*Gasterosteus (Gasterostea) laevis*	*Gasterosteus (Gasterostea) breviceps*	*Gasterosteus (Gasterostea) lotharinga*	Unknown taxon
Moreau (1881)	*Gasterosteus (Gasterostea) pungitia*	*Gasterosteus (Gasterostea) pungitia var. burgundianus*	*Gasterosteus (Gasterostea) pungitia var. laevis*	*Gasterosteus (Gasterostea) pungitia var. breviceps*	*Gasterosteus (Gasterostea) pungitia var. lotharingus*	Unknown taxon
Bertin (1925)	*Pygosteus pungitius*	*Pygosteus burgundianus*	*Pygosteus laevis*	*Pygosteus breviceps*	*Pygosteus lotharingus*	Unknown taxon
Spillmann (1961)	*Pygosteus pungitius*					Unknown taxon
Münzing (1969)	*Pungitius pungitius pungitius*	*Pungitius pungitius laevis*				Unknown taxon
Hureau and Monod (1973)	*Pungitius pungitius pungitius*		*Pungitius pungitius laevis*			Unknown taxon
Kottelat (1997)	*Pungitius pungitius*	*Pungitius laevis*	*Pungitius pungitius laevis*			
Keivany and Nelson (2000)	*Pungitius pungitius pungitius*	*Pungitius pungitius laevis*	*Pungitius pungitius laevis*			
Bănărescu and Paepke (2002)	*Pungitius pungitius*					
This study	*Pungitius pungitius*		*Pungitius laevis*			*Pungitius vulgaris*

northern France and the Netherland where populations include a mix of individuals with and without keel. He considered this area to be a hybridization zone, and, following the Phylogenetic Species Concept sensu Cracraft (1983), recognized two distinct species: *P. pungitius* occurring in Germany to Eastern Europe and *P. laevis* distributed in Western Europe (Kottelat & Freyhof, 2007). Thus, according to this study, *P. laevis* was the only species occurring in France (Keith, Persat, Feunteun, & Allardi, 2011). Recent genetic studies on mitochondrial DNA revealed three distinct lineages of "*Pungitius laevis*" in France, separated from each other most likely since the Pleistocene, and not forming a monophyletic group (Guo, Shikano, Wang, & Merilä, 2016; Wang, Shikano, Persat, & Merilä, 2015, 2017). The three lineages represent populations from Loire (lineage I), Charente-Dordogne (lineage II), and Seine + Meuse + Rhone (lineage III) basins. The high level of divergence between the first two already indicated that they might represent two distinct species (Wang et al., 2015). The third lineage was included within *P. pungitius* from Western Europe, casting doubts on the caudal peduncle keel as a valuable taxonomic character (Wang et al., 2015).

Synonymies add to the already confused situation. *G. vulgaris*, *G. breviceps*, and *G. lotharingus* are considered synonyms of *Pungitius pungitius laevis* (Table 1). Hureau and Monod (1973) relied on the presence of a keel as a major character, whereas other authors took geography into consideration (Keivany & Nelson, 2000; Kottelat, 1997; Münzing, 1969).

The principle of "integrative taxonomy" is to combine findings from the evaluation of several independent characters (morphological, genetic, ecological, biogeographical, etc., eg, Dayrat, 2005; Padial, Miralles, De La Riva, & Vences, 2010; Schlick-Steiner et al., 2010), and constitutes the best practice of modern taxonomy (Padial et al., 2010; Teletchea, 2010).

The aim of this study was to perform an integrative taxonomical review of French ninespined sticklebacks using molecular genetics and morphological data. We analyze DNA sequences of the partial mitochondrial gene coding for *cytochrome C oxidase subunit 1* (*COI*), the reference marker for vertebrate DNA Barcoding (sensu Hebert, Cywinska, Ball, & deWaard, 2003) and use species delineation tools (see Hubert & Hanner, 2015; Teletchea, 2010) in combination with the nuclear *RNF213* marker (Li et al., 2009) to evaluate mitochondrial lineages and to provide an additional source of characters (Vogler & Monaghan, 2007). The cyto-nuclear comparison is especially important as mitochondrial DNA is maternally inherited, and it allows to highlight hybridization and introgression already described among *Pungitius* species (Takahashi & Takata, 2000; Takahashi et al., 2016; Wang et al., 2017; Ziuganov & Gomeluk, 1985). The morphological analysis also assessed specifically whether the morphological characters supported the groups delineated by the DNA sequences analyses.

2 | MATERIALS AND METHODS

2.1 | Sampling

Samples were collected between 2009 and 2013 with the collaborations of the ONEMA, and the FPPMAs of Charente, Cher, Nord,

Pas-de-Calais, and Saone-et-Loire. Specimens were fixed and preserved in 95% EtOH for molecular genetic analysis. In most cases, we managed to lower body shriveling using progressive concentration of EtOH in a few hours. A total of 133 specimens caught mainly by electrofishing in 45 French locations were sequenced for the *COI* and *RNF213* partial genes (Figure 1; Tables 2 and S1). Among the 45 French locations, nine are in common with the study of Wang et al. (2017) (Tables 2 and S1) although different specimens were used. Forty-one additional sequences from other European drainages (FREDIE program; http://www.fredie.eu) and GenBank were included in the analysis (Figure 1; Table 2). Four other gasterosteid species were used as outgroups.

The following type specimens of the described species mentioned by Kottelat (1997) were examined: the syntypes of *Gasterosteus pungitius* (LSL 34–35), the syntypes of *G. laevis* (MNHN 0000-7102 and 0000-7103), the holotype of *G. breviceps* (MNHN 0000-7110), the syntypes of *G. burgundianus* (MNHN 0000-7109), and the syntypes of *G. lotharingus* (MNHN 0000-7090).

2.2 | Species delimitation

The type specimens were collected during the 18th and 19th centuries and are too old to be included in molecular genetic analyses to evaluate to which contemporary clade they would belong to (Jörger & Schrödl, 2013; Kullander, 1999; Schlick-Steiner et al., 2007). The Evolutionary Species Concept (ESC) sensu Wiley and Mayden (2000) was selected to link morphology with DNA sequences data as well as biogeography (Kottelat & Freyhof, 2007; Wiley & Mayden, 2000). We delimit evolutionary lineages as those where the results of the mitochondrial *COI* and nuclear *RNF213* are congruent. Once the lineages were identified, homozygous specimens and type specimens were used for the morphological study. Type specimens were assigned a priori to an evolutionary lineage depending on their origin: *G. pungitius* (Baltic area), *G. laevis* (Seine around Paris and Somme basins), *G. breviceps* (Orne basin in Normandy), *G. burgundianus* (Rhone basin in Burgundy), and *G. lotharingus* (Meuse basin) (Figure 1). The morphological characters identified for each evolutionary lineage were used for the diagnosis.

2.3 | Molecular genetic studies

One hundred and seventy-eight specimens were included in the DNA analyses. For each specimen (Table 2), a small piece of fin was stored in 95% ethanol at 3°C. DNA extraction was performed on an EpMotion Robot using MN Biomedical extraction kits, according to the manufacturer's protocols. DNA amplification was performed by PCR in a final 20 μl volume containing 5% DMSO, 1 μl of dNTP 6.6 mmol/L, 0.15 μl of Qiagen Taq DNA polymerase, using 2 μl of the buffer provided by the manufacturer, and 0.4 μl of each of the two primers at 10 pmol/L; 2.5–10 μl of DNA extract was added. The different primers used are as follows: for *COI* TelF1 5′-TCG ACT AAT CAY AAA GAY ATY GGC AC-3′, TelR1 5′-ACT TCT GGG TGN CCA AAR AAT CAR AA-3′ (Dettai et al., 2011); *RNF213* (984 bp)
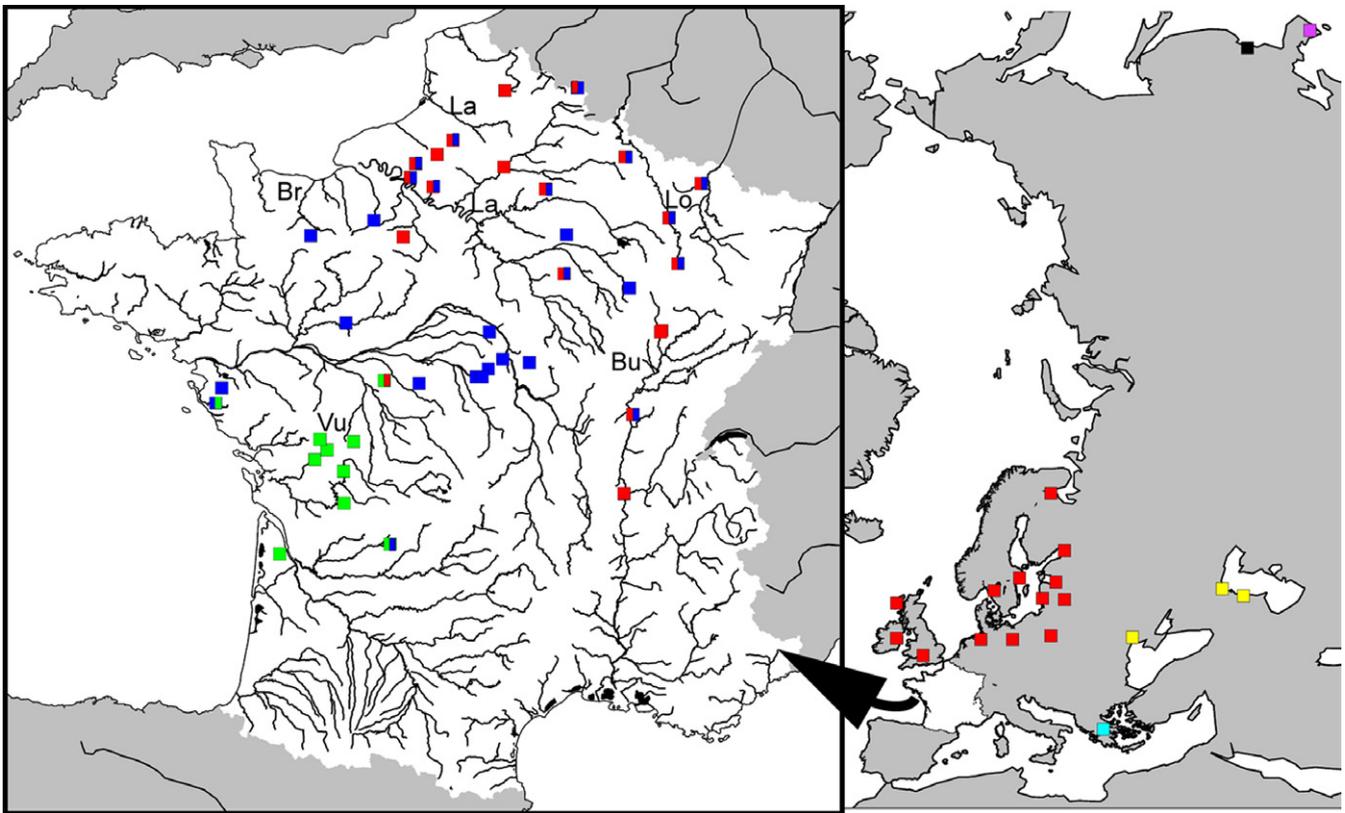
FIGURE 1 Sampling sites of the different species of *Pungitius* in France (map in the left) and in Eurasia (map in the right); see Table S1 for location details. Different color square represent the different clades or species identified with mitochondrial and nuclear markers: *Pungitius pungitius* (North-European clade; red), *Pungitius laevis* (North-Western France clade; dark blue), *Pungitius vulgaris* (South-Western France clade; green), *Pungitius hellenicus* (light blue), *Pungitius platygaster* (yellow), *Pungitius kaibarae* (purple), *Pungitius* sp. (black). Squares with two colors show the presence of hybrids. Type localities of *Gasterosteus breviceps* (Br), *Gasterosteus burgundianus* (Bu), *Gasterosteus laevis* (La), *Gasterosteus lotharingus* (Lo), and *Gasterosteus vulgaris* (Vu) are positioned.

C17 F3111 5′-GCT GAC TGG ATT YAA AAC CTT-3′, C17 R4111 5′-AAC TGT CCA AAR TCC CAC AC-3′ (Li et al., 2009). After denaturation for 2 min, the PCR was run for 60 cycles of (30 s, 94°C; 30 s, 50°C; 1 min to 1 min 30 s, 72°C) on a Biorad applied 2700 cycler. For European samples, DNA extraction, PCR, sequencing, and quality control of the *COI* marker were performed according to Geiger et al. (2014).

The French specimens were sequenced for nuclear *RNF213* marker following the PCR and two-level multiplexing protocols following Hinsinger et al. (2015). Sequencing was performed with the Ion Torrent PGM platform. Assembling and annotations were executed following the approach of Hahn, Bachmann, and Chevreux (2013) with the Geneious version 9.0.5 software (Kearse et al., 2012) using as bait the *RNF213* sequence of *Gasterosteus aculeatus* extracted from the Ensembl genome database (Yates et al., 2016). The other sequences were obtained by Sanger sequencing by Macrogen. Heterozygous sequences of *RNF213* were separated into the two alleles with SEQPHASE (Flot, 2010) and PHASE (Stephens, Smith, & Donnelly, 2001) using as guides homozygote sequences (eg, Denys et al., 2013) from the same drainage.

All new sequences with their voucher information were deposited into GenBank and the Barcode of Life database (BOLD, www.boldsystems.org; Ratnasingham & Hebert, 2007) in the FREPT project

folder. Already published sequences for these species were also integrated in the dataset.

Alignments were performed using Muscle (Edgar, 2004). Phylogenetic analyses were performed with Bayesian inference (MrBayes 3.2, Ronquist et al., 2012), with the GTR+G and HKY+I model selected by jModelTest 2.1.1 (Darriba, Taboada, Dallo, & Posada, 2012) for, respectively, the *COI* and *RNF213*. Two runs of two analyses with 10 million generations and sampling every 200 generations were performed, and 10% of trees were eliminated as burnin after checking for convergence. Intra- and interspecific distances (p-distances) were calculated with the software MEGA 7 (Kumar, Stecher, & Tamura, 2016).

2.4 | Morpho-meristic analysis

The homozygous individuals for the nuclear marker were included in the species descriptions. Counts and measurements were taken from the left side following Kottelat and Freyhof (2007) (Figure 2). We measured the distance between the origin of the second dorsal-fin and the origin of the anal-fin as body depth, because some females were gravid and some type specimens have their morphology so affected that a measurement at the origin of the pelvic fin will introduce a bias. Measurements were taken using an electronic caliper

TABLE 2 Sampling sites and GenBank accession numbers for *COI* and *RNF213* sequences; respectively, 133 and 127 sequences

Basin	Stream	Collection ID	Sample ID	GenBank Accession Number	
				COI	*RNF213*
Charente (France)	Boutonne	MNHN 2014-0007	FFFtag12306	MF123461[a]	
			FFFtag12307	MF123557[a]	
	Lien	MNHN 2013-1308	FFFtag12303	MF123523[a]	
			FFFtag12304	MF123531[a]	
	Touvre[b]	UCBLZ 2012.9.462	EPTK1	MF123488[a]	MF123606[a]
			EPTK2	MF123528[a]	MF123655[a]
			EPTK3	MF123491[a]	MF123609[a]
			EPTK5	MF123509[a]	MF123626[a]
			EPTK6	MF123499[a]	MF123618[a]
			EPTK7		MF123641[a]
			EPTK8		MF123603[a]
Dordogne (France)	Blâme[b]	UCBLZ 2012.9.459	EPTP1		MF123639[a]
			EPTP4		MF123690[a]
			EPTP5		MF123670[a]
			EPTP6		MF123684[a]
			EPTP7		MF123697[a]
			EPTP8		MF123662[a]
			EPTP9		MF123665[a]
	Soue	UCBLZ 2012.9.461	EPTL1	MF123508[a]	MF123625[a]
			EPTL13		MF123656[a]
			EPTL14		MF123585[a]
			EPTL2	MF123457[a]	MF123568[a]
			EPTL4	MF123527[a]	MF123653[a]
			EPTL5	MF123511[a]	MF123630[a]
			EPTL6	MF123479[a]	MF123591[a]
			EPTL7	MF123460[a]	MF123572[a]
			EPTL8		MF123634[a]
Falleron (France)		MNHN 2013-805	FFFtag10986	MF123455[a]	MF123564[a]
			FFFtag10987	MF169498[a]	MF123642[a]
			FFFtag10988		MF123567[a]
Jalle de l'horte (France)		MNHN 2013-803	FFFtag10983	MF169497[a]	MF123632[a]
			FFFtag10984		MF123607[a]
Loire (France)	Belle[b]	UCBLZ 2012.9.465	EPTJ12	MF123482[a]	MF123593[a]
			EPTJ13	MF123497[a]	MF123616[a]
			EPTJ7		MF123601[a]
			EPTJ8		MF123700[a]
	Esves	MNHN 2013-1305	FFFtag12297	MF123558[a]	
			FFFtag12298	MF123484[a]	MF123596[a]
	Fontaine d'enfer[b]	UCBLZ 2012.9.481	EPTQ1		MF123647[a]
			EPTQ2		MF123693[a]
			EPTQ3		MF123651[a]
			EPTQ5		MF123566[a]
			EPTQ7		MF123677[a]
			EPTQ8	MF123543[a]	MF123676[a]
	Nahon	MNHN 2013-1293	FFFtag12274	MF123471[a]	MF123580[a]

(Continues)

TABLE 2 (Continued)

Basin	Stream	Collection ID	Sample ID	GenBank Accession Number COI	GenBank Accession Number RNF213
	Nièvre	MNHN 2013-814	FFFtag12208	MF123514[a]	MF123635[a]
			FFFtag12210	MF123545[a]	MF123679[a]
	Notre-heure	MNHN 2013-801	FFFtag10976	MF123560[a]	MF123698[a]
			FFFtag10977	MF123556[a]	MF123696[a]
	Ouatier	UCBLZ 2012.9.467	EPTO2		MF123590[a]
	Planche-Godard	MNHN 2013-802	FFFtag10979	MF123503[a]	MF123621[a]
			FFFtag10980	MF123470[a]	MF123579[a]
			FFFtag10981		MF123681[a]
	Rampenne	UCBLZ 2012.9.475	EPTI1		MF123680[a]
			EPTI10	MF123505[a]	MF123622[a]
			EPTI3	MF123547[a]	MF123683[a]
			EPTI5		MF123633[a]
			EPTI6		MF123586[a]
			EPTI8		MF123654[a]
			EPTI9	MF123540[a]	MF123673[a]
	Yévrette	MNHN 2013-819	FFFtag12219	MF123538[a]	MF123671[a]
			FFFtag12220	MF123517[a]	MF123643[a]
			FFFtag12221	MF123462[a]	
Meuse (France)	Bar[b]	UCBLZ 2012.9.456	EPTE1	MF123495[a]	MF123614[a]
			EPTE2	MF123456[a]	
			EPTE3	MF123489[a]	MF123608[a]
	Meuse[b]	–	EPT1	MF123478[a]	MF123589[a]
		UCBLZ 2012.9.468	EPTH1	MF123555[a]	
			EPTH2	MF123507[a]	
			EPTH3	MF123535[a]	MF123664[a]
Orne (France)	Rouvre	MNHN 2013-1297	FFFtag12282	MF123553[a]	MF123694[a]
Rhine (France)	Orne[b]	UCBLZ 2012.9.478	EPTN1		MF123623[a]
			EPTN10		MF123686[a]
			EPTN11		MF123640[a]
			EPTN13	MF123516[a]	MF123638[a]
			EPTN14		MF123604[a]
			EPTN15		MF123675[a]
			EPTN16		MF123574[a]
			EPTN2		MF123628[a]
			EPTN3		MF123599[a]
			EPTN5	MF123526[a]	MF123652[a]
			EPTN6		MF123605[a]
			EPTN7	MF123493[a]	MF123611[a]
			EPTN8	MF123476[a]	MF123588[a]
			EPTN9		MF123612[a]
			FFFEPID1	MF123472[a]	MF123581[a]
			FFFEPID2	MF123483[a]	MF123595[a]
			FFFEPID3	MF123494[a]	MF123613[a]

TABLE 2 (Continued)

Basin	Stream	Collection ID	Sample ID	GenBank Accession Number	
				COI	RNF213
Rhone (France)	Saône	–	EPTD1	MF123562[a]	MF123699[a]
			EPTD2	MF123465[a]	MF123576[a]
	Ruisseau de Belle-Fontaine	UCBLZ 2012.9.449	EPTM1	MF123539[a]	MF123672[a]
			EPTM2	MF123521[a]	MF123645[a]
			EPTM3	MF123492[a]	MF123610[a]
			EPTM4	MF123474[a]	MF123584[a]
			EPTM5	MF123468[a]	MF123578[a]
			EPTM6	MF123502[a]	MF123620[a]
			EPTM7	MF123473[a]	MF123583[a]
			EPTM8	MF123464[a]	MF123575[a]
		ZFMK:55646	Ex26F11	KJ554413	MF123600[a]
	Orain[b]	UCBLZ 2012.9.450	EPTC1	MF123559[a]	
			EPTC2	MF123551[a]	MF123688[a]
			EPTC3	MF123533[a]	MF123661[a]
		ZFMK:55648	Ex26G1	KJ554327	MF123692[a]
Scheldt (France)	Canal de la Scarpe	MNHN 2013-614	FFFtag16505	MF123548[a]	
	Trouille[b]	UCBLZ 2012.9.463	EPTR1		MF123669[a]
			EPTR2		MF123587[a]
			EPTR3	MF123501[a]	MF123619[a]
			EPTR4		MF123689[a]
Seine (France)	Ancre	MNHN 2013-1288	FFFtag12264	MF123541[a]	MF123674[a]
	Andelle	MNHN 2013-1298	FFFtag12283	MF123536[a]	MF123666[a]
			FFFtag12284	MF123512[a]	
	Aronde	MNHN 2013-1301	FFFtag12289	MF123458[a]	MF123569[a]
			FFFtag12290	MF123552[a]	MF123691[a]
	Aujon	MNHN 2013-1289	FFFtag12266	MF123522[a]	MF123646[a]
	Blaise	MNHN 2014-0011	FFFtag12315	MF123463[a]	
	Epte	MNHN 2011-1143	FFFtag10873	MF123466[a]	MF123577[a]
	Héron	MNHN 2013-1296	FFFtag12279	MF123480[a]	MF123592[a]
			FFFtag12280	MF123513[a]	MF123631[a]
	Ourcq	MNHN 2013-1302	FFFtag12291	MF123510[a]	MF123627[a]
			FFFtag12292	MF123563[a]	
	Risle	MNHN 2013-1295	FFFtag12277	MF123487[a]	
			FFFtag12278	MF123537[a]	MF123668[a]
	Superbe	MNHN 2013-1283	FFFtag12254	MF123506[a]	
	Thérain	MNHN 2013-1294	FFFtag12275	MF123519[a]	MF123644[a]
Sèvre Niortaise (France)	Chaboussant	MNHN 2014-0008	FFFtag12308	MF123546[a]	MF123682[a]
			FFFtag12309	MF123542[a]	
	Sèvre Niortaise	MNHN 2014-0009	FFFtag12310	MF123504[a]	
			FFFtag12311	MF123524[a]	
			FFFtag12312	MF123550[a]	MF123687[a]
Somme (France)	Evoissons	MNHN 2013-809	FFFtag10997	KR862862	
			FFFtag10998	MF123496[a]	MF123615[a]
			FFFtag10999	MF123486[a]	
Vie (France)	Ligneron	MNHN 2013-806	FFFtag10990	MF123532[a]	MF123660[a]

(Continues)

TABLE 2 (Continued)

Basin	Stream	Collection ID	Sample ID	GenBank Accession Number	
				COI	RNF213
Pungitius hellenicus Sperchios (Greece)		ZFMK:55958	Ex12B5	KJ554130	MF123571[a]
		ZFMK:55959	Ex12B6	KJ554514	
		ZFMK:55960	Ex12B7	KJ554255	MF123667[a]
Pungitius platygaster Bashly-chay (Russia)		ZFMK:ICH:TIS:NB77	Ex23D3	MF123469[a]	
		ZFMK:ICH:TIS:NB77	Ex23D4	MF123485[a]	
		ZFMK:ICH:TIS:NB77	Ex23D5	MF123454[a]	
Pungitius platygaster Kuma (Russia)		ZFMK:ICH:TIS:NB78	Ex23D7	MF123554[a]	
Pungitius platygaster South Bug (Ukraine)		ZIN U110	Ex73G10	KJ554195	MF123649[a]
		ZIN U111	Ex73G11	KJ554378	MF123597[a]
		ZIN U109	Ex73G9	KJ554199	
Pungitius pungitius Karelia (Russia)		ZFMK:53260	Ex66F10	MF123490[a]	
		ZFMK:53261	Ex66F11	MF123467[a]	
Pungitius pungitius Baltic (Russia)		ZFMK:ICH:TIS:20121-4	Ex80G2	MF123520[a]	
Pungitius pungitius Baltic (Latvia)		ZFMK:53791	Ex67G8	MF123525[a]	MF123650[a]
		ZFMK:53792	Ex67G9	MF123518[a]	
Pungitius pungitius Gauja (Latvia)		ZFMK:53895	Ex67H11	MF123459[a]	MF123570[a]
		ZFMK:53906	Ex68A5	MF123481[a]	
Pungitius pungitius Nemunas (Lithuania)		ZFMK:53760	Ex67G1	MF123515[a]	MF123637[a]
		ZFMK:53761	Ex67G2	MF123549[a]	
Pungitius pungitius Husbyan (Sweden)		NRM56751	NRM56751	KJ128594	
Pungitius pungitius Sikhail (Sweden)		NRM52610	NRM52610	KJ128593	
Pungitius pungitius Vistula (Poland)		ZFMK:68702	Ex63D6	MF123498[a]	MF123617[a]
		ZFMK:68701	Ex63D7	MF123544[a]	MF123678[a]
Pungitius pungitius Weser (Germany)		ZFMK:53568	Ex61WH21G5	KM287046	MF123659[a]
		ZFMK:53569	Ex61WH21G6	KM287048	
		ZFMK:53570	Ex61WH21G7		MF123629[a]
Pungitius pungitius Elbe (Germany)		ZFMK:53343	Ex56F12	KM287043	
		ZFMK:53344	Ex56G1	KM287044	MF123598[a]
		ZFMK:53345	Ex56G2	KM287045	MF123582[a]
Pungitius pungitius Suir (Ireland)		ZFMK:54899	Ex79F10	MF123534[a]	MF123663[a]
		ZFMK:54901	Ex79F12	MF123530[a]	MF123658[a]
		ZFMK:54902	Ex79G1	MF123529[a]	
Pungitius pungitius North Sea (United Kingdom)		ZFMK:55472	Ex70G7	MF123561[a]	
Pungitius pungitius Thames (United Kingdom)		ZFMK:53674	Ex67D2	MF123500[a]	
		ZFMK:53675	Ex67D3	MF123477[a]	
Pungitius kaibarae Amur (South Korea)				EU332749	
Pungitius sp. Tumen (Russia)		Hoz-75 (field number)	EEFF083	MF123475[a]	
Apeltes cuadratus				EU524443	
Gasterosteus aculeatus				JX517193	
Culaea inconstans				KR862765	
Spinachia spinachia			BPS1348	MF169496[a]	

[a]New sequences
[b]Common localities with the study of Wang et al. (2017). More details about locations are given in the Table S1
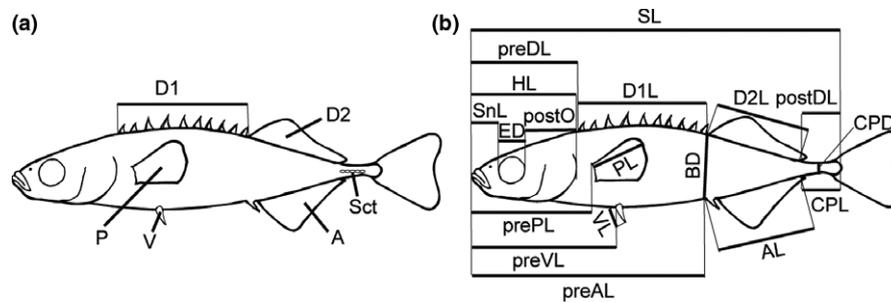
FIGURE 2 Counts (a) and measurements (b) for the morpho-meristic analysis following the method of Kottelat and Freyhof (2007). Characters counted in (a) were: *first Dorsal-fin spines* (D1), *second Dorsal-fin rays* (D2), *Pectoral-fin rays* (P), *Pelvic* (V) and *Anal-fins spines and rays* (A), *scutes forming the keel on the caudal peduncle* (Sct). Characters measured in (b) were: *Standard Length* (SL), *Head Length* (HL), *Snout Length* (SnL), *Eye Diameter* (ED), *post-Orbital Length* (post-O), *pre-Dorsal-fin Length* (pre-DL), *First Dorsal-fin base Length* (D1L), *Second Dorsal-fin base Length* (D2L), *post-Dorsal-fin Length* (post-DL), *pre-Pectoral-fin Length* (pre-PL), *pre-Pelvic fin Length* (pre-VL), *pre-Anal-fin Length* (pre-AL), *Anal-fin base Length* (AL), *Caudal Peduncle Length* (CPL), *Caudal Peduncle Depth* (CPD), *Body Depth* (BD; defined in this study as the distance between the origin of the second dorsal-fin and the origin of the anal-fin), *Pectoral-fin Length* (PL), *Pelvic fin Length* (VL). Note that *Inter-Orbital Width* (distance between the orbits from above the head; IOW) is not shown.

and are expressed to the nearest tenth of a millimeter. All measurements were made point to point, never by projection. The sex of each specimen was determined by observing the urogenital papillae, which are narrow for the males and wide for the females, in order to take into account the possible sexual dimorphism in the comparisons. Thus, 45 females and 35 males were determined within the specimens used for the morphological study as well as two undetermined juveniles. To consider allometry (Keivany & Nelson, 2000), only the individuals longer than 30 mm SL were measured. For smaller specimens, only counts were undertaken. Counts and measurements were also taken on the type specimens in order to check the geographic link to recently collected individuals with genetic information. For the syntypes of *P. pungitius* (LSL 34–35), only the skins are conserved on paper, so no morphometric measurements and counts were possible except the depth and the length of the caudal peduncle. The 72 individuals analyzed were all collected from small brooks, except two of them (ZFMK53791 and ZFMK53760), which, respectively, came from the Baltic Sea and a Lithuanian pond. A principal component analysis (PCA) was conducted in order to morphologically discriminate groups with the measurements. We used the R package (R Core Team 2016) and the ade4 package (Chessel, Dufour, & Thioulouse, 2004).

3 | RESULTS

3.1 | Phylogenetic analysis

The phylogenetic tree based on *COI* sequences (627 bp) of 133 individuals (Figure 3) groups all the examined *Pungitius* species into a monophyletic unit. The first dichotomy separates Asian taxa (*P. kaibarae* and *Pungitius* sp.) from the European species. Within these, we observe three clades. The first is a North-European clade with a mean intra-clade divergence of 0.52% in p-distance. For French populations, this clade is constituted by populations from the Baltic Sea and the North Sea, as well as Meuse, Moselle, Somme, Seine, and Rhone basins. A distinct Baltic subclade has a mean

divergence of 1.07% with the other drainages. This North-European clade is sister group, with a mean divergence of 2.08%, to a South-Western France clade, which has a mean intra-clade divergence of 0.39%. It includes specimens from Loire, Sèvre Niortaise, Charente, Dordogne basins, and Gironde estuary. Finally, we distinguish a separation with low support (ppv 0.61) between Central European taxa (*P. platygaster* and *P. hellenicus*) and a North-Western France clade, which has a mean intra-clade divergence of 0.22%, and which includes specimens from Scheldt, Seine, Orne, costal Atlantic, and Loire basins. The latter French cluster has a mean divergence of 3.89% and 4.42% to the North-European and the South-Western France clades, respectively. This group is composed of two subclades (Seine + Atlantic basins and Loire drainage) separated by 0.37% of divergence from the other drainages.

The phylogenetic tree based on the *RNF213* (937 bp) on 168 distinct alleles from 128 specimens (Figure 4) corroborates the results of the mitochondrial *COI* marker. It first separates *P. hellenicus* from the other taxa. Then a North-European group (A) is distinct from an Atlantic clade (B). The clade A separates *P. platygaster* from a North-European clade supported by 4 diagnostic sites (C vs. T in position 326, 689 and 707, and C vs. G in position 842; Table S2), and including six alleles from Rhone, Meuse, Seine, and Moselle catchments as well as individuals from Ireland, Lithuania, Poland, Germany, and Latvia (Table S3). The clade B displays no resolution except for a South-Western France clade distinct from the other specimens occurring in the North-West of France. The South-Western France clade supported by 1 diagnostic site (C vs. G in position 878; Table S2) includes six alleles from Loire, Charente, Dordogne basins, and Gironde estuary (Table S3). The North-Western France clade contains eight alleles from the Loire, Meuse, Seine, Orne, Scheldt, Moselle, and Falleron catchments (Table S3). They are supported by 1 diagnostic site (T vs. A in position 362; Table S2). Finally, three other haplotypes from the Somme, Loire, Dordogne, and Rhone catchments are not included into either clade because they share common sites with North- and South-Western France clades (Table S2 and 3).
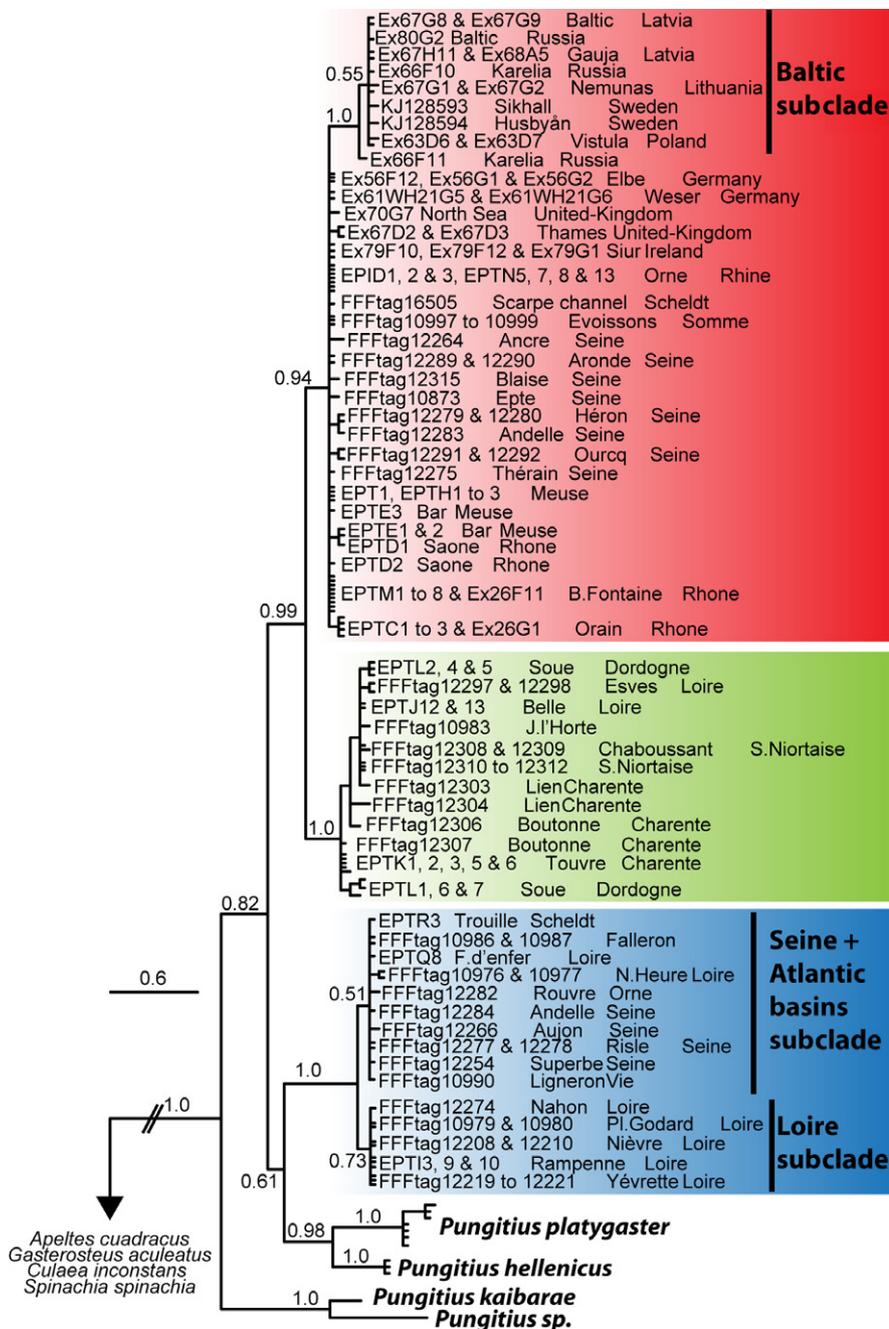
FIGURE 3 Bayesian tree of 627 bp of the cytochrome c oxidase subunit I (*COI*) for 133 individuals of European *Pungtius* spp. and other gasterosteids. The three main lineages are grouped in colored boxes: North-European clade or *Pungitius pungitius* (red), North-Western France clade or *Pungitius laevis* (blue) and South-Western France clade or *Pungitius vulgaris* (green). 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 = 26.091148; alpha = 0.031095, 77.327748, 1.194644.

Comparing the *COI* assignments to the *RNF213* groups, there are some disagreements for North-Western France specimens in the Meuse, Seine, Moselle, and Rhone basins, which are associated to the North-European clade. Similarly, the specimen from the Esves stream in the Loire catchment belongs to the South-Western France clade according to the *COI* marker, but is included among the North-European clade for RNF2013. Examining the *RNF213* alleles for each specimen, many specimens from the Scheldt, Meuse, Moselle, and Rhone drainages are heterozygous, with alleles from both North-European and North-Western France clades. The specimen FFFtag10990 from the Ligneron stream in the Vie basin is heterozygous for the North- and South-Western France clades.

3.2 | Morpho-meristic analysis

The seventy-two specimens both characterized with the *COI* marker and homozygous for the *RNF213* gene were included in the morpho-meristic analysis. Meristic characters were counted for all of them, and 56 specimens were measured. No groups are well separated because of the overlap in all of the characters. However, after grouping specimens by molecular genetic affiliation (*COI* and *RNF213* homozygous sequences), some of the morphological characters were discriminant in combination or between some group pairs. The PCA on the morphometric characters well discriminates two groups, the North-European clade on the one hand and a group formed by the North- and South-Western France clades on the
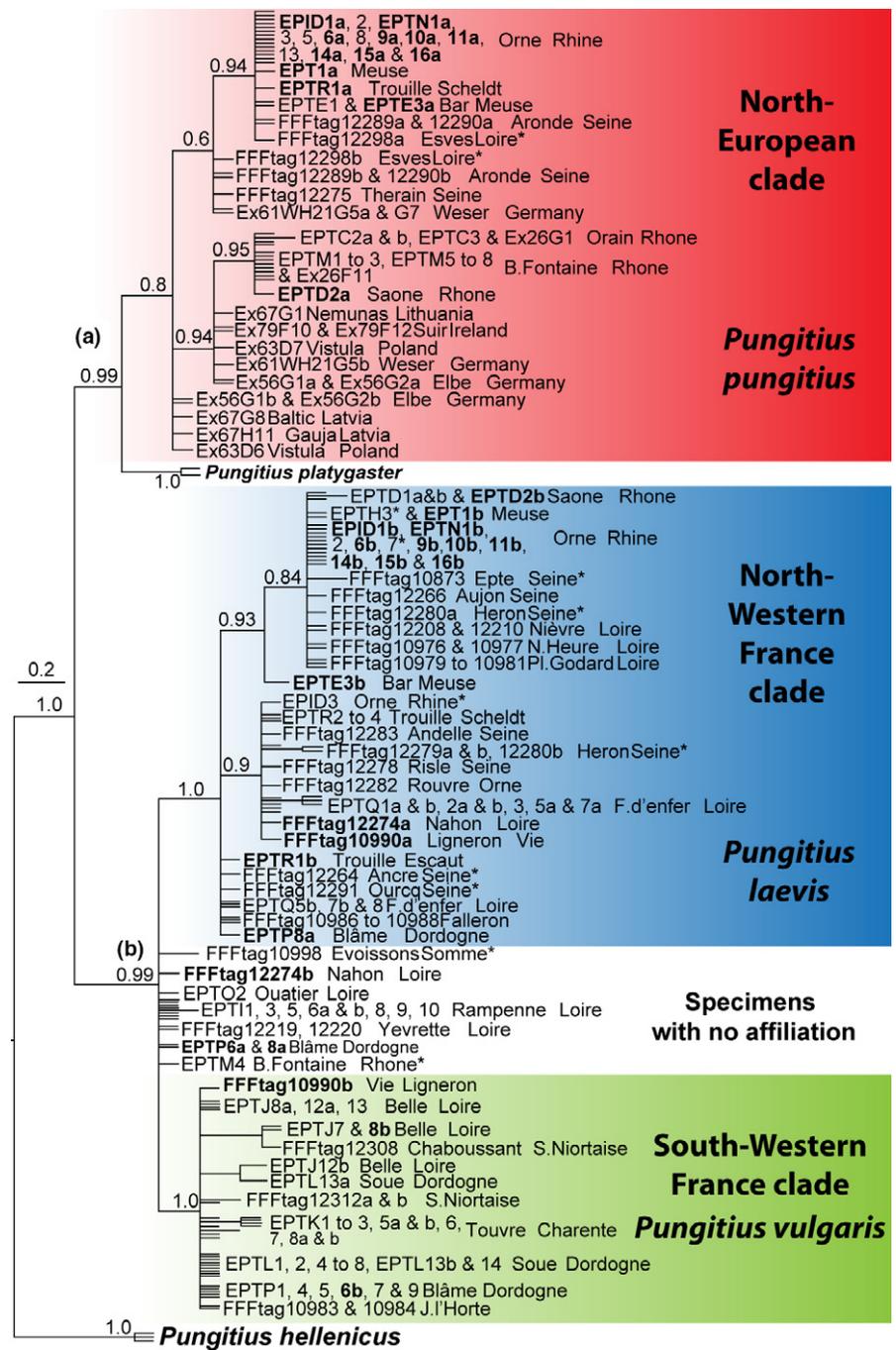
FIGURE 4 Bayesian tree of 937 pb of the *RNF213* marker for 168 alleles of European *Pungitius* spp. and other gasterosteids. Numbers on the nodes represent posterior probabilities. Asterisk point differences with the *COI* affiliation. Heterozygous specimens between clades are in bold. The mean a posteriori values of the parameters are: TL = 31.802139; kappa = 559.816359; pinvar = 0.912518.

other hand (Figure 5). The main contributory morphometric characters are the second dorsal-base length (D2L), the post-dorsal length (post-DL), the pre-pelvic length (pre-VL), the pre-anal length (pre-AL), the caudal peduncle length (CPL), the pelvic fin length (VL), the anal-base length (AL), the body depth (BD), the inter-orbital width (IOW), and the post-orbital length (post-O) (Table 3).

The specimens belonging to the North-European clade differ from those from the North- and South-Western France clades by: a flat head with a straight or slightly concave snout (Figure 6a) for specimens longer than 35 mm SL, typically 9–10 dorsal spines (vs. typically 8–9 for the other groups), typically 10–11 dorsal soft rays (vs. typically 9–10), typically 8–10 anal soft rays (vs. typically 8–9),

0–12 scutes on the caudal peduncle (vs. 0–4) (Table 4), so the keel reaches to just before the last anal-fin ray, a longer post-dorsal length (11.7%–20.6% of the standard length SL, vs. 10.6%–16.2% and 11.1%–15.6% for, respectively, the North and the South Atlantic clades), a shorter pre-anal length (51.1%–65.0% SL, vs. 56.4%–67.4% and 59.5%–65.6%), a longer caudal peduncle length (13.3%–19.9% SL, vs. 10.3%–17.8% and 12.7%–16.9%), longer pectoral-fin lengths (12.8%–16.8% SL, vs. 10.8%–16.6% and 11.6%–15.8%), longer pelvic fin lengths (7.3%–14.4% SL, vs. 6.3%–10.6% and 5.6%–9.6%), a slimmer body (12.5%–21.6% SL, vs. 16.5%–21.9% and 16.1%–19.6%), a slimmer caudal peduncle (2.2%–3.6% SL, vs. 2.6%–3.3% and 2.9%–4.2%), a smaller ratio caudal peduncle depth/length (11.8%–21.9%,
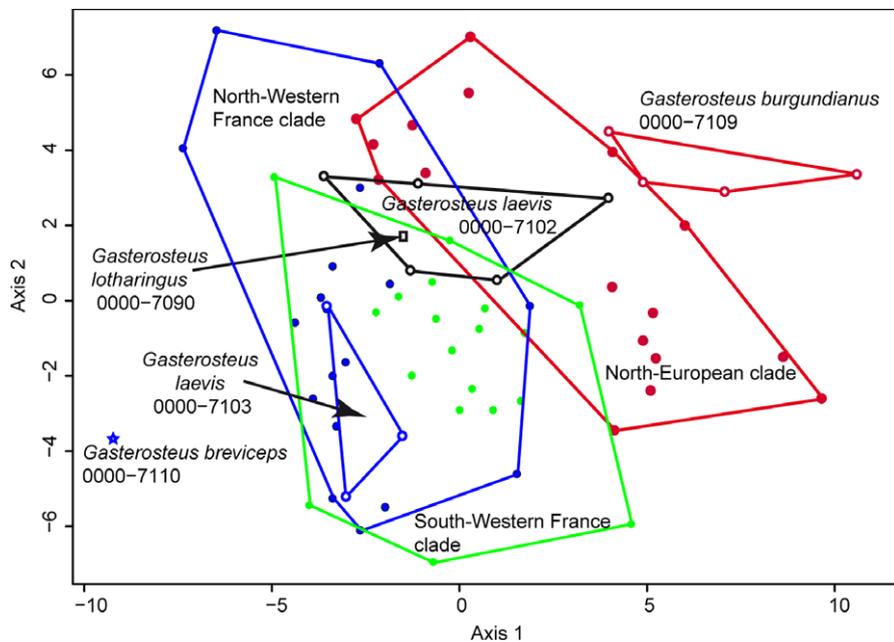
FIGURE 5 Principal Components Analysis with the morphometric measurements of the specimens belonging to the North-European (17 specimens; full red circles), North-Western (18 specimens; full blue circles), and the South-Western (19 specimens; full green circles) clades, as well as the type specimens of *Gasterosteus breviceps* MNHN 0000-7110 (empty blue star), *Gasterosteus burgundianus* MNHN 0000-7109 (empty red circles), *Gasterosteus laevis* MNHN 0000-7102 (empty black circles), MNHN 0000-7103 (empty blue circle), and *Gasterosteus lotharingus* MNHN 0000-7090 (empty black square). Colors correspond to the species delimited: *Pungitius pungitius* (red), *Pungitius laevis* (blue), *Pungitius vulgaris* (green) and hypothetic hybrids *Pungitius pungitius* × *P. laevis* (black).

TABLE 3 Character correlations with each of the two principal components; abbreviations for morphometric characters explained in the Figure 2

Character	PC1	PC2
HL	−0.067632284	0.1059240553
Pre-DL	−0.180068484	−0.0289276024
D1L	0.055411882	−0.2016198805
D2L	−0.375794913	0.2193094156
Post-DL	0.537590977	0.1803073710
Pre-PL	−0.060188217	0.0002747935
Pre-VL	−0.261624605	−0.1408310181
Pre-AL	−0.127429445	−0.5295903516
CPL	0.384744529	0.1035822823
PL	0.032833605	0.1174797047
VL	0.108819583	0.4300752237
AL	−0.264985338	0.4322109855
BD	−0.221987747	0.1291599280
CPD	−0.045149183	−0.0102913437
SnL	0.008657505	−0.1470186907
ED	0.093932701	0.0054482703
IOW	0.347233516	−0.0503176930
Post-O	−0.141227524	0.3365143336
% variance explained	49%	36%

vs. 15.7%–31.3% and 19.1%–27.2%), a longer snout (20.2%–27.6% of the head length HL, vs. 19.8%–26.3% and 20.2%–25.9%), and a longer post-orbital length (43.8%–53.6% HL, vs. 43.3%–50.9% and 41.9%–51.1%) (Table 5).

The fish from the North-Western France clade are characterized by a head rounded with a concave snout (Figure 6b) for specimens longer than 35 mm SL accentuating the impression of fleshy lips, 0–4 scutes on their caudal peduncle (vs. 0–12 and 0 for the North-

European and the South-Western France clades, respectively) (Table 4), as well as a longer first dorsal-fin base length (27.6–36.1% SL, vs. 28.4%–31.7% and 28.9%–34.3%), a longer second dorsal-fin base length (21.3%–29.3% SL, vs. 18.2%–27.4% and 20.5%–27.0%), a shorter post-dorsal length (10.6%–16.2% SL, vs. 11.7%–20.6% and 11.1%–15.6%), a longer pre-pelvic length (40.3%–46.1% SL, vs. 39.8%–43.9% and 38.6%–43.7%), a longer pre-anal length (56.4%–67.4% SL, vs. 51.1%–65.0% and 59.5%–65.6%), a shorter caudal peduncle length (10.3%–17.8% SL, vs. 13.3%–19.9% and 12.7%–16.9%), a longer anal-fin base length (19.6%–29.7% SL, vs. 19.7%–27.7% and 19.9%–26.8%), a higher ratio caudal peduncle depth/length (15.7%–31.3%, vs. 11.8%–21% and 19.1%–27.2%), and a higher inter-orbital width (12.8%–26.5% HL, vs. 15.6%–21.6% and 15.1%–20.0%) (Table 5).

Finally, the specimens from the South-Western France clade differ from the others by a head rounded with a straight or slightly convex snout (Figure 6c), typically 11 pectoral rays (vs. 10–11), no scute on the caudal peduncle (vs. 0–12 and 0–4 for, respectively, the North-European and the North-Western France clades) (Table 4), shorter pelvic fins (5.6%–9.6% SL, vs. 7.3%–14.4% and 6.3%–10.6%), and a deeper caudal peduncle (2.9%–4.2% SL, vs. 2.2%–3.6% and 2.6%–3.3%) (Table 5).

Using these results, we tried to determine to which clade the historical type specimens belong. However, as the long preservation in ethanol has affected their morphology through shrinkage, the shape of the head was not taken into account.

The syntypes of *Gasterosteus pungitius* Linnaeus, 1758 (left half-skins), LSL 34–35, possess a keel on their caudal peduncle, which reaches before the last anal-fin ray, and the ratio for the caudal peduncle depth/length is, respectively, 20.1 and 16.75%. The syntypes of *Gasterosteus burgundianus* Blanchard, 1866, MNHN 0000-7109, have 10 dorsal spines, 5–12 scutes on the caudal peduncle (Table 4), high post-dorsal length (16.0%–21.1% SL), as well as a high
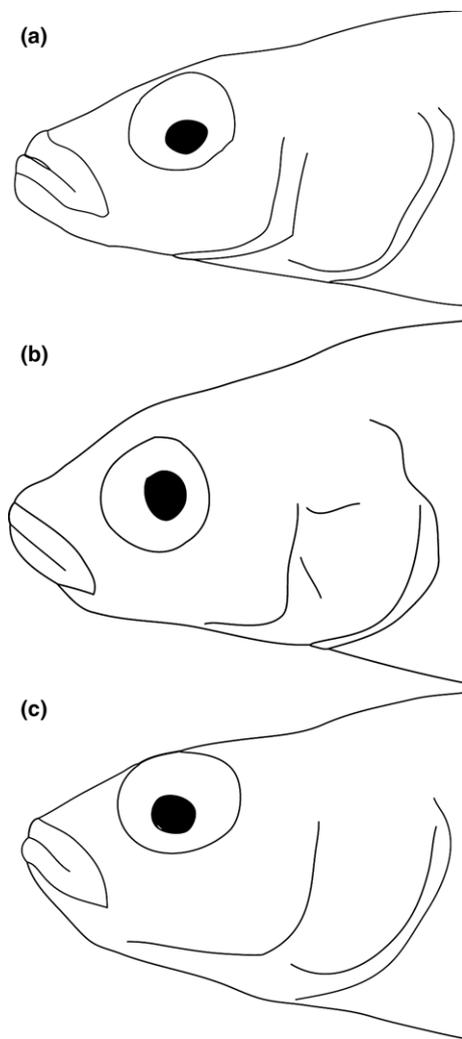
FIGURE 6 Head profile showing the difference of snouts: straight or slightly concave for the North-European clade *Pungitius pungitius* UCBLZ 2012.9.449 (EPTM1), 52.9 mm SL, Ruisseau de Belle-Fontaine (Rhone drainage) at Lyon, Persat, February 23, 2010 (a); concave for the North-Western France clade *Pungitius laevis* MNHN 2013-0814 (FFFtag12210), 39.73 mm SL, Nièvre (Loire drainage) at Dompierre-sur-Nièvre, Denys and ONEMA, June 27, 2013 (b); and straight or slightly convex with a profile rounded for the South-Western France clade *Pungitius vulgaris* UCBLZ 2012-9-459 (EPTP1), 39.25 mm SL, Blâme (Dordogne basin) at Brouchaud, Persat, May 13, 2009 (c)

caudal peduncle length (15.9%–20.0% SL), but a short depth (2.6%–2.7% SL), causing low ratios for the caudal peduncle depth/length (13.0%–17.2%), long pelvic spines (9.2%–13.8% SL), and high post-orbital lengths (46.5%–54.7% HL) characteristic of the North-European clade, despite the high first dorsal-fin base length (32.1%–36.1% SL) (Table 5). We therefore assign the syntypes of both taxa to the North-European clade.

The syntypes of *Gasterosteus laevis* Cuvier, 1829, MNHN 0000-7103, long first dorsal-fin base lengths (31.3%–32.7% SL), short post-dorsal-fin and pectoral-fin lengths (respectively, 9.2%–11.7% and 11.8%–15.8% SL) and high ratios for the caudal peduncle depth/length (18.5%–27.8%) (Table 5). Moreover, as these

specimens are sufficiently well preserved, we can observe a concave snout (Figure 6b). The holotype of *Gasterosteus breviceps* Blanchard, 1866, MNHN 0000-7109 has a high second dorsal-fin base length (28.2% SL), a high pre-pectoral-fin length (38.8% SL), a high pre-pelvic fin length (47.9% SL), and a low post-dorsal-fin and caudal peduncle length (respectively, 10.3 and 10.1% SL) (Table 5). Thus, these specimens best correspond to the North-Western France clade.

Concerning the other syntypes of *G. laevis*, MNHN 0000-7103, it is impossible for us to link their morphological characters unambiguously to any clade, because they exhibit characters corresponding to the North-European clade (ie, keel on the caudal peduncle, the pelvic fin length) and others matching with the North-Western France clade (ie, the ratio depth/length of the caudal peduncle). Similarly, the syntypes of *Gasterosteus lotharingus* Blanchard, 1866 MNHN 0000-7090 show no morphological characters that would permit to clearly place them in any group (see Tables 4 and 5).

Taken together, these results are further evidence for the presence of three species of ninespined sticklebacks in France:

The North-European clade including populations from the Baltic drainage corresponds to *Pungitius pungitius* (Linnaeus, 1758).

The South-Western France clade, which includes specimens from the type locality of *G. vulgaris* (Belle stream at Puy Rabier), is also a distinct evolutionary lineage. *Pungitius vulgaris* (Mauduyt, 1848) is consequently valid.

The hybridization zone is very important in determining the species corresponding to the North-Western France clade. The type localities of *Pungitius laevis* (Seine and Somme drainages) and *Pungitius lotharingus* (Meuse basin) both are in a hybridization zone (Figure 4). As no sequence data exist for these old type specimens because of DNA degradation (Jörger & Schrödl, 2013; Kullander, 1999; Schlick-Steiner et al., 2007) and our morphological analysis does not allow us to assign the type specimens to one of the clades, we consider the syntypes of *G. laevis* (MNHN 0000-7102) and *G. lotharingus* (MNHN 0000-7090) as hybrids of the North-Western France and the North-European taxa. Thus, according to the art. 23 of the ICZN (Ride, 1999), *Gasterosteus lotharingus* Blanchard, 1866 is not valid. However, the type specimens of *G. laevis* (MNHN 0000-7103) and *G. breviceps* correspond to the diagnosis of the North-Western France group. Thus, *Pungitius laevis* (Cuvier, 1829) is valid, and *Gasterosteus breviceps* (Blanchard, 1829) is its junior synonym.

No sexual dimorphism has been observed except the black coloration of the male specimens during the mating period and the shape of the urogenital papillae.

3.3 | Taxonomy

3.3.1 | *Pungitius* Coste, 1848

Pungitius Coste, 1848: 588; Synonyms: *Gasterosteus* Linnaeus, 1758: 296, *Pygosteus* Gill (ex Brevoort) 1861: 39, *Gasterostea* (subgenus) Sauvage, 1874: 729.

TABLE 4 Number of dorsal spines (D1), dorsal soft rays (D2), anal soft rays (A), pectoral-fin rays (P) and scutes (Sct) on the caudal peduncle of the tree clades of French *Pungitius* spp. and the old type specimens

	N	D1				D2					A						P				
		8	9	10	11	8	9	10	11	12	7	8	9	10	11	12	9	10	11	12	13
North-European clade	24	4	8	11	1	1	3	11	7	2	1	4	9	7	1	2	1	18	5		
North-Western France clade	24	14	9	1		4	8	8	4		2	9	9	4				10	12	1	1
South-Western France clade	24	11	11	2		1	11	11	1		1	12	9	2			1	20	2		1
Pungitius breviceps MNHN 0000-7110	1		1						1					1				1			
Pungitius burgundianus MNHN 0000-7109	5			5			3	2					3	2				4	1		
Pungitius laevis MNHN 0000-7102	5	1	3	1				2	3				1	2	2			5			
Pungitius laevis MNHN 0000-7103 and MNHN 2016-0604	3	1	2						3				2	1				1	2		
Pungitius lotharingus MNHN 0000-7090	2	1	1				1		1				1	1					2		

	N	Sct												
		0	1	2	3	4	5	6	7	8	9	10	11	12
North-European clade	24	6	1			2		8		3	2		1	1
North-Western France clade	24	16	4	1	1	2								
South-Western France clade	22	22												
Pungitius breviceps MNHN 0000-7110	1	1												
Pungitius burgundianus MNHN 0000-7109	5						1	1		2		1		
Pungitius laevis MNHN 0000-7102	5	1		1		1		1	1					
Pungitius laevis MNHN 0000-7103 and MNHN 2016-0604	3	3												
Pungitius lotharingus MNHN 0000-7090	2	2												

The genus *Pungitius* is characterized by 7–12 short and isolated dorsal-fin spines except for *Pungitius hellenicus* Stephanidis, 1971 (2–6). The pelvic fin is usually armed by one spine in addition to one soft ray, but the pelvic fin spines are sometimes absent.

3.3.2 | *Pungitius pungitius* (Linnaeus, 1758)

Synonyms (except the North-American taxa) *Gasterosteus pungitius* Linnaeus, 1758: 296, *Gasterosteus burgundianus* Blanchard, 1866: 244, *Gasterosteus (Gasterostea) pungitia* Sauvage, 1874, *Gasterosteus (Pungitius) pungitius* Fatio, 1882, *Pygosteus pungitius* Berg, 1907: 451, *Pygosteus pungitius* forma *carinata* Bertin, 1925: 122, *Pungitius pungitius* Berg, 1932: 169 (Figure 7).

Etymology *Pungitius* means one that pricks (Hertel, 1978; Bănărescu & Paepke, 2002).

Vernacular name Prickly ninespined stickleback, épinochette piquante (in French).

Material examined
Syntypes LSL 34–35 (Figure 7a), 2 left half-skins, Europe, designated by Wheeler (1985: 44).

Bertin (1925) affirmed that the specimens MNHN 0000-7118 (Somme, Abbeville) and MNHN 0000-7197 (Seine drainage, Oise) were designated as types of *P. pungitius* by Cuvier and Valenciennes. But the two specimens LSL 34–35 came from the personal collection of Linnaeus and were the basis of his personal knowledge of the fish. Therefore, Wheeler (1985) considered these specimens as types of *P. pungitius*.

FRANCE: Meuse drainage: UCBLZ 2012.9.456 (EPTE1 24.13 mm SL), Bar at Sauville, Mougenez, August 31, 2009; Moselle basin: UCBLZ 2012.9.418 (EPID2 ♀ 30.75 mm SL, EPTN3 ♂ 32.53 mm SL, EPTN5 ♂ 31.59 mm SL, EPTN8 ♂ 37.15 mm SL, EPTN13 ♂ 37.02 mm SL), Orne at Rosselange, Colas and Lafon, September 21, 2009; Rhone drainage: MNHN 0000-7109, 5 specimens (4 ♀ 28.02, 33.35, 35.95 et 37.58 mm SL et 1 ♂ 32.21 mm SL), Dijon, Brulle, 1872; UCBLZ 2012.9.449 (EPTM1 ♂ 52.90 mm SL, EPTM2 ♀ 51.92 mm SL, EPTM3 ♀ 44.55 mm SL, EPTM5 ♀ 42.43 mm SL, EPTM6 ♂ 42.95 mm SL, EPTM7 ♀ 37.71 mm SL, EPTM8 ♂ 46.66 mm SL) and ZFMK:55646 (Ex26F11 ♀ 41.44 mm SL), Ruisseau de Belle-Fontaine at Lyon, Persat, February 23, 2010; UCBLZ 2012.9.450 (EPTC3 27.11 mm SL) and ZFMK:55648 (Ex26G1 ♀ 44.08 mm SL), Orain at Orain, Persat and Bouchard, June 11, 2009; Seine drainage: MNHN 2013-1294 (tag 12275 ♀ 36.61 mm SL), Thérain at Fontenay-Torcy, ONEMA, September 24, 2013; GERMANY: Weser drainage: ZFMK:53570 (Ex61WH21G7 28.69 mm SL), Wettern, behind Holler Church, Hunte stream, June 2004; IRELAND: Siur drainage: ZFMK:54899 (Ex79F10 juvenile specimen with caudal peduncle cut), Grand Canal, Circular Line L7 Harolds Cross Bridge, November 21, 2012; LATVIA: Baltic Sea: ZFMK:53791 (Ex67G8 ♂ 42.68 mm SL), small ditch in swampy area at border between Latvia and Lithuania, September 05, 2011; Gauja drainage: ZFMK:53895 (Ex67H11 ♂ 27.30 mm SL), Gauja River NW of Sigulda

TABLE 5 Morphometric measurements of the tree clades of French *Pungitius* spp. and the old type specimens

	North-European clade	North-Western France clade	South-Western France clade	Pungitius breviceps 0000-7110	Pungitius burgundianus MNHN 0000-7109	Pungitius laevis MNHN 0000-7102	Pungitius laevis MNHN 0000-7103 and MNHN 2016-0604	Pungitius lotharingus MNHN 0000-7090
Number of specimens	17	20	19	1	4	5	3	1
Standard length (mm)	30.75–52.90	32.09–47.28	30.02–47.06	30.14	32.21–37.58	30.06–34.00	32.16–38.81	30.66
In percent of standard length								
Head length	26.1–30.3 (28.5)	24.9–33.2 (28.9)	25.6–30.6 (28.4)	28.4	26.0–28.4 (27.5)	27.6–30.5 (29.1)	26.3–28.0 (27.4)	29.6
Pre-dorsal-fin length	29.9–35.5 (32.4)	31.1–35.4 (33.7)	30.1–35.5 (33.3)	32.7	24.0–32.7 (30.1)	32.5–34.4 (33.1)	32.1–33.2 (32.6)	31.5
First dorsal-fin base length	28.4–31.7 (30.8)	27.6–36.1 (31.5)	28.9–34.3 (31.8)	32.3	32.1–36.1 (33.5)	29.4–31.5 (30.6)	31.3–32.7 (31.8)	30.0
Second dorsal-fin base length	18.2–27.4 (23.6)	21.3–29.3 (25.3)	20.5–27.0 (23.5)	28.2[c]	21.7–23.0 (22.7)	22.6–26.5 (24.5)	24.2–26.3 (25.0)	23.2
Post-dorsal-fin length	11.7–20.6 (16.4)	10.6–16.2 (13.1)	11.1–15.6 (13.5)	10.3[c]	16.0–21.1 (18.0)[a]	11.3–14.9 (13.2)	9.2–11.7 (10.8)[c]	14.5
Pre-pectoral-fin length	33.2–36.1 (34.3)	31.2–36.4 (33.7)	31.2–35.9 (34.0)	38.8	32.7–34.5 (33.3)	32.2–35.6 (34.1)	31.8–35.2 (33.4)	39.3
Pre-pelvic fin length	39.8–43.9 (42.1)	40.3–46.1 (43.3)	38.6–43.7 (42.0)	47.9[c]	37.7–41.4 (39.3)	39.4–42.6 (41.2)	42.1–43.3 (42.6)	46.7[c]
Pre-anal-fin length	51.1–65.0 (60.7)	56.4–67.4 (62.3)	59.5–65.6 (61.9)	67.8[c]	57.5–63.1 (59.7)	59.9–63.3 (61.7)	61.4–64.5 (63.1)	61.9
Caudal peduncle length	13.3–19.9 (16.3)	10.3–17.8 (14.7)	12.7–16.9 (15.2)	10.1[c]	15.9–20.0 (17.8)[c]	12.0–17.8 (15.0)[c]	12.0–15.5 (14.0)	17.2
Pectoral-fin length	12.8–16.8 (14.5)	10.8–16.6 (14.5)	11.6–15.8 (13.6)	12.5	13.5–16.5 (15.2)	15.7–18.2 (16.8)	11.8–15.8 (13.8)	11.2
Pelvic fin length	7.3–14.4 (10.4)	6.3–10.6 (8.1)	5.6–9.6 (7.6)	6.9[b]	9.2–13.8 (10.9)[a]	6.7–11.0 (8.3)[b]	5.3–8.9 (7.0)[b]	11.0[a]
Anal-fin base length	19.7–27.7 (24.0)	19.6–29.7 (24.7)	19.9–26.8 (24.1)	26.9	24.0–25.3 (24.7)	23.2–28.9 (25.8)[c]	20.8–27.5 (23.6)	24.8
Body depth	12.5–21.6 (17.2)	16.5–21.9 (19.0)	16.1–19.6 (18.1)	18.5	16.7–18.9 (17.4)	18.0–20.0 (18.8)	16.3–17.9 (16.9)	18.6
Caudal peduncle depth	2.2–3.6 (2.7)	2.6–3.3 (3.0)	2.9–4.2 (3.4)	3.5	2.6–2.7 (2.6)[a]	2.7–3.3 (3.0)	2.9–3.4 (3.2)	2.8
Ratio depth/length caudal peduncle	11.8–21.9 (16.7)	15.7–31.3 (20.7)	19.1–27.2 (22.4)	34.5[c]	13.0–17.2 (14.8)[a]	16.8–25.6 (20.7)[b]	18.5–27.8 (23.1)[c]	16.3
In percent of head length								
Snout length	20.2–27.6 (24.0)	19.8–26.3 (23.6)	20.2–25.9 (23.6)	21.2	20.5–24.3 (23.0)	20.0–21.7 (21.1)	23.9–27.3 (26.1)	24.3
Eye diameter	27.3–32.2 (29.4)	24.1–33.3 (28.1)	25.3–33.9 (29.5)	33.1	28.2–33.1 (30.5)	28.6–37.7 (29.7)	27.0–31.0 (29.3)	31.1
Post-orbital length	43.8–53.6 (47.7)	43.3–50.9 (47.8)	41.9–51.1 (46.1)	48.9	46.5–54.7 (49.3)[a]	45.9–52.0 (49.8)[a]	44.4–50.8 (48.1)	50.7
Inter-orbital width	15.6–21.6 (18.9)	12.8–26.5 (16.4)	15.1–20.0 (17.7)	17.7	19.4–24.1 (20.9)	19.4–21.9 (20.7)	16.3–18.1 (17.4)	15.0[b]

Values in parentheses: mean

[a] Character corresponding to the North-European clade

[b] Character not corresponding to the North-European clade

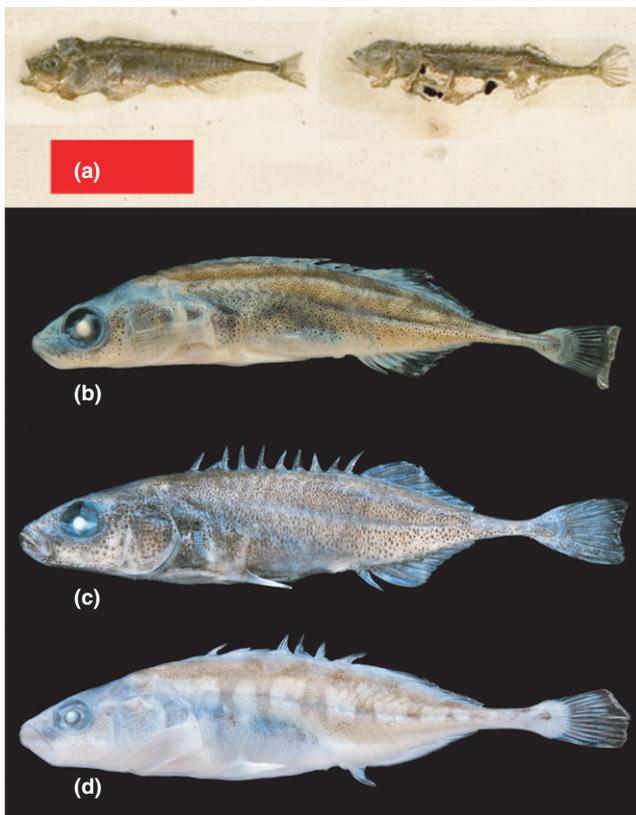[c] Character corresponding exclusively to the North-Western France clade

FIGURE 7 Specimens of *Pungitius pungitius*: left half-skins of the type specimens (LSL 34 and 35, respectively, 34 and 35 mm SL; a) with the agreement of the Linnean Society of London; ZFMK:53791, Ex67G8 ♂ 42.68 mm SL, Baltic, Latvia (b); UCBLZ 2012.9.449, EPTM6 ♂ 42.95 mm SL, Ruisseau de Belle-Fontaine (Rhone basin) at Lyon, France (c); MNHN 2013-1294, tag 12275 ♀ 36.61 mm SL, Thérain (Seine basin) at Fontenay-Torcy, France (d)

village at bridge of motorway P8, September 09, 2011; LITHUANIA: Nemunas drainage: ZFMK:53760 (Ex67G1 ♂ 32.20 mm SL), small pond at motorway 4313, bus-station Kaimynai village, September 11, 2011; POLAND: Vistula drainage: ZFMK:68701 (Ex63D7 ♀ 29.77 mm SL) and ZFMK:68702 (Ex63D6 23.49 mm SL), Pilica stream N of Mysiakowiec, July 07, 2011.

Diagnosis *Pungitius pungitius* is distinguished from the two other French species by a flat head with a straight or slightly concave snout (Figure 6a) in specimens longer than 35 mm SL, and the combination of following morpho-meristic characters: typically 9–10 dorsal spines (vs. typically 8–9 for the other groups), typically 10–11 dorsal soft rays (vs. 9–10), typically 9–10 anal soft rays (vs. 8–9), 0–12 scutes on the caudal peduncle (vs. 0–4) (Table 4) so the keel reaches before the last anal-fin ray, a longer post-dorsal length (11.7%–20.6% of the standard length SL, vs. 9.2%–16.2% and 11.1%–15.6% for, respectively, *P. laevis* and *P. vulgaris*), a longer caudal peduncle length (13.3%–20.0% SL, vs. 10.1%–17.8% and 12.7%–16.9%), the smallest ratio caudal peduncle depth/length (11.8%–21.9% vs. 15.7%–34.5% and 19.1%–27.2%), and longer pelvic fin lengths (7.3%–14.4% SL, vs. 5.3%–10.6% and 5.6%–9.6%) (Table 5).

Redescription

The specimens belonging to *Pungitius pungitius* have a flat head with a straight or slightly concave snout (Figure 6a) for specimens longer than 35 mm SL, 8–11 dorsal-fin spines (usually 9–10), (8) 9–12 dorsal-fin soft rays (usually 10–11), (7) 8–12 anal-fin soft rays (usually 9–10), 0–12 scutes on the caudal peduncle so the keel reaches before the last anal-fin ray, and (9) 10–11 pectoral-fin rays (Table 4). Concerning the morphometric data, the head length measures 26.1%–30.3% of the standard length SL, the pre-dorsal length 24.0%–35.5% SL, the first dorsal-fin base length 28.4%–36.1% SL, the second dorsal-fin base length 18.2%–27.4% SL, the post-dorsal length 11.7%–20.6% SL, the pre-pectoral length 32.7%–36.1% SL, the pre-pelvic length 37.7%–43.9% SL, the pre-anal length 51.1%–65.0% SL, the caudal peduncle length 13.9%–20.0% SL, the pectoral fin length 12.8%–16.8% SL, the pelvic fin length 7.3%–14.4% SL, the anal-fin base length 19.7%–27.7% SL, the body depth 12.5%–21.6% SL, the caudal peduncle depth 2.2%–3.6% SL. The caudal peduncle depth represents 11.8%–21.9% of its length. The snout length measures 20.2%–27.6 of the head length HL, the eye diameter 27.3%–33.1% HL, the post-orbital length 43.8%–54.7% HL, and the inter-orbital width 15.6%–24.1% HL (Table 5).

Color pattern of live specimens The coloration varies depending on the environment and season. The body is generally yellow green or olive with brownish gray blotches arranged irregularly or forming a regular pattern on the flanks, and the belly is white (see also Bănărescu & Paepke, 2002).

Color pattern of preserved specimens Specimens preserved in ethanol have a white belly. Their flanks are pale yellow, and the blotches are composed by brownish or gray dots.

Biology/ecology *Pungitius pungitius* is a primary freshwater fish. But as it tolerates a more elevated salinity, it also occurs in brackish and marine water (see Bănărescu & Paepke, 2002). In inland waters, it is an ariadnophylic and eurytopic fish, occurring as well in upstream as downstream habitats (Fieseler & Wolter, 2006), but it prefers living in the small chalky and silty tributaries (Belliard, Boët, & Tales, 1997). During the spawning season, the males have a black coloration especially on the throat and abdomen often extending over much of the body. The pelvic spines are bright turquoise according to Bănărescu and Paepke (2002), but rather white in the Rhone basin. Contrary to the males, the females do not change coloration keeping their color pattern, but they have well-rounded bodies when there are full of eggs (Bănărescu & Paepke, 2002). This species can hybridize with the two other French species *P. laevis* and *P. vulgaris*.

Distribution *Pungitius pungitius* has a circumpolar distribution (Bănărescu & Paepke, 2002; Kottelat & Freyhof, 2007): North America, Asia, and the North of Europe. In France, this species is rare in the Rhone drainage (Carrel, 2002) with some isolated populations. It occurs also in the Moselle, Meuse, Somme, and Seine

drainages but these populations are often hybridized with *P. laevis*. Based on nuclear data and also naturalist records, it also occurs in the Loire drainage: Vienne basin and Anjou county (De Soland, 1869; Mauduyt, 1848) (Figure 1).

3.3.3 | *Pungitius laevis* (Cuvier, 1829)

Synonyms *Gasterosteus lotharingus* Blanchard, 1866: 245, *Gasterosteus (Gasterostea) breviceps* Sauvage, 1874, *Gasterosteus (Gasterostea) laevis* Sauvage, 1874, *Gasterosteus (Gasterostea) lotharinga* Sauvage, 1874, *Gasterosteus (Gasterostea) pungitia* var. *Breviceps* Moreau, 1881, *Gasterosteus (Gasterostea) pungitia* var. *Laevis* Moreau, 1881, *Gasterosteus (Gasterostea) pungitia* var. *Lotharingus* Moreau, 1881 (Figure 8).

Etymology *Laevis* means smooth in Latin, referencing to the absence of keel on the caudal peduncle.

Vernacular name Smooth-tail stickleback, épinochette à queue lisse (in French).

Material examined

Lectotype According to Hureau and Monod (1973) and Kottelat (1997), MNHN 0000-7102 and 0000-7103 are the syntype specimens of *Gasterosteus laevis* Cuvier, 1829. The type localities are, respectively, the Somme and the Seine (around Paris) basins which are inside the hybridization zone between *P. pungitius* and *P. laevis* in the North of France highlighted by the DNA sequence data (Figures 3 and 4). The morphological analysis (Figure 5; Tables 4–5) assigned the syntype lot MNHN 0000-7103 to the North-Western France clade, whereas the other syntype specimens MNHN 0000-7102 have morphological characters of both taxa. We conclude these last syntypes are probably hybrids between *P. pungitius* and *P. laevis*.

Thus, a lectotype designation is needed. We designate the specimen MNHN 2016-0604 (extracted from MNHN 0000-7103), ♂ 38.81 mm SL, Seine basin at Bobigny (Figure 7a) as lectotype of *Pungitius laevis* (Cuvier, 1829). The two other specimens from the lot MNHN 0000-7103 (♀ 37.40 mm SL and ♂ 32.16 mm SL) are the paralectotypes for this species.

FRANCE: Scheldt drainage: UCBLZ 2012.9.463 (ETPR2 ♂ 34.45 mm SL, EPTR3 ♀ 36.70 mm SL and EPTR4 ♀ 41.71 mm SL), Trouille at Vieux Rang, Persat, September 11, 2007; Falleron drainage: MNHN 2013-0805 (tag 10986 ♀ 47.28 mm SL, 10987 ♀ 33.07 mm SL and 10988 25.36 mm SL), Falleron at Paulx, ONEMA, June 27, 2013; Loire drainage: MNHN 2013-0801 (tag 10976 ♀ 46.80 mm SL and 10977 ♀ 38.01 mm SL), Notre-heure at Autry-le-Châtel, ONEMA, June 5, 2013; MNHN 2013-0802 (tag 10979 ♀ 40.09 mm SL, 10980 ♂ 43.19 mm SL and 10981 ♀ 40.45 mm SL), Planche-Godard à Vinon, ONEMA, June 07, 2013; MNHN 2013-0814 (tag 12208 ♀ 37.24 mm SL and 12210 ♀ 39.73 mm SL), Nièvre à Dompierre-sur-Nièvre, Denys and ONEMA, June 27, 2013; MNHN 2013-0819 (tag 12220 ♀ 42.70 mm SL), Yévrette at Osmoy, Denys and ONEMA, June 28, 2013; UCBLZ 2012.9.475 (EPTI1 ♂ 39.50 mm SL, EPTI3 ♀ 32.53 mm SL, EPTI5 ♂ 40.51 mm SL, EPTI8 ♀ 33.96 mm SL, EPTI9 ♀ 29.92 mm SL and EPTI10 ♀ 26.68 mm SL), Rampennes at Bourges, Rousseau, January 08, 2010; UCBLZ 2012.9.481 (EPTQ3 ♀ 32.09 mm SL and EPTQ8 ♂ 38.17 mm SL), Fontaine d'enfer at Aubigné-Racan, Lefrançois, December 2009; Orne drainage : MNHN 0000-7110 ♂ 30.14 mm SL, Caen, De l'hopital; MNHN 2013-1297 (tag 12282 ♂ 29.37 mm SL), Rouvre at Faverolles, ONEMA, September 06, 2013; Seine drainage: MNHN 2013-1295 (tag 12278 ♀ 34.28 mm SL), Risle at Ambenay, ONEMA, October 10, 2013; MNHN 2016-0604 ♂ 38.81 mm SL and MNHN 0000-7103 (♀ 37.40 mm SL and ♂ 32.16 mm SL), Bobigny, Bocourt.

Diagnosis *Pungitius laevis* differs from the other species occurring in France by a head rounded with a concave snout (Figure 6b) for specimens longer than 35 mm SL accentuating the impression of fleshy lips, 0–4 scutes on their caudal peduncle (vs. 0–12 and 0 for respectively *P. pungitius* and *P. vulgaris*) (Table 4), as well as the higher ratio caudal peduncle depth/length (15.7%–34.5%, vs. 11.8%–21% and 19.1%–27.2%) (Table 5).

Redescription

Cuvier (1829) delimited this species based on the absence of keel as the only diagnostic character. Blanchard (1866) noticed this species has a lower number of dorsal spines than *P. pungitius* (9 *vs.* 10). He also described another species *G. breviceps* characterized mainly by a large and mildly elongated caudal-peduncule and a short head. However, this last description corresponds to juvenile specimens (Bertin, 1925). This explains why it was considered rare, whereas many
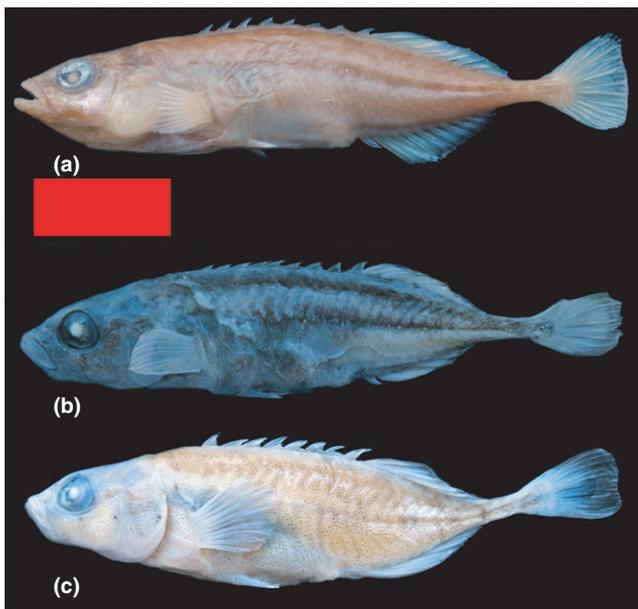

FIGURE 8 Specimens of *Pungitius laevis*: lectotype MNHN 2016-0604 ♂ 38.81 mm SL, Bobigny, France (a); MNHN 2013-0802, tag 10980 ♂ 43.19 mm SL, Planche-Godard (Loire drainage) at Vinon, France (b); UCBLZ 2012.9.481, EPTQ8 ♂ 38.17 mm SL, Fontaine d'enfer (Loire drainage) at Aubigné-Racan, France (c)

populations of *P. laevis* were observed (De Soland, 1869). We provide here a complete description.

Pungitius laevis specimens have a head rounded with a concave snout (Figure 6b) for specimens longer than 35 mm SL accentuating the impression of fleshy lips, 8–9 (10) dorsal spines (lectotype 8), 8–11 dorsal soft rays (lectotype 11), 7–11 anal soft rays (lectotype 10), 0–4 scutes on the caudal peduncle (lectotype 0), and 10–11 (12–13) pectoral-fin rays (lectotype 11) (Table 4). Concerning the morphometric data, the head length measures 24.9%–33.2% of the standard length SL (lectotype 27.8%), the pre-dorsal length 31.1%–35.4% SL (lectotype 32.1%), the first dorsal-base length 27.6%–36.1% SL (lectotype 31.5%), the second dorsal-base length 21.3%–29.3% SL (lectotype 26.3%), the post-dorsal length 9.2%–16.2% SL (lectotype 11.6%), the pre-pectoral length 31.2%–38.8% SL (lectotype 35.2%), the pre-pelvic length 40.3%–47.9% SL (lectotype 42.1%), the pre-anal length 56.4%–67.8% SL (lectotype 61.4%), the caudal peduncle length 10.1%–17.8% SL (lectotype 14.7%), the pectoral fin length 10.8%–16.6% SL (lectotype 13.6%), the pelvic fin length 5.3%–10.6% SL (lectotype 5.3%), the anal-fin base length 19.6%–29.7% SL (lectotype 27.5%), the body depth 16.3%–21.9% SL (lectotype 17.9%), the caudal peduncle depth 2.6%–3.5% SL (lectotype 3.4%). The caudal peduncle depth represents 15.7%–34.5% of its length (lectotype 23.0%). The snout length measures 19.8%–27.3 of the head length HL (lectotype 27.0%), the eye diameter 24.1%–33.3% HL (lectotype 29.9%), the post-orbital length 43.3%–50.9% HL (lectotype 49.2%), and the inter-orbital width 12.8%–26.5% HL (lectotype 18.1) (Table 5).

Color pattern of live specimens According to Blanchard (1866), the general coloration of the body is bright green with dark tints. Although the body is covered by blackish blotches, which are absent on the pectoral and abdominal regions.

Color pattern of preserved specimens *Pungitius laevis* specimens preserved in ethanol have the same color pattern as *P. pungitius*: white belly, pale yellow flanks, and brownish or gray dots on the flecks.

Biology/ecology *Pungitius laevis* inhabits streams and tributaries and has a tolerance to salinity like the other species of *Pungitius* (Feunteun et al. 1992). It occurs in lowland and sand brooks rather than warmer streams (H. Persat, personal observation). In the Seine drainage, this species might have a preference for limestone or chalky sediments (Belliard et al., 1997).

Distribution The type locality of *Pungitius laevis* is the Seine basin around Paris. It occurs also mostly in the Southern and the Eastern slopes of this catchment as well as in the small coastal rivers of Normandy, in the Scheldt, the coastal Atlantic basins, and the Loire drainage (except the Vienne basin) where it is more abundant than *G. aculeatus* (Bertin, 1925). Nuclear data indicate its presence also in Meuse and Moselle basins, which might mean that its distribution could have been further east in the past, and also in Saone basin (Figure 1).

3.3.4 | *Pungitius vulgaris* (Mauduyt, 1848)

Synonyms *Pygosteus pungitius* forma *hologymna* Bertin, 1925: 122 (Figure 9).

Etymology *Vulgaris* means common in Latin.

Vernacular name Poitevin stickleback, épinochette poitevine or épinochette marichaud (in French).

Material examined

Neotype Mauduyt (1848) mentioned Cuvier as descriptor of *Gasterosteus vulgaris* (in "Ency., pl. 57, fig. 225″). But this reference cannot be found and might be a confusion of Mauduyt with *Gasterosteus laevis* Cuvier, 1829;. Kottelat (1997) validated Mauduyt (1848: 19) as the species descriptor of *Pungitius vulgaris*. According to Mauduyt (1848), the type localities of this species are in the Vienne basin (Loire drainage), in the streams, tributaries and ponds at la Vergne and around Poitiers, la Bergue (Magné town near of Gençay), Les Aiffes (Saint-Maurice town near of La Ressonnière), and La Séguinière (Saint-Julien-Lars town). Nevertheless, Mauduyt (1848) distinguished another form of ninespined stickleback with a keel on the caudal peduncle and called it *G. pungitius*. No specimens from these localities were deposited into the MNHN collections (P. Pruvost, personal communication), nor the museums of Nantes and Bordeaux (M.-L. Guérin, personal communication; L. Charles,
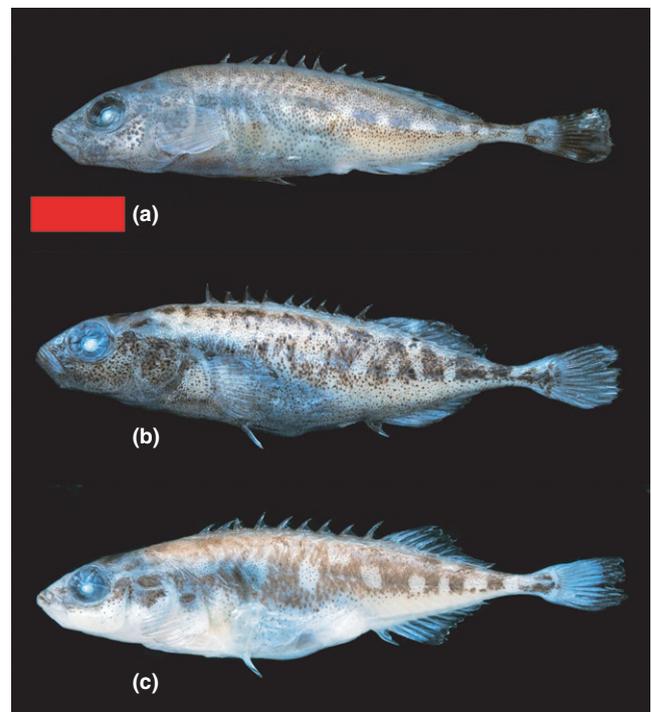

FIGURE 9 Specimens of *Pungitius vulgaris*: Neotype UCBLZ 2012.9.1010, ♂ 32.24 mm SL, Belle at Puy-Rabier, France (a); UCBLZ 2012.9.459, EPTP7 ♂ 30.02 mm SL, Blâme (Dordogne basin) at Brouchaud, France (b); UCBLZ 2012.9.461, EPTL5 ♀ 33.63 mm SL, Soue (Dordogne basin) at Brouchaud, France (c)

personal communication). So we consider the types of *P. vulgaris* unknown. Moreover, Mauduyt (1848) did not give any picture of *P. vulgaris* with its species description.

Thus, the identity of *P. vulgaris* needs to be clarified by the designation of a neotype, according to the art. 75.3.1 of the ICZN. We designate the specimen UCBLZ 2012.9.1010, ♂ 32.24 mm SL, Belle at Puy-Rabier (Figure 8) as neotype of *Pungitius vulgaris* (Mauduyt, 1848).

FRANCE: Charente drainage: MNHN 2013-1308 (tag 12303 ♀ 40.53 mm SL and 12304 ♂ 35.43 mm SL), Lien at Condac, Denys and FPPMA 16, September 10, 2013; MNHN 2014-0008 (tag 12308 ♀ 40.53 mm SL), Chaboussant at Lezay, Denys and Persat, September 09, 2013; UCBLZ 2012.9.462 (EPTK1 ♂ 38.91 mm SL, EPTK2 ♂ 38.57 mm SL, EPTK3 ♀ 46.02 mm SL and EPTK6 ♀ 39.92 mm SL), Touvre at Magnac sur Touvre, Charneau, July 15, 2009; Dordogne drainage: UCBLZ 2012.9.459 (EPTP1 ♂ 39.25 mm SL, EPTP4 ♀ 29.16 mm SL, EPTP5 ♂ 30.82 mm SL, EPTP7 ♂ 30.02 mm SL and EPTP9 ♀ 24.54 mm SL), Blâme at Brouchaud, Terrier, May 13, 2009; UCBLZ 2012.9.461 (EPTL1 ♂ 30.94 mm SL, EPTL2 ♂ 33.97 mm SL, EPTL4 ♂ 31.39 mm SL, EPTL5 ♀ 33.63 mm SL, EPTL6 ♂ 30.65 mm SL, EPTL7 ♀ 32.54 mm SL, EPTL8 ♂ 34.61 mm SL and EPTL14 ♀ 28.87 mm SL), Soue at Brouchaud, Persat, May 13, 2009; Gironde estuary: MNHN 2013-0803 (tag 10983 and 10984, juveniles specimens with caudal peduncle cut), Jalle de l'Horte at Saint-Laurent-en-Médoc, Denys, July 08, 2013; Loire drainage: UCBLZ 2012.9.465 (EPTJ10 ♀ 35.43 mm SL) and UCBLZ 2012.9.1010 (EPTJ13 ♂ 32.24 mm SL), Belle at Puy-Rabier, Derrouch, February 25, 2010.

Diagnosis *Pungitius vulgaris* is differentiated from the other species occurring in France by a head rounded with a straight or slightly convex snout (Figure 6c), typically 11 pectoral rays (vs. typically 10–11) and no scute on the caudal peduncle (vs. 0–12 and 0–4 for, respectively, *P. pungitius* and *P. laevis*) (Table 4).

Redescription

The specimens belonging to *Pungitius vulgaris* have a head rounded with a straight or slightly convex snout (Figure 6c), 8–9 (10) dorsal spines (neotype 9), (8) 9–10 (11) dorsal soft rays (neotype 10), (7) 8–9 (10) anal soft rays (neotype 9), no scute on the caudal peduncle, and (10) 11 (12–13) pectoral fin rays (neotype 11) (Table 4). Concerning the morphometric data, the head length measures 25.6%–30.6% of the standard length SL (neotype 30.3%), the pre-dorsal-fin length 30.1%–35.5% SL (neotype 35.4%), the first dorsal-fin base length 28.9%–34.3% SL (neotype 31.3%), the second dorsal-fin base length 20.5%–27.0% SL (neotype 21.7%), the post-dorsal-fin length 11.1%–15.6% SL (neotype 13.9%), the pre-pectoral-fin length 31.2%–35.9% SL (neotype 35.0%), the pre-pelvic fin length 38.6%–43.7% SL (neotype 42.3%), the pre-anal-fin length 59.5%–65.6% SL (neotype 59.7%), the caudal peduncle length 12.7%–16.9% SL (neotype 15.3%), the pectoral-fin length 11.6%–15.8% SL (neotype 14.3%), the pelvic fin length 5.6%–9.6% SL (neotype 7.2%), the anal-fin base length 19.9%–26.8% SL (neotype 24.8%), the body depth

16.1%–19.6% SL (neotype 18.2%), the caudal peduncle depth 2.9%–4.2% SL (neotype 2.9%). The caudal peduncle depth represents 19.1%–27.2% of its length (neotype 19.3%). The snout length measures 20.2%–25.9 of the head length HL (neotype 24.0%), the eye diameter 25.3%–33.9% HL (neotype 30.4%), the post-orbital length 41.9%–51.1% HL (neotype 45.4%), and the inter-orbital width 15.1%–20.0% HL (neotype 15.1) (Table 5).

Color pattern of live specimens Mauduyt (1848) described adult specimens with a yellowish coloration with very thin black dots, whereas the young ones are blueish green. But the color pattern is very variable between individuals from a same population, or having the same age or sex; this is clearly shown on the drawings of specimens from Angoulême (Charente basin) by Bertin (1925: 146) for instance.

Color pattern of preserved specimens Preserved specimens in ethanol of *P. vulgaris* have the same color pattern as *P. pungitius*: white belly, pale yellow flanks, and brownish or gray dots on the flecks.

Biology/ecology *Pungitius vulgaris* inhabits streams, tributaries, and ponds (Mauduyt, 1848). As alleles for *RNF213* characteristic of this species were encountered in small coastal Atlantic basins (this study), this species may also have a tolerance of salinity like the other species of *Pungitius*. In the Garonne drainage, it has a very restrictive habitat with low elevation and short distance from the source (Santoul, Figuerola, Mastrorillo, & Céréghino, 2005). Mauduyt (1848) observed in 1813 the monogamy and nest building for this species, but the original publication cannot be found. However, its monogamy needs to be verified because Vallot (1850) doubted this observation, noting that, ordinarily, the ninespined-sticklebacks are polygamous according to Coste (1848). This species can hybridize with the two other species where they are in sympatry: with *P. pungitius* in the Vienne basin and *P. laevis* in the coastal Atlantic basins.

Distribution The type locality of *Pungitius vulgaris* is the Vienne basin (more precisely the Clain sub-basin) belonging to the Loire drainage. It occurs also in the Gironde estuary streams, in some tributaries of the Dordogne basin as well as in the coastal Atlantic basins between the Loire and Garonne estuaries like Charente, Sèvre Niortaise, and the Ligneron where it is in sympatry with *P. laevis* (Figure 1).

4 | DISCUSSION

4.1 | Three distinct evolutionary lineages of ninespined-sticklebacks in France

The *COI* sequences discriminate three clades within French *Pungitius*: a North-European clade including also Baltic populations, a South-Western France clade, and a North-Western France clade. This

separation is in accordance with other studies (Guo, Shikano, et al., 2016; Guo, Toli, & Merilä, 2016; Wang et al., 2015, 2017). The North-Western France clade, here identified as *P. laevis*, corresponds to the *P. laevis* Lineage I of Wang et al. (2015). The South-Western France clade, here identified as *P. vulgaris*, correlates with their *P. laevis* Lineage II, and the North-European clade, here identified as *P. pungitius*, corresponds to their Eastern (Baltic) and Western European *P. pungitius* + *P. laevis* Lineage III.

Our study includes a nuclear marker (*RNF213*), which distinguishes three clades and corroborates the *COI* results. But like the Asian *Pungitius* (Takahashi et al., 2016), this marker points to hybridizations between these clades, both through heterozygous specimens and through individuals assigned to different clades depending on the marker analyzed. Moreover, our nuclear results highlight a hybridization zone in the North of France (North Sea, English Channel, Seine, Meuse, and Rhine drainages) between *P. laevis* and *P. pungitius*. Kottelat (1997) already suspected this hybridization zone from Northern France to Netherlands based on the number of scutes in the caudal peduncles from Münzing (1969). Wang et al. (2017) also confirm a historical introgression in this area.

4.2 | Integrative taxonomy of the French ninespined-sticklebacks

The traditional taxonomy of *Pungitius* based only on morphological characters has been repeatedly discussed since the 18th century (Table 1). No morphological study was able to clearly discriminate the groups (Keivany & Nelson, 2000). But associated with DNA sequences data, some of the morphological characters support the molecular-determined evolutionary lineages (Figure 5; Tables 4 and 5). Characters like the number of scutes on the caudal peduncle or the length of the pelvic fin spines, which were considered to be dependent on the environment (ie, the salinity) as they constitute a defense against predators (Herczeg, Turtiainen, & Merilä, 2010; Mobley, Lussetti, Johansson, Englund, & Bokma, 2011; Välimäki, Herczeg, & Merilä, 2012), are also taxonomic criteria for these species (Cuvier, 1829; Blanchard, 1866; Mauduyt, 1848; Kottelat, 1997; Keivany & Nelson, 2000; Kottelat & Freyhof, 2007) unlike for the three-spined stickleback *Gasterosteus aculeatus* (Denys et al. 2015). Nevertheless, the absence of a keel on the caudal peduncle is not a relevant taxonomic character (Wang et al., 2015), because it might be due to introgression (Wang et al., 2017). The pelvic girdle was also used as a taxonomic character (Blanchard, 1866; Keivany & Nelson, 2000). Indeed, Keivany and Nelson (2000) observed differences on the pelvic girdle of *P. pungitius* from Germany, Poland, Baltic Sea, and Japan, and *P. laevis* from Ireland, which is reduced. But our molecular genetic results showed populations from these localities belong to the same North-European clade (Figure 3). The pelvic reduction is controlled by the *Pituitary homeobox transcription factor 1* gene (Pitx1), and its expression is linked to the freshwater habitat (see Shikano, Laine, Herczeg, Vilkki, & Merilä, 2013).

The counts and measurements between the different groups overlap. This could be explained by both hybridization and environment. We applied a filter excluding the heterozygous specimens, but this method allowed only to rule out hybrid F1 but not following generations. So, some homozygous specimens could still be introgressed having morphological characters of another group (ie, short caudal peduncle for specimens belonging to the North-European lineage). Concerning especially the North-Western and the South-Western France clades, there might be another explanation which would be the environmental conditions. All specimens from these groups were caught in small brooks, with sometimes the presence of predators (ie, pikes *Esox* spp.) and competitors (*G. aculeatus*), which affect the morphology of gasterosteids inducing parallel evolution (Gray, Robinson, & Parsons, 2005; Herczeg et al., 2010; Mobley et al., 2011; Thompson, Taylor, & McPhail, 1997; Välimäki et al., 2012). Similar environment induces similar morphological constraints and thus an incidence on the morphological species delineation (Ravinet, Ishikawa, & Kitano, 2016).

The combination of DNA sequences and morphological data is in agreement with delimit three species of *Pungitius* spp. in France. Molecular genetic data were helpful in this study in which the morphological data are overlapping. Among the French sculpins *Cottus* spp., DNA sequences data allowed to delineate species, which are morphologically close (Freyhof, Kottelat, & Nolte, 2005). These two types of datasets are complementary (eg, Boero & Bernardi, 2014). Concerning *P. laevis*, a lectotype from the Seine basin at Bobigny (around Paris) has been designated. But its type locality was strongly damaged by urbanization and pollution, considerably reducing the number of species occurring in this area (Belliard, Boët, & Allardi, 1995). Now the only waterway is the Canal de l'Ourcq, which, to our knowledge, does not provide any suitable habitat for the ninespined sticklebacks. So, the presence of this species today in this area cannot be checked by new specimens and molecular genetic data.

These delineations are also congruent with some ecological data. In the Seine drainage, *P. pungitius* seems to occur preferentially in chalky or silty streams, whereas *P. laevis* would prefer limestone as well as chalky sediments (Belliard et al., 1997). More investigations are needed, including the study of the mating behaviors and the trophic niches. These are particularly interesting as they distinguish two Japanese cryptic ninespined stickleback species (Meguro et al., 2016; Ravinet et al., 2016).

4.3 | Biogeography

Three species of *Pungitius* spp. occur in France. *P. pungitius* is located in the Rhone, Rhine, Meuse, Somme, and Seine drainages. *P. laevis* extends from the Scheldt, Seine, and Loire drainages, in the Normandy and Atlantic coastal basins, and occurred also in the Meuse and Moselle drainages. And finally, *P. vulgaris* is endemic to the Vienne, Dordogne, and some coastal Atlantic basins of the South-West of France (Figure 1).

The distribution pattern of the different *Pungitius* species is not identical to any other French genus of fishes revised recently. If the occurrence of an endemic species is not surprising (Costedoat,

Chappaz, Barascud, Guillard, & Gilles, 2006; Denys, 2015; Denys, Dettai, Persat, Hautecœur, & Keith, 2014; Keith et al., 2011; Kottelat & Freyhof, 2007; Kottelat & Persat, 2005), the presence of another endemic species in the Seine-Loire-Meuse-Rhine drainages is unique. It was already admitted that the Ice Age of the Pleistocene has had a major impact on the European fauna (Hewitt, 2000). The French territory in the North of the Garonne drainage was covered by permafrost (Bertran et al., 2014; Vanderberghe et al., 2014), and all fish species, which were not sufficiently cryophilic, disappeared, and were replaced by another ichthyofauna from septentrional Europe (see Denys, 2015; Keith et al., 2011). The nine-spined sticklebacks have a high tolerance for low temperatures (Bănărescu & Paepke, 2002). So we hypothesize that *P. laevis* and *P. vulgaris* were sufficiently cryophilic to find refuge during the last quaternary ice age.

The distributions of *P. laevis* and *P. vulgaris* are a reminiscence of the hydrographic map of France during the early Pliocene (−5.3 to 3.6 Ma) (see Persat & Keith, 2011; Figure 10). The Loire basin belonged to the Seine (Etienne & Larue, 2011; Larue, 2003). The Vienne basin was an isolated Atlantic basin but was also linked to the Charente and Aquitanian (Dordogne and Gironde estuary) basins (Bichot, 2004; Passerat, 1911). During the Late Pliocene, a tectonic event caused the Loire deviation to the Atlantic coast (Etienne & Larue, 2011; Larue, 2003) connecting the Vienne basin to this new drainage. Since this geological event, the Seine and Loire populations would have been isolated and this could be the origin of the two subclades in the *COI* tree within the North-Western France group (Figure 3). Wang et al. (2015) did a time estimation with mitochondrial data using the oldest fossil record of *Gasterosteus aculeatus* as the only calibration point. They estimated the age of the common ancestor of *P. laevis* and the two other species at 2.26 Ma, and those of *P. vulgaris* and *P. pungitius* at 1.38 Ma. They, however, expressed concern that their time estimation might be an underestimation. According to our results, the speciation events might indeed have been older, and we suggest revising the biogeographical scenario and the time divergences including the biogeographic data.

From the phylogenetic tree of Wang et al. (2015), *P. pungitius* sensu stricto probably has a Siberian origin like some Salmoniformes and non-vertebrate groups (Makhrov & Bolotov, 2006). This taxon might have arrived in Europe in the Pleistocene, between 660 000 (Wang et al., 2015) and 390 000 years ago (Aldenhoven, Miller, Showers Corneli, & Shapiro, 2010). During the last glacial episodes, the Channel was a drainage with the Seine, Rhine, Meuse, Thames, Oder, Elbe, Weser, and Vistula basins as tributaries (Hantke, 1993). *Pungitius pungitius* might have taken advantage of the different floods at the end of each glacial episode (Craw, Burridge, Anderson, & Waters, 2006) to invade the North-West of Europe like many septentrional fish taxa (Bănărescu, 1992; Costedoat & Gilles, 2009; Le Gall, 2010). With the melting glaciers during the end of the Würm (about 10 000 years ago), there were some connections between the Seine, the Meuse, and the Rhone drainages (Wang et al., 2017) (Figures 3 and 4). During this period, some populations of *P. laevis* from the Seine drainage have also colonized the Loire mouth and the Atlantic coastal basins around, as well as the Saone basin like the three-spined stickleback *G. aculeatus* (Mäkinen and Merilä, 2008) (Figure 4).

This scenario needs to be assessed with additional studies and genetic markers, especially more variable nuclear markers. Nevertheless, the ninespined sticklebacks might be quite good witnesses of the last geologic and climatic events.
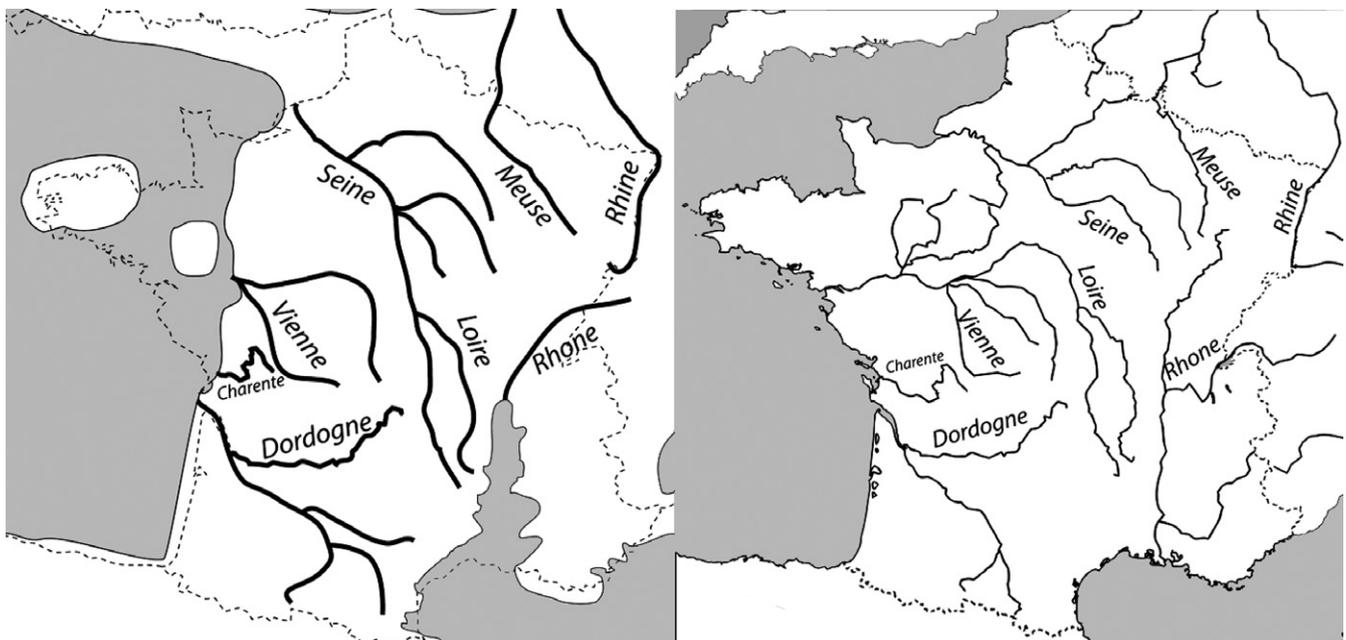

FIGURE 10 Hydrographical map of France during the early Pliocene (left; adapted and modified from Persat & Keith, 2011) and today (right); sea water is in gray

4.4 | Consequences for conservation and biology

This study has consequences on conservation and biology.

For many decades, ichthyologists thought there was only one species of *Pungitius* in France, a pattern also found for multiple other genera (Costedoat et al., 2006; Denys, 2015; Denys et al., 2013, 2014, 2015; Doadrio, Kottelat, & De Sostoa, 2007; Doadrio & Madeira, 2004; Freyhof et al., 2005; Geiger et al., 2014; Keith et al., 2011; Kottelat, 2007; Kottelat & Freyhof, 2007; Kottelat & Persat, 2005; Sideleva, 2009). But the present revision confirms that three species of *Pungitius* are present in France, and two of them are endemic. Thus, it is necessary to study all ecological characteristics of both species (habitat, life traits, behaviour, etc.. . .) in order to update biotic indexes like the French "Fish River Index" (FRI; Oberdorff et al., 2002), as well as the status of these species on the Red List of the threatened species (Collares-Pereira, Cowx, & Coelha, 2002; Keith & Marion, 2002). The best conservation measures should be evaluated and enforced (Dudgeon et al., 2006; Hey, Waples, Arnold, Butlin, & Harrison, 2003) for these two species, which now have a patrimonial interest (Maire, Buisson, Biau, Canal, & Laffaille, 2013). The type locality of *P. laevis* was strongly damaged (Belliard et al., 1995), and needs to be restored. For these taxa without commercial or angling interest, the priority for conservation measures is the protection and restoration of habitats (Maire, Laffaille, Maire, & Buisson, 2016; Maire et al., 2013, 2015), specifically in the most upstream and downstream areas of large catchments, as well as in small coastal basins of the English Channel and the Atlantic Ocean (Maire et al., 2016). Nevertheless the application of the restoration and conservation protocol by Maire et al. (2013) in the North of France had a negative effect on the ninespined sticklebacks populations (Maire et al., 2015), and most populations living in coastal basins are sympatric with another species or impacted by hybridization. So a better protocol is needed.

This study highlighted some cases of hybridization between the three species of *Pungitius* in France, and helped select which described species was valid, as we considered two of the three possibilities (*G. lotharingus*) as hybrids of *P. pungitius* and *P. laevis*. The ninespined stickleback is also an emerging model for evolutionary biology research (Merilä, 2013). Hybridization is now considered as a possible cause of speciation in some cases by creating more competitive individuals than parental species (see Abbott et al., 2013; Nolte & Tautz, 2010). The North of France is a hybridization zone between these two species. It might have led to a hybrid swarm which might be more competitive, like the hybrid between *Cottus perifretum* Freyhof et al. 2005 and *Cottus rhenanus* Freyhof et al. 2005 (eg, Nolte, Freyhof, Stemshorn, & Tautz, 2005). Thus, the study of the hybridizations between *P. pungitius*, *P. laevis*, and *P. vulgaris* is strongly needed to understand the mechanisms of speciation by hybridization.

REFERENCES

Abbott, R., Albach, D., Ansell, S., Arntzen, J. W., Baird, S. J. E., Bierne, N., . . . Zinner, D. (2013). Hybridization and speciation. *Journal of Evolutionary Biology*, *26*, 229–246.

Aldenhoven, J. T., Miller, M. A., Showers Corneli, P., & Shapiro, M. D. (2010). Phylogeography of ninespine sticklebacks (*Pungitius pungitius*) in North America: Glacial refugia and the origins of adaptive traits. *Molecular Ecology*, *19*, 4061–4076.

Bănărescu, P. M. (1992). *Zoogeography of freshwater. Distribution and dispersal of freshwater animals in North America and Eurasia. Tome II*. Wiesbaden, Germany: Aula-Verlag.

Bănărescu, P. M., & Paepke, H.-J. (2002). *The Freshwater Fishes of Europe. Vol. 5/III: Cyprinidae 2, Part III: carassius to Cyprinus*, Gasterosteidae. Wiebelsheim, Germany: Aula-Verlag.

Belliard, J., Boët, P., & Allardi, J. (1995). Evolution à long terme du peuplement piscicole du bassin de la Seine. *Bulletin Français de la Pêche et de la Pisciculture*, *337*(338/339), 83–91.

Belliard, J., Boët, P., & Tales, E. (1997). Regional and longitudinal patterns of fish community structure in the Seine River basin, France. *Environmental Biology of Fishes*, *50*, 133–147.

Berg, L. S. (1949). Freshwater fishes of the U.S.S.R. and adjacent countries. Vol. III. Translated from Russian in 1965. Israel Program for Scientific Translations, Jerusalem, Israel.

Bertin, L. (1925). Recherches bionomiques, biométriques et systématiques sur les épinoches (Gastérostéidés). *Ann Inst Océanogr Monaco*, *2*, 1–204.

Bertran, P., Andrieux, E., Antoine, P., Coutard, S., Deschodt, L., Gardère, P., . . . Van Vliet-Lanoë, B. (2014). Distribution and chronology of Pleistocene permafrost features in France: Database and first results. *Boreas*, *43*, 699–711.

Bichot, F. (2004). Les relations entre le bassin de la Dive et celui de la Haute Sèvre Niortaise – Synthèse des connaissances. BRGM RP-52865-FR report.

Blanchard, E. (1866). *Les poissons des eaux douces de la France: Anatomie, physiologie, description des espèces, mœurs, instincts, industrie, commerce, ressources alimentaires, pisciculture, législation concernant la pêche*. Paris, France: Baillère.

Boero, F., & Bernardi, G. (2014). Phenotypic vs genotypic approaches to biodiversity, from conflict to alliance. *Marine Genomics*, *17*, 63–64.

Carrel, G. (2002). Prospecting for historical fish data from the Rhone River basin: A contribution to the assessment of reference condition. *Archiv fur Hydrobiologie*, *155*, 273–290.

Chessel, D., Dufour, A. B., & Thioulouse, J. (2004). The ade4 package-I-One-table methods. *R News*, *4*, 5–10.

Collares-Pereira, M. J., Cowx, I. G., & Coelha, M. M. (2002). *Conservation of freshwater fishes: Options for the future*. Oxford, UK: Fishing News Books.

Coste, P. (1848). Nidification des épinoches et des épinochettes. *Mémoires présentés par divers savants à l'Académie des Sciences de l'Institut de France, Sciences mathématiques et physiques*, *10*, 574–588.

Costedoat, C., Chappaz, R., Barascud, B., Guillard, O., & Gilles, A. (2006). Heterogeneous colonization pattern of European Cyprinids, as highlighted by the dace complex (Teleostei: Cyprinidae). *Molecular Phylogenetics and Evolution*, *41*, 127–148.

Costedoat, C., & Gilles, A. (2009). Quaternary pattern of freshwater fishes in Europe: Comparative phylogeography and conservation perspective. *The Open Conservation Biology Journal*, *3*, 36–48.

Cracraft, J. (1983). Species concepts and speciation analysis. In R. F. Johnston (Ed.), *Current ornithology* (pp. 159–187). New York, USA: Plenum Press.

Craw, D., Burridge, C., Anderson, L., & Waters, J. M. (2006). Late Quaternary river drainage and fish evolution, Southland, New Zealand. *Geomorphology*, *84*, 98–110.

Cuvier, G. (1829). *Le Règne Animal, distribué d'après son organisation, pour servir de base à l'histoire naturelle des animaux et d'introduction à l'anatomie comparée*. Paris, France: Deterville.

Darriba, D., Taboada, G. L., Dallo, R., & Posada, D. (2012). jModeltest 2: More models, new heuristics and parallel computing. *Nature Methods*, *9*, 772.

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, *85*, 407–415.

De Soland, A. (1869). *Etude sur les poissons de l'Anjou*. Angers, France: Imprimerie P. Lachèse, Belleuvre et Dolbeau.

Denys, G. (2015). Taxonomie integrative des poissons d'eau douce de France métropolitaine. PhD thesis, Muséum national d'Histoire naturelle, Paris, France.

Denys, G. P. J., Dettai, A., Persat, H., Doadrio, I., Cruaud, C., & Keith, P. (2013). Status of the Catalan chub *Squalius laietanus* (Actinopterygii, Cyprinidae) in France: Input from morphological and molecular data. *Knowledge and Management of Aquatic Ecosystems*, *408*(4), 1–13.

Denys, G. P. J., Dettai, A., Persat, H., Hautecœur, M., & Keith, P. (2014). Morphological and molecular evidence of three species of pikes *Esox spp* (Actinopterygii, Esocidae) in France, including the description of a new species. *Comptes Rendus Biologies*, *337*, 521–534.

Denys, G. P. J., Geiger, M. F., Persat, H., Keith, P., & Dettai, A. (2015). Invalidity of *Gasterosteus gymnurus* (Cuvier, 1829) (Actinopterygii, Gasterosteidae) according to integrative taxonomy. *Cybium*, *39*, 37–45.

Dettai, A., Lautredou, A.-C., Bonillo, C., Goimbault, E., Busson, F., Causse, R., ... 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 Part II: Topical Studies in Oceanography*, *58*, 250–263.

Doadrio, I., Kottelat, M., & De Sostoa, A. (2007). *Squalius laietanus*, a new species of cyprinid fish from north-eastern Spain and southern France (Teleostei: Cyprinidae). *Ichthyological Exploration of Freshwaters*, *18*, 247–256.

Doadrio, I., & Madeira, M. J. (2004). A new species of the genus *Gobio* Cuvier, 1816 (Actinopterigii, Cyprinidae) from the Iberian Peninsula and southwestern France. *Graellsia*, *60*, 107–116.

Dudgeon, D., Arthington, A. H., Gessner, M. O., Kawabata, Z.-I., Knowler, D. J., Lévêque, C., ... Sullivan, C. A. (2006). Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biological Reviews*, *81*, 163–182.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research*, *32*, 1792–1797.

Etienne, R., & Larue, J.-P. (2011). Contribution à l'étude des liaisons Loire-Seine: Mise en évidence par l'étude des minéraux lourds de l'antécédence de la Loire en Sologne (Bassin Parisien, France). *Physio-Géo*, *5*, 269–291.

Feunteun, E., Rigaud, C., Elie, P., & Lefeuvre, J. C. (1992). Le marais doux endigué de Bourgneuf-Machecoul (Pays de Loire). Premiers éléments de connaissance du peuplement piscicole. Relation ichtyofaune-habitat et problèmes majeurs de gestion. *Revue des Sciences de l'Eau*, *5*, 509–528.

Fieseler, C., & Wolter, C. (2006). A fish-based typology of small temperate rivers in the northeastern lowlands of Germany. *Limnologica*, *36*, 2–16.

Flot, J.-F. (2010). SEQPHASE: A web tool for interconverting PHASE input/output files and FASTA sequence alignments. *Molecular Ecology Resources*, *10*, 162–166.

Freyhof, J., Kottelat, M., & Nolte, A. (2005). Taxonomic diversity of European *Cottus* with description of eight new species (Teleostei: Cottidae). *Ichthyological Exploration of Freshwaters*, *16*, 107–172.

Geiger, M. F., Herder, F., Monaghan, M. T., Almada, V., Barbieri, R., Bariche, M., ... Freyhof, J. (2014). Spatial heterogeneity in the Mediterranean Biodiversity Hotspot affects barcoding accuracy of its freshwater fishes. *Molecular Ecology Resources*, *14*, 1210–1221.

Gray, S. M., Robinson, B. W., & Parsons, K. J. (2005). Testing alternative explanations of character shifs against ecological character displacement in brook sticklebacks (*Culaea inconstans*) that coexist with ninespine sticklebacks (*Pungitius pungitius*). *Oecologia*, *146*, 25–35.

Gross, H. P. (1979). Geographic variation in European ninespine sticklebacks, *Pungitius pungitius*. *Copeia*, *1979*, 405–412.

Guo, B., Shikano, T., Wang, C., & Merilä, J. (2016). Complete mitochondrial genomes of the smooth tail nine-spined stickleback *Pungitius laevis* (Gasterosteifromes, Gasterosteidae). *Mitochondrial DNA Part B Resource*, *1*, 70–71.

Guo, B., Toli, E.-A., & Merilä, J. (2016). Complete mitochondrial genome of the nine-spined stickleback *Pungitius pungitius* (Gasterosteifromes, Gasterosteidae). *Mitochondrial DNA Part B Resuorce*, *1*, 72–73.

Hahn, C., Bachmann, L., & Chevreux, B. (2013). Reconstructing mitochondrial genomes directly from genomic next-generation sequencing reads – a baiting and iterative mapping approach. *Nucleic Acids Research*, *41*, e129.

Hantke, R. (1993). *Flussgeschichte Mitteleuropas*. Stuttgart, Germany: Ferdinand Enke Verlag.

Hebert, P. D. N., Cywinska, A., Ball, S. L., & deWaard, J. R. (2003). Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London B: Biological Sciences*, *270*, 313–321.

Herczeg, G., Turtiainen, M., & Merilä, J. (2010). Morphological divergence of North-European nine-spined sticklebacks (*Pungitius pungitius*): Signatures of parallel evolution. *Biological Journal of the Linnean Society*, *101*, 403–416.

Hertel R (1978). Über die "Ichtyographie der Elbe" des Johannes Kentmann. *Zoologische Abhandlunger (Dresden) 35*:75–100.

Hewitt, G. (2000). The genetic legacy of the Quaternary ice ages. *Nature*, *405*, 907–913.

Hey, J., Waples, R. S., Arnold, M. L., Butlin, R. K., & Harrison, R. G. (2003). Understanding and confronting species uncertainty in biology and conservation. *Trends in Ecology & Evolution*, *18*, 597–603.

Hinsinger, D. D., Debruyne, R., Thomas, M., Denys, G. P. J., Mennesson, M., Utge, J., & Dettai, A. (2015). Fishing for barcodes in the Torrent: From COI to complete mitogenomes on NGS platforms. *DNA Barcodes*, *3*, 170–186.

Hubert, N., & Hanner, R. (2015). DNA Barcoding, species delineation and taxonomy: A historical perspective. *DNA Barcodes*, *3*, 44–58.

Hureau, J. C., & Monod, T. (1973). *Check-list of the fishes of the Northeastern Atlantic and of the Mediterranean (Clofnam)*. Paris, France: Unesco.

Jörger, K. M., & Schrödl, M. (2013). How to describe a cryptic species? Practical challenges of molecular taxonomy. *Frontiers in Zoology*, *10*, 59.

Kawahara, R., Miya, M., Mabuchi, K., Near, T. J., & Nishida, M. (2009). Stickleback phylogenies resolved: Evidence from mitochondrial genomes and 11 nuclear genes. *Molecular Phylogenetics and Evolution*, *50*, 401–404.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., ... Drummond, A. (2012). Geneious basic: An integrated and

extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, 28, 1647–1649.

Keith, P., & Marion, L. (2002). Methodology for drawing up a Red List of threatened freshwater fish in France. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 12, 169–179.

Keith, P., Persat, H., Feunteun, E., & Allardi, J. (2011). *Les Poissons d'eau douce de France*. Paris, France: Collection Inventaires et Biodiversités, Biotope Editions, Mèze, Publications scientifiques du Muséum.

Keivany, Y., & Nelson, J. S. (2000). Taxonomic review of the genus *Pungitius*, ninespine sticklebacks (Gasterosteidae). *Cybium*, 24, 107–122.

Keivany, Y., & Nelson, J. S. (2004). Phylogenetic relationships of sticklebacks (Gasterosteidae), with emphasis on ninespine sticklebacks (*Pungitius* spp.). *Behaviour*, 141, 1485–1497.

Kottelat, M. (1997). European freshwater fishes. An heuristic checklist of the freshwater fishes in Europe (exclusive of former USSR), with an introduction for non-systematists and comments on nomenclature and conservation. *Biologia, Bratislava* 52(5): 1–271.

Kottelat, M. (2007). Three new species of *Phoxinus* from Greece and southern France (Teleostei: Cyprinidae). *Ichthyological Exploration of Freshwaters*, 18, 145–162.

Kottelat, M., & Freyhof, J. (2007). *Handbook of European freshwater fishes*. Cornol, Switzerland: Publication Kottelat.

Kottelat, M., & Persat, H. (2005). The genus *Gobio* in France, with reedescription of *G. gobio* and description of two new species (Teleostei: Cyprinidae). *Cybium*, 29, 211–234.

Kullander, S. O. (1999). Fish species – how and why. *Reviews in Fish Biology and Fisheries*, 9, 325–352.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger data sets. *Molecular Biology and Evolution*, 33, 1870–1874.

Larue, J.-P. (2003). L'encaissement inégal de la Seine et de la Loire dans le Bassin parisien (France). *Géographie Physique et Quaternaire*, 57, 21–36.

Le Gall, O. (2010). Influences des glaciaires-interglaciaires sur les ichtyofaunes des eaux douces européennes. *Quaternaire*, 21, 203–214.

Li, B., Dettai, A., Cruaud, C., Couloux, A., Desoutter-Meniger, M., & Lecointre, G. (2009). RNF213, a new nuclear marker for acanthomorph phylogeny. *Molecular Phylogenetics and Evolution*, 50, 345–363.

Maire, A., Buisson, L., Biau, S., Canal, J., & Laffaille, P. (2013). A multifaceted framework of diversity for prioritizing the conservation of fish assemblages. *Ecological Indicators*, 34, 450–459.

Maire, A., Buisson, L., Canal, J., Rigault, B., Boucault, J., & Laffaille, P. (2015). Hindcasting modelling for restoration and conservation planning: Application to stream fish assemblages. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 25, 839–854.

Maire, A., Laffaille, P., Maire, J. F., & Buisson, L. (2017). Identification of priority areas for the conservation of stream fish assemblages: Implications for river management in France. *River Research and Applications*, 33, 524–537.

Makhrov, A. A., & Bolotov, I. N. (2006). Dispersal routes and species identification of freshwater animals in Northern Europe: A review of molecular evidence. *Russian Journal of Genetics (Translation of Genetika (Moscow, Russian Federation))*, 42, 1101–1115.

Mäkinen, H. S., & Merilä, J. (2008). Mitochondrial DNA phylogeography of the three-spined stickleback (*Gasterosteus aculeatus*) in Europe – Evidence for multiple glacial refugia. *Molecular Phylogenetics and Evolution*, 46, 167–182.

Mattern, M. Y. (2004). Molecular phylogeny of the Gasterosteidae: The importance of using multiple genes. *Molecular Phylogenetics and Evolution*, 30, 366–377.

Mattern, M. Y., & McLennan, D. A. (2004). Total evidence phylogeny of Gasterosteidae: Combining molecular, morphological and behavioural data. *Cladistics*, 20, 14–22.

Mauduyt, F. (1848). Ichthyologie de la Vienne, ou tableau méthodique et descriptif des poissons qui vivent habituellement dans les eaux de ce département ou qui y remontent périodiquement et accidentellement. *Bulletin de la Société académique d'agriculture, belles-lettres, sciences et arts, établie à Poitiers*, 3(9–12), 8–49.

McLennan, D. A., & Mattern, M. Y. (2001). The phylogeny of the gasterosteidae: combining behavioral and morphological data sets. *Cladistics*, 17, 11–27.

Meguro, Y., Takahashi, H., Machida, Y., Shirakawa, H., Gaither, M. R., & Goto, A. (2016). Assortative mating and divergent male courtship behaviours between two cryptic species of nine-spined sticklebacks (genus *Pungitius*). *Behaviour*, 153, 1879–1911.

Merilä, J. (2013). Nine-spined stickleback (*Pungitius pungitius*): An emerging model for evolutionary biology research. *Annals of the New York Academy of Sciences*, 1289, 18–35.

Mobley, K. B., Lussetti, D., Johansson, F., Englund, G., & Bokma, F. (2011). Morphological and genetic divergence in Swedish postglacial stickleback (*Pungitius pungitius*) populations. *BMC Evolutionary Biology*, 11, 287.

Moreau, E. (1881). *Histoire naturelle des poissons de la France*. Paris, France: Masson.

Münzing, J. (1969). Variabilität, verbreitung und systematik der arten und unterarten der Gattung *Pungitius* Coste, 1848 (Pisces, Gasterosteidae). *Journal of Zoological Systematics and Evolutionary Research*, 7, 208–233.

Nolte, A. W., Freyhof, J., Stemshorn, K. C., & Tautz, D. (2005). An invasive lineage of sculpins, *Cottus* sp. (Pisces, Teleostei) in the Rhine with new habitat adaptations has originated from hybridization between old phylogeographic groups. *Proceedings of the Royal Society B*, 272, 2379–2387.

Nolte, A. W., & Tautz, D. (2010). Understanding the onset of hybrid speciation. *Trends in Genetics*, 26, 54–58.

Oberdorff, T., Pont, D., Hugueny, B., Belliard, J., Berrebi dit Thomas, T., & Porcher, J. P. (2002). Adaptation et validation d'un indice poisson (FBI) pour l'évaluation de la qualité biologique des cours d'eau français. *Bulletin Français de la Pêche et de la Pisciculture*, 365(366), 405–433.

Padial, J. M., Miralles, A., De La Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, 7, 1–16.

Passerat, C. (1911). Les origines de la vallée de la Charente. *Annales de Géographie*, 20, 213–232.

Persat, H., & Keith, P. (2011). Biogéographie et historique de la mise en place des peuplements ichtyologiques de France métropolitaine. In P. Keith, H. Persat, E. Feunteun, & J. Allardi (Eds.), *Les Poissons d'eau douce de France* (pp. 37–93). Mèze and Paris, France: Collection Inventaires & biodiversité, Biotope Editions, Publications scientifiques de Muséum.

R Core Team (2016). *R: A language and environment for statistical computing*. Vienna: R Development Core Team. Retrieved from: http://www. R-project.org/. Accessed 31th October 2016.

Ratnasingham, S., & Hebert, P. D. N. (2007). BOLD: The barcode of life data system (www.barcodinglife.org). *Molecular Ecology Notes*, 7, 355–364.

Ravinet, M., Ishikawa, A., & Kitano, J. (2016). Trophic niche differentiation and phenotypic divergence among cryptic species of Japanese ninespine sticklebacks. *Evolutionary Ecology Research*, 17, 505–523.

Reshetnikov, Y. S., Bogutskaya, N., Vasilieva, E. D., Dorofeyeva, E. A., Naseka, A., Popova, O. A., . . . Sokolov, L. I. (1997). An annotated check-list of the freshwater fishes of Russia (in Russian). *Journal of Ichthyology*, 37, 723–771.

Ride, W. (1999). *International code of zoological nomenclature*. London, UK: International Trust for Zoological Nomenclature.

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., . . . Huelsenbeck, J. P. (2012). MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, 61, 539–542.

Santoul, F., Figuerola, J., Mastrorillo, S., & Céréghino, R. (2005). Patterns of rare fish and aquatic insects in a southwestern French river catchment in relation to simple physical variables. *Ecography*, *28*, 307–314.

Sauvage, H. E. (1874). Révision des espèces du groupe des Epinoches. *Nouvelles archives du Muséum d'histoire naturelle de Paris*, *10*, 5–38.

Schlick-Steiner, B. C., Seifert, B., Stauffer, C., Christian, E., Crozier, R. H., & Steiner, F. M. (2007). Without morphology, cryptic species stay in taxonomic crypsis following discovery. *Trends in Ecology & Evolution*, *22*, 391–392.

Schlick-Steiner, B. C., Steiner, F. M., Seifert, B., Stauffer, C., Christian, E., & Crozier, R. H. (2010). Integrative taxonomy: a multisource approach to exploring biodiversity. *Annual Review of Entomology*, *55*, 421–438.

Shikano, T., Laine, V. N., Herczeg, G., Vilkki, J., & Merilä, J. (2013). Genetic architecture of parallel pelvic reduction in ninespine sticklebacks. *G3: Genes, Genomes, Genetics*, *3*, 1833–1842.

Sideleva, V. G. (2009). A new sculpin species *Cottus sabaudicus* sp. nova (Scorpaeniformes: Cottidae) from the Savoy District, France. *Journal of Ichthyology*, *49*, 209–214.

Spillmann, C. J. (1961). *Faune de France. Poissons d'Eau douce*, Vol. 65. Paris, France: Lechevalier.

Stephens, M., Smith, N. J., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics*, *68*, 978–989.

Takahashi, H., Møller, P. R., Shedko, S. V., Ramatulla, T., Joen, S.-R., Zhang, C.-G., . . . Nishida, M. (2016). Species phylogeny and diversification process of Northeast Asian *Pungitius* revealed by AFLP and mtDNA markers. *Molecular Phylogenetics and Evolution*, *99*, 44–52.

Takahashi, H., & Takata, K. (2000). Multiple lineages of the mitochondrial DNA introgression from *Pungitius pungitius* (L.) to *Pungitius tymensis* (Nikolsky). *Canadian Journal of Fisheries and Aquatic Science*, *57*, 1814–1833.

Teletchea, F. (2010). After 7 years and 1000 citations: Comparative assessment of the DNA barcoding and the DNA taxonomy proposals for taxonomists and non-taxonomists. *Mitochondrial DNA*, *21*, 206–226.

Thompson, C. E., Taylor, E. B., & McPhail, J. D. (1997). Parallel evolution of lake-stream pairs of threespine sticklebacks (*Gasterosteus*) inferred from mitochondrial DNA variation. *Evolution*, *51*, 1955–1965.

Välimäki, K., Herczeg, G., & Merilä, J. (2012). Morphological anti-predator defences in the nine-spined stickleback: Constitutive, induced or both? *Biological Journal of the Linnean Society*, *107*, 854–866.

Vallot, J. N. (1850). *Supplément à l'Ichthyologie française: et tableau général des poisons d'eau douce de la France*. Dijon, France: Imprimerie de E. Trichault.

Vanderberghe, J., French, H. M., Gorbunov, A., Marchenko, S., Velichko, A. A., Jin, H., . . . Wan, X. (2014). The Last Permafrost Maximum (LPM) map of the Northern Hemisphere: Permafrost extent and mean annual air temperatures, 25-17 ka BP. *Boreas*, *43*, 652–666.

Vogler, A. P., & Monaghan, M. T. (2007). Recent advances in DNA taxonomy. *Journal of Zoological Systematics and Evolutionary Research*, *45*, 1–10.

Wang, C., Shikano, T., Persat, H., & Merilä, J. (2015). Mitochondrial phylogeography and cryptic divergence in the stickleback genus *Pungitius*. *Journal of Biogeography*, *42*, 2334–2348.

Wang, C., Shikano, T., Persat, H., & Merilä, J. (2017). Phylogeography and historical introgression in smoothtail nine-spined sticklebacks, *Pungitius laevis* (Gasterosteiformes: Gasterosteidae). *Biological Journal of the Linnean Society*, *121*, 340–354.

Wheeler, A. C. (1985). The Linnaean fish collection in the Linnean Society of London. *Zoological Journal of the Linnean Society*, *84*, 1–76.

Wiley, E. O., & Mayden, R. L. (2000). The evolutionary species concept. In Q. D. Wheeler, & R. Meier (Eds.), *Species concepts and phylogenetic theory – a debate* (pp. 70–89). New York, USA: Columbia University Press.

Wootton, R. J. (1984). *The functional biology of the sticklebacks*. London, UK: Academic Press.

Yates, A., Akanni, W., Amode, M. R., Billis, K., Carvalho-Silva, D., Cummins, C., . . . Flicek, P. (2016). Ensembl 2016. *Nucleic Acids Research*, *44*, 710–716.

Ziuganov, V. V., & Gomeluk, V. Y. (1985). Hybridization of two forms of ninespine stickleback, *Pungitius pungitius* and *P. platygaster*, under experimental conditions and an attempt to predict the consequences of their contact in nature. *Environmental Biology of Fishes*, *13*, 241–251.
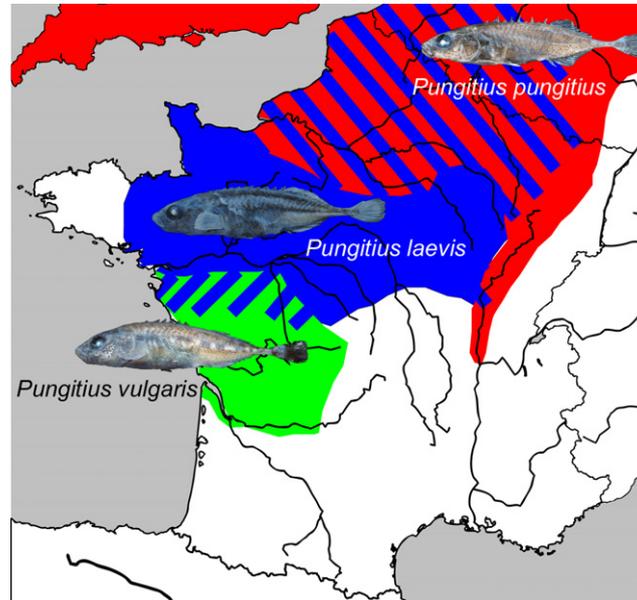
SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

Graphical Abstract

The contents of this page will be used as part of the graphical abstract of html only. It will not be published as part of main article.

This study is an integrative taxonomical review of French nine-spined sticklebacks (*Pungitius* spp.): morphological, mitochondrial (*COI*), and nuclear (*RNF213*) data. The results highlight three species in France: *Pungitius pungitius* (North of France and Rhone basin; red), *Pungitius laevis* (English Channel basins and Loire drainage; blue), and the endemic *Pungitius vulgaris* (from the Vienne River to the Garonne estuary; green). Our results confirm the existence of a hybridization zone in the North of France between *P. pungitius* and *P. laevis*.