Knowl. Manag. Aquat. Ecosyst. 2020, 421, 20
© R. Maciaszek *et al.*, Published by EDP Sciences 2020
https://doi.org/10.1051/kmae/2020014

www.kmae-journal.org

Knowledge & Management of Aquatic Ecosystems

Journal fully supported by Office français de la biodiversité

SHORT COMMUNICATION OPEN ACCESS

First record and DNA barcodes of the invasive blue-coloured spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) (Decapoda: Cambaridae)

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Received: 14 February 2020 / Accepted: 8 April 2020

Abstract – This contribution presents first record of a blue colour morph of spiny-cheek crayfish, *Faxonius limosus* (Rafinesque, 1817). Two unusually coloured individuals were caught in Poland, in two different locations, separated from each other by approximately 500 km and belonging to different river catchments (Oder and Vistula), within the range of the species occurrence in Europe. Taxonomic identification of collected crayfish has been confirmed by the standard DNA barcoding procedure. Although blue coloured forms of crayfish have been previously described within the Cambaridae family, collected individuals are the first records of blue colouration within *F. limosus*. It is recommended to supplement identification keys with a new colour form of the species, which, while characteristic dark red bands are not clear, may cause mistakes in recognition of exotic, aquarium as well as native species.

Keywords: Astacidae / alien species / ornamental pet trade / inland waters / Central Europe

Résumé – Premier signalement et code-barres ADN de l'écrevisse américaine *Faxonius limosus* (Rafinesque, 1817) (Decapoda: Cambaridae) de couleur bleue, espèce envahissante. Cette contribution présente le premier signalement d'une forme de couleur bleue de l'écrevisse américaine, *Faxonius limosus* (Rafinesque, 1817). Deux individus de couleur inhabituelle ont été capturés en Pologne, dans deux endroits différents, séparés l'un de l'autre d'environ 500 km et appartenant à des bassins versants différents (Oder et Vistule), dans l'aire de répartition de l'espèce en Europe. L'identification taxonomique des écrevisses collectées a été confirmée par la procédure standard de codage à barres de l'ADN. Bien que des formes d'écrevisses de couleur bleue aient déjà été décrites au sein de la famille des Cambaridés, les individus collectés sont les premiers à présenter une coloration bleue chez *F. limosus*. Il est recommandé de compléter les clés d'identification par une nouvelle forme de couleur de l'espèce, qui, alors que les bandes rouge foncé caractéristiques ne sont pas claires, peut entraîner des erreurs dans la reconnaissance des espèces exotiques, d'aquarium et indigènes.

Mots clés : Astacidae / espèces exotiques / commerce d'animaux / eaux intérieures / Europe centrale

Crayfish are crustaceans valued in aquaculture, where they are bred for food (Holdich, 1993). They are characterized by widespread variety of colour which encourages their participation at global exhibitions and ornamental pet competitions (Chucholl and Wendler, 2017). In both cases, the most commonly used are representatives of Cambaridae family (Holdich, 1993; Souty-Grosset *et al.*, 2016). On the other hand, their high resistance to adverse environmental conditions and relatively low breeding requirements have contributed to uncontrolled expansion of their populations around the world due to escaping from the aquaculture or intentional introductions (Chucholl, 2013; Patoka *et al.*, 2014; Oficialdegui *et al.*, 2019; Maciaszek *et al.*, 2019).

The spiny-cheek crayfish, *Faxonius limosus* (Rafinesque, 1817), previously known as *Orconectes limosus* (Crandall and De Grave, 2017), is a representative of the Cambaridae family, naturally occurring in freshwaters of north-eastern USA (Souty-Grosset *et al.*, 2006). It was intentionally introduced in

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Europe into a breeding pond in Barnówko (nowadays North-Western Poland) in the 1890s. From that locality the species entered open waters and began its expansion which was additionally supported by subsequent introductions (Filipová *et al.*, 2011). The justification for the release of *F. limosus* in European waters was its resistance to crayfish plague caused by *Aphanomyces astaci* (Schikora, 1906), a parasitic water mould causing crayfish diseases that drastically reduced the population of species native to Europe (such as the one of noble crayfish *Astacus astacus* (Linnaeus, 1758) (Svoboda *et al.*, 2017; Śmietana *et al.*, 2018). Unfortunately, the new species proved to be of a smaller size and less palatable than the native species therefore it was considered inferior from a commercial aspect (Nolfi, 1980). Although, as to the latter, newer studies are not necessarily consistent (Krzywosz, 1999; Stanek *et al.*, 2010).

F. limosus is currently found in most of Central and Western European freshwaters (Kouba *et al.*, 2014; Seprős *et al.*, 2018), where it prevents effective reintroduction of native crayfish species due to being a potential plague vector and a competitive invasive species. It also contributes to acquired aquatic ecosystems degradation process (Pârvulescu *et al.*, 2009; Manenti *et al.*, 2019). Despite imposed ban based on the species being listed among invasive in Poland, *F. limosus* is regularly sold on zoological and aquatic shows and markets as a crustacean proposed for private ponds (Śmietana *et al.*, 2018). *F. limosus* can be also found in aquaculture ponds, where it gets to from adjacent waterbodies (Patoka *et al.*, 2014; Śmietana *et al.*, 2018). On a small scale this species is also observed in the so-called aquatic biotope sector due to being the most common crayfish in Polish waters (Śmietana *et al.*, 2018), where *F. limosus* is introduced as representative of crustaceans.

Fortunately, *F. limosus* is a species relatively easy to determine thanks to the presence of characteristic multiple spines located on hepatic (cheek) region of carapace, not occurring in other representatives of the family Cambaridae observed in European waters (Svecker *et al.*, 2019). This typically brown or grey-coloured crayfish can be also distinguished from native crayfish by the presence of dark red bands on the abdomen segments, orange claw tips and simultaneously the lack of rostral cresta median (Füreder and Machino, 2002; Souty-Grosset *et al.*, 2006; Śmietana *et al.*, 2018). However, none of the recognisable features seem to have such a significance as those dark red bands. They seem to be the most notable feature often used for marking by anglers, aquarists or veterinary inspectors. Difficulties in assessing all the other characteristics could contribute to erroneous identification, which then could result in further spread of unwanted, invasive crayfish in Europe.

In this study, we provide the first report on the occurrence of the blue coloured *Faxonius limosus*.

The total of 380 crayfish were collected in two sampling sites (Fig. 1). Among gathered specimens two were characterized by the blue colouration of the carapace and terga (Fig. 2).

First of untypically coloured specimens was found on May 1, 2018. Observation of colour durability of this particular individual was carried on for a year in aquarium conditions. It was collected from Dziewoklicz city beach, situated in southern part of Szczecin, north-west Poland (53.3788° N, 14.5379° E) located on the Oder River, ca. 9.5 km from the Poland-Germany border. The beach is a valued place of rest and recreation among the local community, and throughout its entire area, it is included in the buffer zone of the Landscape Park "Lower Oder Valley" and Natura 2000 project. Additional inspections at that site were carried out in May 2018 and 2019, however, no more blue specimens were found.

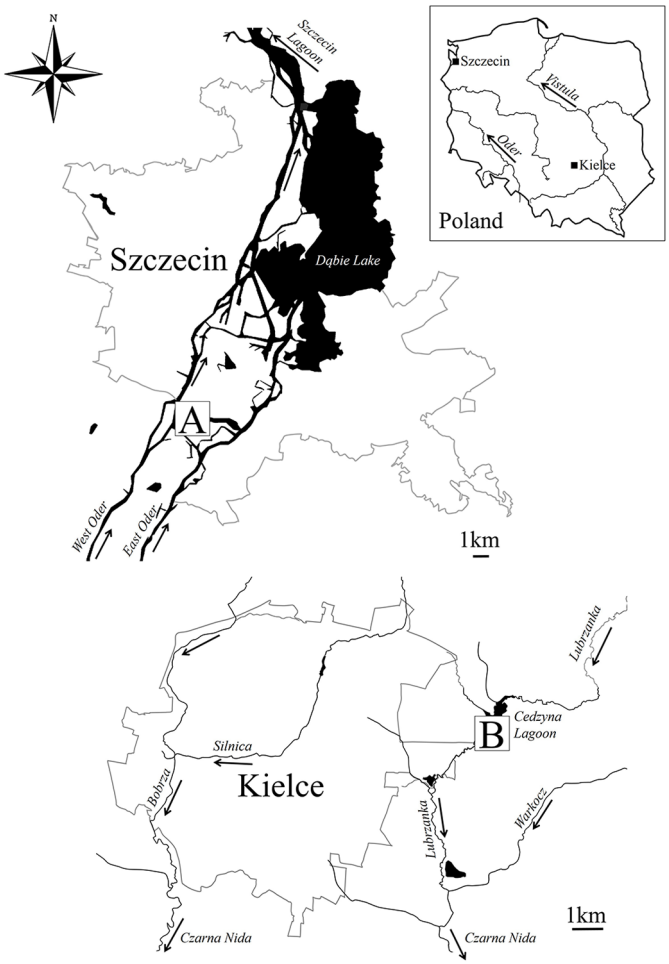

Fig. 1. Localisation of sampling sites in Szczecin, city beach of Dziewoklicz (A) and Cedzyna Lagoon near Kielce (B).

The second individual was observed on October 14, 2019 during regular catches of *F. limosus* crayfish conducted from November the 10th to October the 30th, 2019 in the Cedzyna Lagoon near Kielce (Central and Eastern Poland) (50.8770° N, 20.7346° E).

Due to its natural values, the entire reservoir is included in Podkieleckie Protected Landscape Area. It is also used by local hydroelectric plant as well as provides recreational activities.

Both studied crayfish were caught using the hydrobiological hand net. After being transported to the laboratory, collected individuals were identified, measured and photographed. For an observation of the colour stability they were placed into an aerated aquaria with water set to room temperature for at least 3 weeks. At the time specimens were fed with food rich in astaxanthin.

Both specimens were barcoded with the use of the cytochrome C oxidase subunit I (COI) marker. DNA was

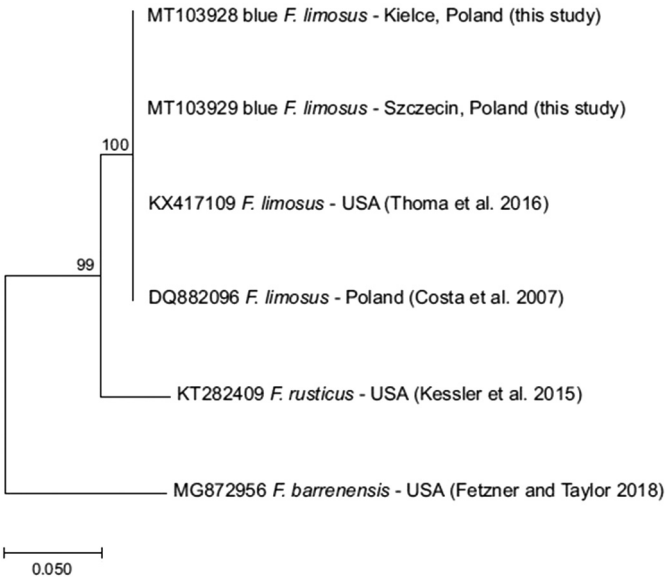

Fig. 2. *Faxonius limosus*. A. Blue morph (female) – Szczecin; B. Typical morph (male) – Szczecin.

extracted from the claw muscle tissue using the Chelex method (Casquet *et al.*, 2012). The polymerase chain reaction (PCR) followed the protocol provided by Hou *et al.* (2007). The primer pair HCOJJ/LCOJJ (Astrin and Stuben, 2008) was adapted. PCR products were purified with Exonuclease I and FastAP alkaline phosphatase (Werle *et al.*, 1994). Then they were sent for sequencing in Macrogen Inc., Korea. Obtained sequences were aligned, trimmed to the same length (627 bp) and deposited in the GenBank data base (Benson *et al.*, 2005) with accession numbers, MT103928 and MT103929. COI sequences *of F. limosus* were also acquired from GenBank data base for the comparison. Their accession numbers were obtained as follows: KX417109 (Thoma *et al.*, 2016) and DQ882096 (Costa *et al.*, 2007). The phylogenetic tree was built with Maximum Likelihood method using MEGA 7.0 (Kumar *et al.*, 2016). As an outgroups the sequences of *Faxonius rusticus*, deposited in GenBank by Kessler *et al.* (2015) with the accession number KT282409, as well as *Faxonius barrenensis*, deposited in GenBank (accession number MG872956) by Fetzner and Taylor (2018), were used. Haplotypes were checked in DnaSP software (Librado and Rozas, 2009).

For qualitative assessment of the pigmentation confirming mutation in astaxanthin-based pigment complexes or possible difference in pigment quantity, separate pereiopod samples were taken. Samples were placed in ethyl alcohol as well as in boiling water for an observation. In result protein denaturation was observed. Control sample contained pereiopods typically coloured. In both cases, the leg samples taken after placing in boiling water turned orange-red, however much less intense than in the individuals with the original colouration.

Collected specimens were identified as representatives of *F. limosus*. Taxonomic affiliation to the species was confirmed by the DNA Standard Barcoding procedure study. Moreover, all the studied individuals represented only one haplotype (both blue specimens as well as the sequences taken from

Fig. 3. Neighbour-Joining tree for blue *Faxonius limosus* and GenBank-stored sequences.

GenBank collection) (Fig. 3). Individual from Szczecin was identified as female, while the one from Kielce, was a male. Crayfish were characterised by total length of 97 and 40 mm respectively. Differences in the coloration of the blue individual and the typical coloration of the crayfish are shown in Figure 2.

Collected individuals are the first records of blue colour forms of the *F. limosus*, although such colouration has already been reported in other species within the Cambaridae family (Fitzpatrick, 1987; Secker, 2013), as well as in the genus *Faxonius* (Momot and Gall, 1971; Thacker *et al.*, 1993). Despite the small sample, it can be estimated that blue pigmentation may occur in males and females as representatives of both sexes were caught and it is possible to recognize it already in non-reproductive phase as one of the specimens shown the characteristics typical for form II (Berrill and Arsenault, 1984; Chybowski and Juchno, 2002). Obtained results suggest that blue-coloured crayfish occur in no more than 0.5% of the population of this species (1/180 and 1/200) which is similar to what was stated by Momot and Gall (1971) in other species of the genus *Faxonius*.

Blue colouration is most probably genetic-based as it was already suggested by *e.g.* Dowell and Winier (1969), as well as Momot and Gall (1971). However, environmental factors should be also considered (Bowman, 1942; Kaldre *et al.*, 2015; Fingerman, 2016). The basis of crayfish colouration are

pigment-protein complexes in which the colouring agents are most often astaxanthin containing compounds (Higuera-Ciapara *et al.*, 2006). Temporary blue colouration is often observed in aquarium crustaceans, including the Cambaridae family (Umbers *et al.*, 2014; Kaldre *et al.*, 2015) and may occur due to a change in dispersion of pigment grain in chromatophores. In this case, the concentration of the exemplary black colorant in the cell gives the visual effect of blue glow, while its dispersion presents black colour. Most often it occurs in response to stress caused by transport, change in environmental conditions (including physical and chemical parameters *e.g.* such as the light and background) and may be food dependent (Thacker *et al.*, 1993; Kaldre *et al.*, 2015; Fingerman, 2016). In the latter case, astaxanthin deficiency may give the effect of blue coloured individuals, as demonstrated by Kaldre *et al.* (2015). This is probably due to the impossibility of forming astaxanthin-protein complexes. In result the chromatophores contain less pigment grains, which, regardless of dispersion, is responsible for the visual effect of blue colouration (free spaces between existing pigment grains). However, this is a temporary condition that may change when astaxanthin enriched food is provided. Similar, but long-lasting effect can be obtained by selection, therefore, on the basis of genetic factors. In this case, regardless of the amount of astaxanthin provided in food, the colour remains blue (Fingerman, 2016). This conclusion is supported by the invariability of the colouration of the collected blue *F. limosus*, despite providing rich in astaxanthin food servings, as well as by bigger number of standard coloured individuals present in the same environmental conditions (Secker, 2013). However, as only 2 specimens were observed, more detailed investigations should be conducted comparing histological and molecular methods. The argument supporting this hypothesis will be also, the less intense red colouration of the sampled pereiopod fragments of crayfish with blue and standard colouration. Paler colouration is most likely the result of a lower content of astaxanthin-containing compounds (Parisenti *et al.*, 2011). The above conclusions require confirmation by performing additional research focused on determining the exact biochemical changes of carotenoproteins and taking into account the genetic basis of these changes.

Presence of new colouration in *F. limosus* followed by the lack of distinctive dark red bands on the abdomen terga may cause misidentification, especially in species native to Europe and associated with blue colour variety such as in the Astacidae family (Rivas *et al.*, 1988).

It could result in misidentification of individuals being released into natural environment. However, in Europe, blue colour may also occur in alien crayfish such as *Pacifastacus leniusculus* (Dana, 1852) (Fitzpatrick, 1987), as well as in other representatives of the Cambaridae family, including the aquarium forms of *Procambarus clarkii*, which have already been recorded in Polish inland waters (Maciaszek *et al.*, 2019).

We recommend introducing to the crayfish identification keys a possibility of occurrence of blue colouration in *F. limosus* which may disrupt classification due to undetermined taxonomical features. Although atypical individuals are observed very rarely, information regarding potential occurrence of other colouration should be provided to enable public to comply effectively with the law. It is also recommended to emphasize that the identification of the crayfish should not be solely based on its colouration.

Acknowledgments. We thank Wiktor Liwoch for his valuable help in collecting crayfish.

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Cite this article as: Maciaszek R, Bonk M, Sosnowski W, Jabłońska A. 2020. First record and DNA barcodes of the invasive blue-coloured spiny-cheek crayfish *Faxonius limosus* (Rafinesque, 1817) (Decapoda: Cambaridae). *Knowl. Manag. Aquat. Ecosyst.*, 421, 20.