Invalidity of *Gasterosteus gymnurus* (Cuvier, 1829) (Actinopterygii, Gasterosteidae) according to integrative taxonomy

by

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Abstract. – The three-spined stickleback *Gasterosteus* spp. is a model organism largely used in biology. Four species have been described in Europe: *G. aculeatus*, *G. gymnurus*, *G. islandicus* and the extinct *G. crenobiontus*. Our integrative taxonomy study, including 194 specimens, demonstrates that these taxa are distinguishable neither with the mitochondrial COI marker nor the morphological characters like lateral plates, supposed to differentiate them. Thus, we invalidate *Gasterosteus gymnurus*, and we consider it as junior synonym of *Gasterosteus aculeatus*. The taxonomical status of *Gasterosteus islandicus* still needs to be clarified.

Résumé. – Invalidité de *Gasterosteus gymnurus* (Cuvier, 1829) (Actinopterygii, Gasterosteidae) confirmée par la taxonomie intégrative.

L'épinoche *Gasterosteus* spp. est un organisme modèle utilisé en biologie. Quatre espèces sont connues en Europe : *G. aculeatus*, *G. gymnurus*, *G. islandicus* et *G. crenobiontus* qui est éteinte. Notre étude de taxonomie intégrative, comprenant 194 spécimens, démontre que ces taxons ne peuvent être distingués ni avec le marqueur mitochondrial COI, ni avec les données morphologiques comme les plaques latérales. Nous invalidons donc l'espèce *Gasterosteus gymnurus*, et nous la considérons comme synonyme junior de *Gasterosteus aculeatus*. Le statut taxonomique de *Gasterosteus islandicus* nécessite d'être clarifié.

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Gasterosteus islandicus
Integrative taxonomy
Cytochrome C oxidase subunit 1

The three-spined stickleback *Gasterosteus aculeatus* Linnaeus, 1758 (Actinopterygii, Gasterosteidae) has for decades been a model organism for evolutionary, behavioural, developmental and ecological research, which qualified it as "supermodel" (Merilä, 2013). It inhabits sea or freshwater, and has a very large distribution in circumarctic and temperate regions (Froese and Pauly, 2014). Its taxonomy has often been discussed as it is very polymorphic, especially regarding plate morphs. 47 species were described throughout the world (Eschmeyer, 2014), and have been mainly considered to be synonyms to the species present in Western Europe, *G. aculeatus* and *Gasterosteus gymnurus* Cuvier, 1829 (Kottelat, 1997). Four other species are currently considered valid: *Gasterosteus wheatlandi* Putnam, 1867, *Gasterosteus islandicus* Sauvage, 1874, *Gasterosteus crenobiontus* Bacescu and Mayer, 1956 (extinct), and *Gasterosteus nipponicus* Higuchi *et al*., 2014, respectively endemic to North-West America, Iceland, Central of Europe and Japan. Considering morphological and molecular studies (*e.g*. Münzing, 1962; Mäkinen and Merilä, 2008), Kottelat and Freyhof (2007) changed the assignation of West European sticklebacks into *G. gymnurus*, because of the low plate numbers on the flanks and the absence of a keel on the caudal peduncle. Keith *et al*. (2011) considered it to be the only species of three-spined stickleback in France, whereas Iglésias (2012) recognized both species. However, several sticklebacks "forms" in France, especially in the coastal basins of the Channel (North-West of France), display a variability in their lateral plate numbers, as already noted by Bertin (1925). This is of particular importance, as *G. gymnurus* was described solely on the character "presence of plates only on pectoral region" (translated from French: "*G. gymnurus* (…) n'a de ces plaques que dans la région pectorale"; Cuvier 1829). A morphological and morphometric study (Woltmann and Berg, 2013) on more than 5000 German individuals belonging to both forms found no difference between *G. aculeatus* and *G. gymnurus*.

The aim of this study is to verify the status of *G. aculeatus* and *G. gymnurus* using DNA taxonomy *sensu* Tautz *et al*. (2003), combining morphological characters and the mito-

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chondrial gene coding for cytochrome C oxidase subunit 1 (COI) on a large circumpolar sampling, in order to evaluate both species in an integrative taxonomy approach (Padial *et al.*, 2010). It occurs in a context where the taxonomy of European ichthyofauna undergoes many discoveries and changes (Kottelat and Freyhof, 2007; Denys *et al.*, 2014; Geiger *et al.*, 2014).

MATERIALS AND METHODS

Sampling

Samples were collected between 2003 and 2013 with collaborations of the FREDIE program (http://www.fredie.eu), the French National Agency for Water and Aquatic Environments (Onema), and the Fédération nationale de la pêche en France (FNPF). A total of 174 specimens were caught, mainly by electrofishing, in 30 locations in the major French drainages. Ninety-five individuals from 37 other European sites were added (Fig. 1; Annexe 1). Specimens were fixed in 95% EtOH for later fin clipping. We did all morphological identification (juveniles excepted): *G. aculeatus* has a complete series of 29-35 bony scutes covering the trunk and the caudal peduncle (Fig. 2A), whereas *G. gymnurus* has only 2-10 scutes on the anterior part of the trunk (Fig. 2B), and *G. islandicus* has a deep notch on the anterior margin of the pelvic girdle (*vs.* straight or rounded for the other taxa) (Kottelat and Freyhof, 2007). One hundred sixty two specimens were used for DNA analyses.

DNA extraction, PCR, sequencing and quality control were performed according to Geiger *et al.* (2014) and for French samples Denys *et al.* (2014), yielding a dataset of partial COI sequences (627 bps). It was completed by sequences from other *Gasterosteus* species with locality and photo vouchers available on the Barcode of Life database (BOLD, www.boldsystems.org; Ratnasingham and Hebert, 2007) and GenBank. Several other gasterosteid species were used as outgroups (Fig. 1; Annexe 1). All new COI sequences were deposited in the EUEPI project folder in the BOLD with their voucher information. Alignment was performed manually, as neither marker includes indels. Phylogenetic analyses were performed with Bayesian inference (MrBayes 3.2, Ronquist *et al.*, 2012), with the GTR+I+G model selected by JModelTest 2.1.1 (Darriba *et al.,* 2012). 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 inter-specific distances (p-distances) were calculated with MEGA 6 (Tamura *et al.*, 2013).

RESULTS

The morphological identification according to Kottelat and Freyhof (2007) of the 159 non-juvenile three-spined sticklebacks (*i.e.* over 30 mm SL) placed *G. islandicus* (one population, four specimens) in Iceland, *G. gymnurus* (40 populations, 100 specimens) from Portugal to Turkey including United Kingdom and Mediterranean drainages, and *G. aculeatus* (28 populations, 51 specimens) from Channel coast and east of Europe to Alaska and Canada. Mediterranean and all French populations were determined as *G. gymnurus*, except one from Taute stream in Normandy (MNHN 2013-1299), which was identified as *G. aculeatus*. The pop-

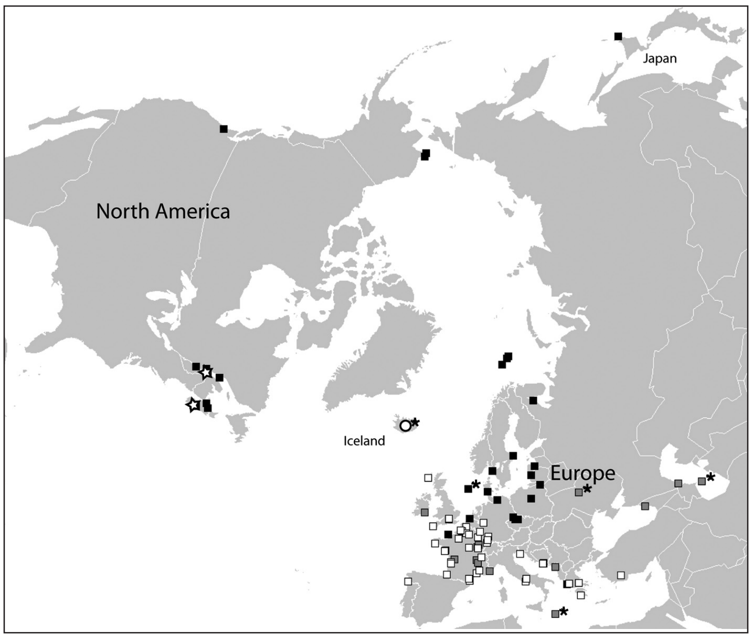

Figure 1. - Geographical distribution of the vouchers used in the analysis in polar view. *Gasterosteus aculeatus* (black squares), *G. gymnurus* (white squares), *G. islandicus* (white circle), *G. wheatlandi* (white stars) and unidentified morphologically specimens (grey squares). Asterisks mean approximative locations.

ulations from the North Sea (ZFMK:ICH:55427), Acheron in Greece (ZFMK:ICH:55875 to 55879), and the Japanese specimen (UW 41883) were also identified as *G. aculeatus*.

Genetic analyses were performed on 194 individuals for the COI marker. Twenty-four specimens belonging to *G. aculeatus* have identical sequences to specimens identified as *G. gymnurus*.

In the Bayesian phylogenetic tree reconstruction (Fig. 2), a deep divergence (12.62% mean pairwise divergence) separates *G. wheatlandi* from the strongly supported (1 ppv) clade including *G. aculeatus*, *G. gymnurus* and *G. islandicus*. The clade is a large polytomy; the variability within it is 0.36% pairwise divergence, and it has no taxonomical structure. Two *G. gymnurus* individuals from the United Kingdom (North Sea: Ex70G2 and Ex70G4) are included into a Canadian subclade of *G. aculeatus* supported by one synapomorphy (A in position 401). However, several subclades are geographically homogeneous, like a Western Mediterranean subclade from France to Italy (except for Brague stream) supported by two synapomorphies (G in positions 14 and 547), with moderate support (0.87 ppv) and mean divergence from the others of 0.5%. On the other hand, some localities from surrounding areas or even areas within the geographic areas of these subclades, have different haplotypes.

DISCUSSION

Münzing (1962) drew a map of Europe with the repartition of the different plate morphs. He distinguished clearly the plateless morph "*leiurus*" in the West part of Europe, whereas the morph with plates "*trachurus*" occurs from Channel to Baltic Sea. Our morphological identifications demonstrate globally the same pattern. But one population in Channel drainage is fully plated; other French and English populations have just the lower part of the flank covered. Bertin (1925) noticed that "forms" covered by plates "*trachura*" occurred only in costal basin of Channel, but during their growth, they passed by the other forms having less plates: "*hologymna*", "*gymnura*" and "*semiarmata*" (see Bakker and Sevenster, 1988 for the terminology review). This is why care must be taken to compare only adults, which is the case of our specimens. Moreover, one Greek population (Acheron drainage) is fully plated, whereas all other Mediterranean populations have a few plates. Thus, the morphological identification demonstrates that the species distinction between *G. aculeatus* and *G. gymnurus* using plate numbers and arrangement as key character does not lead to a clear biogeographic pattern.

The lateral plate number is controlled by the ectodysplasin-A *Eda* gene, with a stronger expression in adults than juveniles (see Barrett, 2010). Plate presence is strongly correlated to the habitat: marine specimens are totally covered with plates, whereas freshwater populations are less shielded (Barrett 2010; Merilä 2013). A coastal-inland gradient as positive relationship between salinity and plate numbers was documented (Raeymaekers *et al.*, 2007), activating firstly the anterior plates development, followed by posterior plates and finally middle plates (Bell, 2001). So plate reduction probably constitutes an adaptation to freshwater habitat (Le Rouzic *et al.*, 2011) colonized by fully plated ancestral marine populations, and often occurring in numerous cases through allelic substitution in the *Eda* gene (Leinonen *et al.*, 2012). The main explanation for these phenotypes is that lateral plates serve as defensive structures against the teeth of predatory fish in marine environments, but they alter the escape capacity in freshwater (Barrett, 2010).

Density of plates is therefore not an appropriate taxonomical criterion for European three-spined sticklebacks, but represents an intraspecific polymorphism induced by habitat, contrary to *G. nipponicus*, which has a characteristic plate morph and is supported by ecological, chromosomal

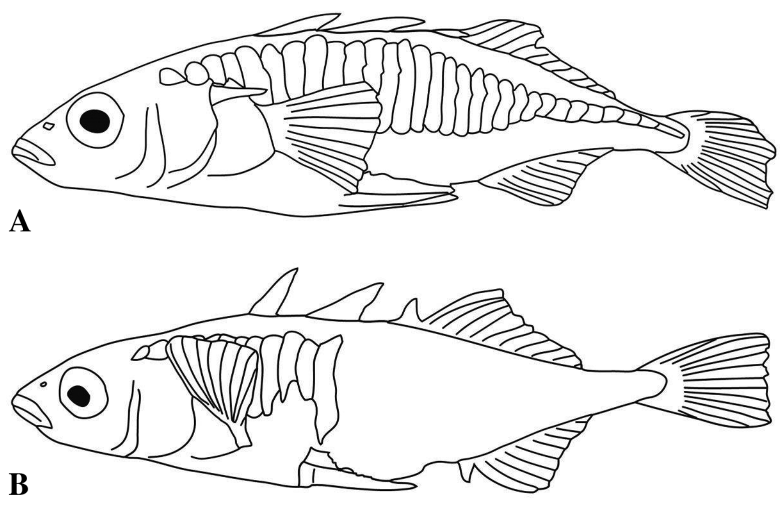

Figure 2. - **A**: *Gasterosteus aculeatus*, MNHN 2013-1299, FFFtag12285, 35 mm SL, Taute (Douve drainage) at Tribehou, France, 17 Sep. 2013; **B**: *G. gymnurus*, MNHN 2014-0010, FFFtag12314, 42 mm SL, Blaise (Seine drainage) at Saint-Ange-et-Torçais, France, 24 Sep. 2013.

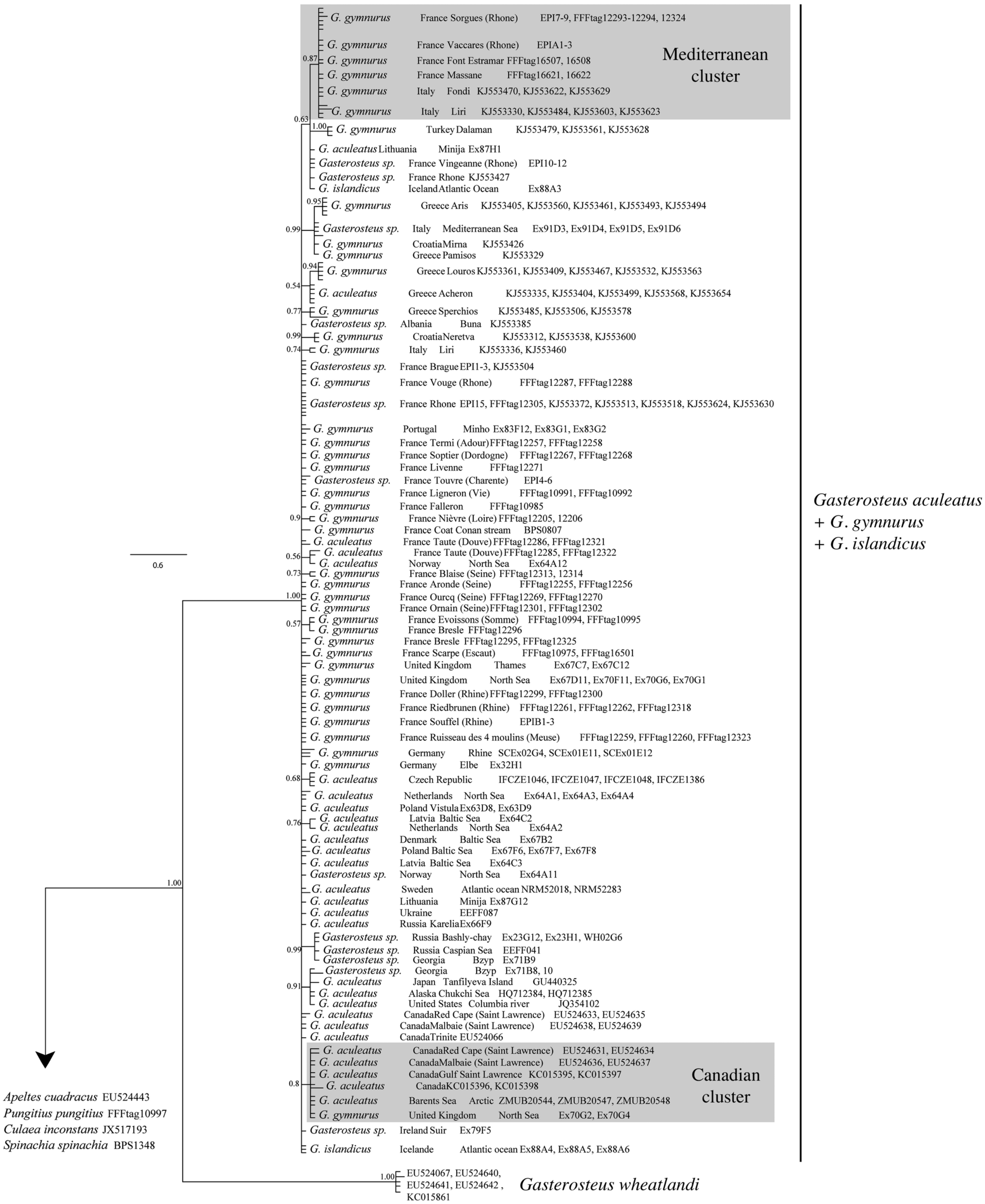

Figure 3. - Bayesian tree of the cytochrome c oxidase subunit I (COI) for 194 individuals of *Gasterosteus* spp. and other gasterosteids. The mean *a posteriori* values of the parameters are: TL = 38.329096; alpha = 0.076817; pinvar = 0.308267. As a reminder, *G. aculeatus* is full plated, whereas *G. gymnurus* has two to 10 lateral plates on both sides.

and reproduction data (Higuchi *et al.*, 2014). Besides, the plateless morph has been observed from Norway to Alaska *via* Iceland (Barrett, 2010), and is not characteristic to any European area. "Presence of plates only on pectoral region" is the sole character in the original description of *G. gymnurus*. Moreover, as no type specimen is currently known (Kottelat, 1997), no taxonomical review on a reference individual is possible.

The DNA taxonomy analysis with the COI marker does not differentiate *G. aculeatus* from *G. gymnurus* and *G. islandicus*. Geographically, a few divergences were observed on the mitochondrial cytochrome b and control region markers between European, American and Japanese populations, because of recent colonisations estimated at 90,000 to 260,000 years from Alaska and British Columbia to Atlantic (Ortí *et al.*, 1994; Mäkinen and Merilä, 2008). Several transatlantic exchanges have taken place during this period (Mäkinen and Merilä, 2008). In addition, Mäkinen *et al.* (2006) demonstrate the absence of a relationship between phylogeny and habitat. While this lack of differentiation could be due to insufficient variability of the marker, it is here combined to the lack of morphological characters other than the highly plastic plate numbers.

Mediterranean populations are distinct from West European populations, however the dataset is not well representative of the area (Mäkinen *et al.*, 2006; Cano *et al.*, 2008). Consequently, Bianco (2014) proposed that Mediterranean populations constitute another lineage. Our results also recover a Western Mediterranean subclade, but it does not include all Mediterranean specimens. Furthermore, the type locality of *G. gymnurus* is in England and North of France (Cuvier, 1829).

In consequence, we consider *Gasterosteus gymnurus* Cuvier, 1829 as a junior synonym of *G. aculeatus* Linnaeus, 1758.

G. islandicus is endemic to Iceland, and is recognizable by its notch in pelvic girdle (Kottelat and Freyhof, 2007). The four specimens in our dataset are also included in the same cluster as *G. aculeatus* and *G. gymnurus*. As some populations possibly belonging to this species were found in Norway and Finland (in sympatry with *G. aculeatus*; Kottelat and Freyhof, 2007), the presence of the notch in pelvic girdle might also be a polymorphic character. More investigations are needed in order to clarify this species delineation.

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Annexe 1 - List of vouchers used with BOLD and GenBank Accession numbers.

Morphological identification	Country	Locality (Drainage)	Collection Number	BOLD samples Ids (for new sequences)	GenBank accession numbers	Presence of plates
Gasterosteus aculeatus	Alaska	Chuckchi Sea	CAS 230129-01 and 230129-02		HQ712384-712385	yes
Gasterosteus aculeatus	Arctic	Barents Sea	ZMUB20544, 20547 and 20548			yes
Gasterosteus aculeatus	Canada	Red Cape (Saint Lawrence)	ROM-80233		EU524631, EU524633, EU524634, EU524635	yes
Gasterosteus aculeatus	Canada	Malbaie (Saint Lawrence)	ROM-80264		EU524636, EU524637, EU524638, EU524639	yes
Gasterosteus aculeatus	Canada	Trinite	ROM-80256		EU524066	yes
Gasterosteus aculeatus	Canada	Gulf Saint Lawrence	ARC 26106 and 26590		KC015395, KC015397	yes
Gasterosteus aculeatus	Canada				KC015396, KC015398	yes
Gasterosteus aculeatus	Czech Republic			IFCZE1046-1048, 1386		yes
Gasterosteus aculeatus	Denmark	Baltic Sea	ZFMK:ICH:53588	Ex67B2		no
Gasterosteus aculeatus	France	Taute (Douve)	MNHN 2013-1299	FFFtag12285-12286, 12321-12322		yes
Gasterosteus aculeatus	Germany	Rhine	ZFMK:ICH:58260	SCEx01E12		yes
Gasterosteus aculeatus	Germany	Elbe	FSJF:1215	Ex32H1		yes
Gasterosteus aculeatus	Japan	Tanfilyeva Island	UW 41883		GU440325	yes
Gasterosteus aculeatus	Latvia	Baltic Sea	ZFMK:ICH:50541-50542	Ex64C2-3		yes
Gasterosteus aculeatus	Lithuania	Minja	ZFMK:ICH:59256-59257	Ex87G12, Ex87H1		yes
Gasterosteus aculeatus	Netherlands	North Sea	ZFMK:ICH:50077 to 50080	Ex64A1; Ex64A2; Ex64A3; Ex64A4		no
Gasterosteus aculeatus	Poland	Vistula	ZFMK:ICH:P124	Ex63D8		yes
Gasterosteus aculeatus	Poland	Vistula	ZFMK:ICH:P123	Ex63D9		no
Gasterosteus aculeatus	Poland	Baltic Sea	ZFMK:ICH:53736 to 53738	Ex67F6-8		yes
Gasterosteus aculeatus	Russia	Karelia	ZFMK:ICH:53254	Ex66F9		yes
Gasterosteus aculeatus	Sweden	Atlantic Ocean	NRM52018, NRM52283	NRM52018, 52283		yes
Gasterosteus aculeatus	UK	North Sea	ZFMK:ICH:55427	Ex70G2, Ex70G3		yes
Gasterosteus aculeatus	United States	Columbia River	UW 49025		JQ354102	yes
Gasterosteus aculeatus	Greece	Acheron	ZFMK:ICH:55875 to 55879		KJ553335, KJ553404, KJ553499, KJ553568, KJ553654	yes
Gasterosteus gymnurus	Croatia	Mirna	FSJF:198		KJ553426	no
Gasterosteus gymnurus	Croatia	Neretva	FSJF:2258 and 2261		KJ553312, KJ553538, KJ553600	no
Gasterosteus gymnurus	France	Sorgues (Rhone)	MNHN 2013-1303	EPI7-9, FFFtag12293-12294, 12324		no
Gasterosteus gymnurus	France	Vaccarès (Rhone)	UCBLZ 2012.9.498	EPIA1-3		no
Gasterosteus gymnurus	France	Font Estramar	MNHN 2013-0615	FFFtag16507-16508		no
Gasterosteus gymnurus	France	Massane	MNHN 2013-0672	FFFtag16621-16622		no
Gasterosteus gymnurus	France	Brague	ZFMK:ICH:55611		KJ553504	no
Gasterosteus gymnurus	France	Vouge (Rhone)	MNHN 2013-1300	FFFtag12287-12288		no

Annexe 1 - Continued.

Morphological identification	Country	Locality (Drainage)	Collection Number	BOLD samples Ids (for new sequences)	GenBank accession numbers	Presence of plates
Gasterosteus gymnurus	France	Rhone	MNHN 2013-1309; ZFMK:ICH:55616	FFFtag12305	KJ553372, KJ553513, KJ553518, KJ553624, KJ553630	no
Gasterosteus gymnurus	France	Termi (Adour)	MNHN 2013-1285	FFFtag12257-12258		no
Gasterosteus gymnurus	France	Soptier (Dordogne)	MNHN 2013-1290	FFFtag12267-12268		no
Gasterosteus gymnurus	France	Livenne	MNHN 2013-1292	FFFtag12271		no
Gasterosteus gymnurus	France	Ligneron (Vie)	MNHN 2013-807	FFFtag10991-10992		no
Gasterosteus gymnurus	France	Falleron	MNHN 2013-804	FFFtag10985		no
Gasterosteus gymnurus	France	Nièvre (Loire)	MNHN 2013-813	FFFtag12205-12206		no
Gasterosteus gymnurus	France	Blaise (Seine)	MNHN 2014-0010	FFFtag12313-12314		no
Gasterosteus gymnurus	France	Coat Conan stream (Brittany)		BPS-0807		no
Gasterosteus gymnurus	France	Aronde (Seine)	MNHN 2013-1284	FFFtag12255-12256		no
Gasterosteus gymnurus	France	Ourcq (Seine)	MNHN 2013-1291	FFFtag12269-12270		no
Gasterosteus gymnurus	France	Ornain (Seine)	MNHN 2013-1307	FFFtag12301-12302		no
Gasterosteus gymnurus	France	Evoissons (Somme)	MNHN 2013-0808	FFFtag10994-10995		no
Gasterosteus gymnurus	France	Bresle	MNHN 2013-1304	FFFtag12295-12296, 12325		no
Gasterosteus gymnurus	France	Scarpe (Escaut)	MNHN 2013-0613	FFFtag10975, 16501		no
Gasterosteus gymnurus	France	Ruisseau des 4 Moulins (Meuse)	MNHN 2013-1286	FFFtag12259-12260, 12323		no
Gasterosteus gymnurus	France	Doller (Rhine)	MNHN 2013-1306	FFFtag12299-12300		no
Gasterosteus gymnurus	France	Riedbrunen (Rhine)	MNHN 2013-1287	FFFtag12261-12262, 12318		no
Gasterosteus gymnurus	France	Souffel (Rhine)	UCBLZ 2012.9.521	EPIB1-3		no
Gasterosteus gymnurus	Germany	Rhine	ZFMK:ICH:58255	SCEx02G4, SCEx01E11		no
Gasterosteus gymnurus	Greece	Aris	ZFMK:ICH:55737 to 55741		KJ553405, KJ553461, KJ553493, KJ553494, KJ553560	no
Gasterosteus gymnurus	Greece	Pamisos	ZFMK:ICH:55869		KJ553329	no
Gasterosteus gymnurus	Greece	Louros	ZFMK:ICH:55870 to 55874		KJ553361, KJ553409, KJ553467, KJ553532, KJ553563	no
Gasterosteus gymnurus	Greece	Sperchios	ZFMK:ICH:55880 to 55882		KJ553485, KJ553506, KJ553578	no
Gasterosteus gymnurus	Italy	Fondi	ZFMK:ICH:59082 to 59084		KJ553470, KJ553622, KJ553629	no
Gasterosteus gymnurus	Italy	Liri	ZFMK:ICH:59101 to 59106		KJ553330, KJ553336, KJ553460, KJ553484, KJ553603, KJ553623	no
Gasterosteus gymnurus	Portugal	Minho	ZFMK:ICH:54867 to 54869	Ex83F12, EX83G1-2		no
Gasterosteus gymnurus	Turkey	Dalamian	FSJF:2585		KJ553479, KJ553561, KJ553628	no
Gasterosteus gymnurus	UK	Thames	ZFMK:ICH:53637 and 53668	Ex67C7, Ex67C12		no
Gasterosteus gymnurus	UK	North Sea	ZFMK:ICH:55426-55427 and 53692	Ex67D11, Ex70F11, Ex70G1, Ex70G6		no
Gasterosteus islandicus	Iceland	Atlantic Ocean	ZFMK:ICH:59275-59276	Ex88A3-4		no
Gasterosteus islandicus	Iceland	Atlantic Ocean	ZFMK:ICH:59277-59278	Ex88A5-6		yes

Annexe 1 - Continued.

Morphological identification	Country	Locality (Drainage)	Collection Number	BOLD samples Ids (for new sequences)	GenBank accession numbers	Presence of plates
Gasterosteus sp.	Albania	Buna	P6V 83689 (field label, in NM collections)		KJ553385	
Gasterosteus sp.	France	Vingeanne (Rhone)	MNHN 2014-0017	EPI10-12		
Gasterosteus sp.	France	Brague	MNHN 2014-0018	EPI1-3		
Gasterosteus sp.	France	Rhone	MNHN 2014-0019	EPI15		
Gasterosteus sp.	France	Touvre (Charente)	MNHN 2014-0020	EPI4-6		
Gasterosteus sp.	Georgia	Bzyp	K13-3 (field label, in ZIN collections)	Ex71B8-10		
Gasterosteus sp.	Ireland	Suir	ZFMK:ICH:54896	Ex79F5		
Gasterosteus sp.	Italy	Mediterranean Sea	GAS1 to 4 (field label, in ZFMK collections)	Ex91D3-6		
Gasterosteus sp.	Norway	North Sea	ZFMK:ICH:50107-50108	Ex64A11-12		
Gasterosteus sp..	Russia	Bashly-chay	Dar 66 (field label, in ZIN collections)	Ex23G12, Ex23H1, WH02G6		
Gasterosteus sp.	Russia	Caspian Sea	ZIN 54178	EEFF041		
Gasterosteus sp.	Ukraine	Dniestr	MY-1 G.a. (field label, in ZIN collections)	EEFF087		
Gasterosteus wheatlandi					EU524067, EU524640, EU524641, EU524642, KC015861	
Apeltes cuadracus					EU524443	
Pungitius pungitius	France	Evoissons (Somme)	MNHN 2013-0809	FFFtag10997		
Culaea inconstans					JX517193	
Spinachia spinachia				BPS-1348		