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First record of *Phoxinus csikii* Hankó, 1922 (Actinopterygii, Cypriniformes) in France

by

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Résumé. – Premier signalement de *Phoxinus csikii* Hankó, 1922 (Actinopterygii, Cypriniformes) en France.

Une identification moléculaire par barcoding (marqueur du *COI*, 651 pb) a été réalisée sur un vairon capturé dans le Rupt de Mad à Essey-et-Maizerais (bassin du Rhin/Moselle). Ainsi, ce spécimen appartient à l'espèce récemment revalidée *Phoxinus csikii* Hankó, 1922. Il s'agit ainsi du premier signalement de cette espèce en France, et de la quatrième espèce de vairon connue dans ce pays.

Key words. – France – Rhine basin – First record – Molecular identification – *Phoxinus csikii*.

European minnows *Phoxinus* are small leuciscids with an Eurasiatic distribution. They are rheophilic and cryophilic fishes living in cold streams and clear mountain lakes (Kottelat and Freyhof, 2007; Keith *et al.*, 2011). The taxonomical knowledge on this genus has progressed a lot since 2007 with the description of 7 new nominal species (*Phoxinus apollonicus* Bianco & De Bonis, 2015, *P. bigerri* Kottelat, 2007, *P. karsticus* Bianco & De Bonis, 2015, *P. ketmaieri* Bianco & De Bonis, 2015, *P. likai* Bianco & De Bonis, 2015, *P. septimaniae* Kottelat, 2007 and *P. strymonicus* Kottelat, 2007), 5 revalidated species (*P. colchicus* Berg, 1910, *P. csikii* Hankó, 1922, *P. lumaireul* (Schinz, 1840), *P. marsilii* Heckel, 1836 and *P. strandjae* Drensky, 1926) and the documentation of 8 other

evolutionary lineages in Europe (see Palandačić *et al.*, 2017a). Southern French catchments were also related to the description of two new species in addition to *P. phoxinus* (Linnaeus, 1758) in the French Northern drainages (Kottelat, 2007; Kottelat and Freyhof, 2007; Keith *et al.*, 2011): *P. bigerri* Kottelat, 2007 in the Adour drainage and *P. septimaniae* Kottelat, 2007 in the Mediterranean basin and also in the Rhône drainage considering molecular data (*e.g.* Palandačić *et al.*, 2017a).

Following these studies, the Rhine system harbours the following four minnow taxa: *Phoxinus phoxinus* (from where its neotype specimen (NRM 55108 from the River Agger) was designated by Kottelat (2007)), *P. csikii*, *P. cf. morella* (Leske, 1774) and *P. septimaniae* with potential cases of hybridization (Kneibelsberger *et al.*, 2015; Palandačić *et al.*, 2017a). *P. phoxinus* would be the native species whereas the three others would likely have an anthropogenic origin (Kneibelsberger *et al.*, 2015).

On 27th of September 2011, the French national agency for water and aquatic environments (Office National de l'Eau et des Milieux Aquatiques, ONEMA) did an inventory in a location on the Moselle catchment, the Rupt-de-Mad stream at Essey-et-Maizerais (Fig. 1), by electrofishing. The minnows caught were collected and examined in the Muséum national d'Histoire naturelle of Paris (MNHN). The aim of this study is to present the results of the molecular identification.

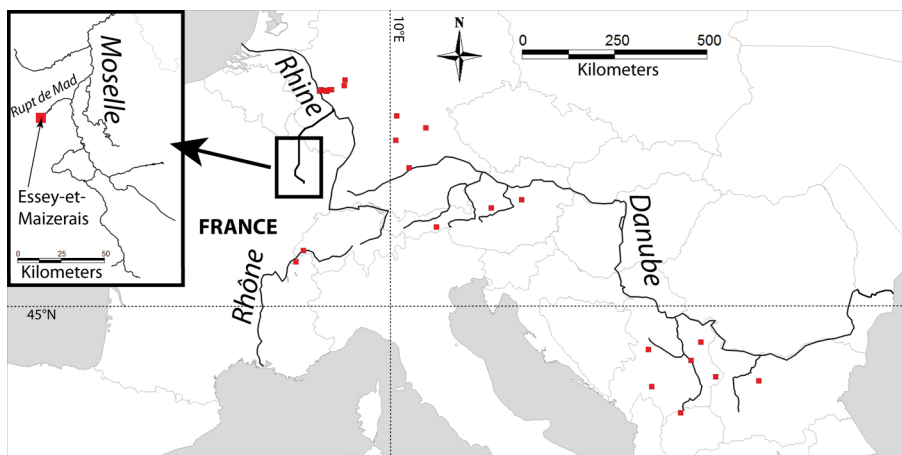


Figure 1. – Distribution of *Phoxinus csikii* Hankó, 1922 (red squares) in Europe with its first occurrence in France at Essey-et-Maizerais (top left box); modified and adapted from Palandačić *et al.* (2017a).

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Figure 2. – Two specimens of *Phoxinus csikii* Hankó, 1922 caught in the Rupt de Mad stream at Essey-et-Maizerais (MNHN 2011-0899): 57 mm SL ♀ (top) and 50.9 mm SL ♂ (bottom). Scale bar = 1 cm.

MATERIAL AND METHODS

The Rupt-de-Mad stream at Essay-et-Maizerais is located at 24 km from the source, in the Woëvre plains and near the Madine reservoir. The average width of the stream is 6.5 m for an average depth of 0.67 m with a clay and silty substrate. The aquatic vegetation is mainly composed of water lily. Finally, its minor bed is partially artificial due to old hydraulic works related to land consolidation. This location is monitored every two years since 2007.

Fishes were caught by electrofishing with two anodes and three nets carriers going up the stream by feet along 110 m.

Specimens were fixed and preserved in EtOH, concentrating it progressively from 40% to 95% in the span of a few hours in order to reduce shrinking effects. Some studies gave morphological criteria to identify minnow species (*e.g.* Kottelat, 2007); however, some authors do not find them sufficiently discriminant (Bianco, 2014; Palandačić *et al.*, 2017a; Denys, pers. obs.; Doadrio, pers. com.), or no morphological character was found to distinguish *P. phoxinus* from *P. csikii* (Walter, 2013). Moreover, the redescription of *P. csikii* was made only on its neotype specimen (NMW 98673) from Ibar River (Montenegro) designated by Palandačić *et*

al. (2017b). Accordingly, a DNA Barcoding analysis *sensu* Hebert *et al.* (2003), with a mitochondrial marker (cytochrome c oxidase subunit 1: *COI*), appears to be ideal for identification. From a fin clip, DNA extraction, PCR products and sequencing were performed according to Dettai *et al.* (2011). *COI* sequence (651 bp) was deposited in GenBank (accession number MK310229).

This sequence was integrated with 384 available sequences of *Phoxinus* spp. from GenBank (Appendix 1). Aligning, p-distances and NJ-tree reconstruction based on the DNA barcodes were performed under MEGA 7 (Kumar *et al.*, 2016) with the Kimura 2 parameter model (K2P; Kimura, 1980). Bootstrap values (Felsenstein, 1985) with 1000 replicates, were also calculated for evaluating the robustness of clusters.

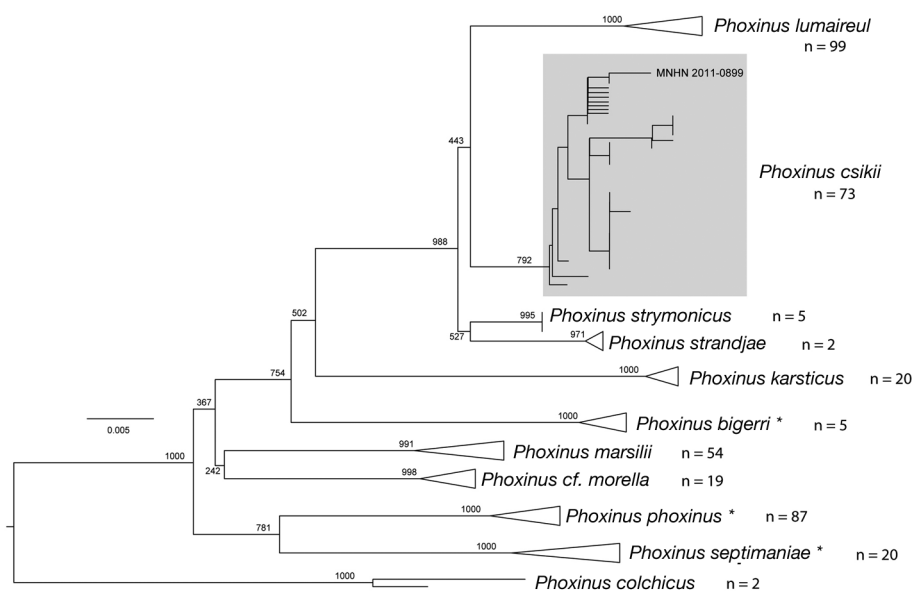
Finally, an observation of morphological characters was made and compared to the other species descriptions (Kottelat, 2007; Palandačić *et al.*, 2017a, b).

RESULTS AND DISCUSSION

Twenty-five minnows were caught among the following species: *Rutilus rutilus* (Linnaeus, 1758), *Squalius cephalus* (Linnaeus, 1758), *Cottus rhenanus* Freyhof *et al.*, 2005, *Gobio gobio* (Linnaeus, 1758), *Esox lucius* Linnaeus, 1758, *Barbatula barbatula* (Linnaeus, 1758), *Cobitis taenia* Linnaeus, 1758, *Tinca tinca* (Linnaeus, 1758), *Scardinius erythrophthalmus* (Linnaeus, 1758), *Leuciscus leuciscus* (Linnaeus, 1758) and the invasive decapod *Orconectes limosus* (Rafinesque, 1817). Two specimens of minnows, one male, 50.9 mm SL, and one female, 57 mm SL (catalog number MNHN 2011-0899; Fig. 2), were fixed and preserved for morphological and molecular identification (the female).

The DNA barcoding analysis discriminates 10 clusters already characterized by Palandačić *et al.* (2017a) with intraspecific distances from 0 to 1.5% and supported by bootstraps values from 792 to 1000. The female specimen was put into the *Phoxinus csikii* cluster (Fig. 3). The mean p-distance to the other sequences of this taxon is 0.7%, and from 2.2% to 6.2% to the other *Phoxinus* species. Thus, with clear evidence between intra- and interspecific distances, molecular data allows the identification of this specimen as *Phoxinus csikii*.

Figure 3. – Neighbour-Joining barcoding tree with a K2P model based on *COI* data (651 bp) of European minnows *Phoxinus* spp. (385 specimens) identifying the specimen caught at Essay-et-Maizerais (MNHN 2011-0899). Numbers at nodes correspond to bootstrap values (1000 replicates). Grey box refers to the *Phoxinus csikii* cluster. Asterisks designate species occurring in France.



Observing the morphological characters (Fig. 2), the specimen has a very stout and blunt snout, a slightly convex anal-fin margin and two scale patches separated by unscaled area on the breast. This diagnosis does not correspond to two *Phoxinus* species occurring in France (*P. phoxinus* and *P. septimaniae*), which have slightly pointed snouts, straight to slightly concave anal-fin margins for both species and a breast completely scaled for *P. septimaniae* (Kottelat, 2007). However, these characters are shared by *P. csikii* (Palandačić *et al.*, 2017a, b) and *P. bigerri* (Kottelat, 2007). Nevertheless, it has 64 lateral line scales (51 for the neotype of *P. csikii*; vs. 76–82 for *P. bigerri*). Thus, the diagnosis of the specimen corresponds to the redescription of *P. csikii* (Palandačić *et al.*, 2017b). Walter (2013) did not find morphological characters discriminating *P. csikii* from *P. phoxinus* living in sympatry in the Sieg River, this could be explained by the hybridization of both species (Palandačić *et al.*, 2017a).

Molecular and morphological data agree to highlight the first occurrence of *Phoxinus csikii* Hankó, 1922 in France, in the Moselle basin. According to Palandačić *et al.* (2017a, b), it could also be found in Upper Savoy, in the Arve stream; specimens were caught in its Swiss part. *P. csikii* lives in the Danube drainage, the Rhine basin co-occurring with essentially *P. phoxinus* and around the Geneva Lake in Switzerland co-occurring with *P. septimaniae* (Fig. 1; Palandačić *et al.*, 2017a). According to Kneibelsberger *et al.* (2015), this species (called in this study *P. phoxinus* D) would not be native in the Rhine basin. But this species may have taken advantage of the catch of watershed heads and the post-glaciation events since the Pleistocene to colonize the Rhine and the Geneva Lake systems from the Danube (see Persat and Keith, 2011). It then would be native in France.

Phoxinus csikii is then the fourth known minnow species in this country. A study on several populations in the Rhine drainage is needed for detecting sympatry with *P. phoxinus*, as well as a taxonomical revision in order to know if there might be other minnow species in France.

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Appendix 1. – GenBank accession numbers of COI sequences of *Phoxinus* species used for DNA Barcoding analysis.

Phoxinus bigerri Kottelat, 2007: KJ554128, KJ554275, KJ554288, KU729252, KU729253; *Phoxinus colchicus* Berg, 1910: KU729254, KU729255; *Phoxinus csikii* Hankó, 1922: MK310229 (this study), KM286805, KM286809, KM286824, KM286833, KM286836, KM286847, KM286848 to KM286850, KM286853 to KM286857, KM286861, KM286862, KM286873, KM286916, KM286918, KM286921, KM286935, KM286951, KM286964, KM286967, KM286973 to KM286975, KM286977, KM286978, KM286981, KM286983, KM286988, KM286995, KM287000,

KM287003, KM287005, KM287006, KM287008, KM287013, KM287017, KM287019, KM287020, KM287022, KM287025, KM373651, KM373657, KM373662, KM373664, MF407712 to MF407722, MF407724 to MF407728, MF407731 to MF407738; *Phoxinus karsticus* Bianco and De Bonis, 2015: MF407745 to MF407764; *Phoxinus lumaireul* Schinz, 1840: KJ554125, KJ554282, KJ554304, MF407787 to MF407822, MF407824 to MF407840, MF407842 to MF407884; *Phoxinus marsilii* Heckel, 1836: HQ960812 to HQ960816, HQ960893, HQ960910,

HQ960931, HQ960937, HQ960939, MF407956 to MF407999; *Phoxinus cf. morella*: HQ960469 to HQ960471, HQ960477, HQ960538, HQ960606, HQ960697 to HQ960700, HQ960952, HQ960953, HQ960970, HQ961030, HQ961031, KM286877, KM286879 to KM286881; *Phoxinus phoxinus* (Linnaeus, 1758): KM286804, KM286811 to KM286823, KM286825 to KM286831, KM286832, KM286834, KM286835, KM286837, KM286840 to KM286846, KM286851, KM286852, KM286858, KM286863, KM286864, KM286866, KM286868, KM286874, KM286882 to KM286884, KM286886 to KM286889, KM286900 to KM286903, KM286906, KM286908, KM286909, KM286911, KM286912,

KM286914, KM286922, KM286926, KM286930, KM286936, KM286938, KM286940 to KM286943, KM286946, KM286949, KM286962, KM286969, KM286992, KM287014, KU729256 to KU729258, MF407768 to MF407778; *Phoxinus septimaniae* Kottelat, 2007: KJ554137, KJ554141, KJ554383, KJ554474, KJ554499, KJ554504, KJ554507, KJ554531, KM286838, KM286839, KM286867, KM286955, MF407779 to MF407786; *Phoxinus strandjae* Drensky, 1926: KJ554187, KU729264; *Phoxinus strymonicus* Kottelat, 2007: KJ554101, KJ554135, KJ554301, KJ554359, KJ554386.