Further steps of *Cryptorchestia garbinii* invasion in Polish inland waters with insights into its molecular diversity in Central and Western Europe

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Abstract – *Cryptorchestia garbinii* Ruffo, Tarocco and Latella, 2014 (Amphipoda: Talitridae), a semiterrestrial amphipod, reported (as *Orchestia cavimana* Heller, 1865) from the Polish inland waters, e.g., the lowermost Vistula River, for the first time in 2009. Now, we have reported this species from the upper Oder River, which is c.a. 350 km SE from the closest known localities near Berlin, Germany. We confirmed its identity by the DNA barcodes and showed that all individuals found so far in other countries in Central and Western Europe represent the same haplotype, excluding a singleton from the *locus typicus* of this species in Lake Garda, Italy.

Keywords: Amphipoda / Talitridae / beach hoppers / non-indigenous species / DNA barcoding

Résumé – Nouvelles étapes de l'invasion de *Cryptorchestia garbinii* dans les eaux intérieures polonaises avec un aperçu de sa diversité moléculaire en Europe centrale et occidentale. *Cryptorchestia garbinii* Ruffo, Tarocco et Latella, 2014 (Amphipoda : Talitridae), un amphipode semi-terrestre, signalé (sous le nom d'*Orchestia cavimana* Heller, 1865) dans les eaux intérieures polonaises, par exemple, dans la Vistule la plus aval, pour la première fois en 2009. Nous avons maintenant signalé cette espèce dans le cours supérieur de l'Oder, qui se trouve à environ 350 km au sud-est des localités connues les plus proches, près de Berlin, en Allemagne. Nous avons confirmé son identité par les codes barres ADN et montré que tous les individus trouvés jusqu'à présent dans d'autres pays d'Europe centrale et occidentale représentent le même haplotype, à l'exception d'un singleton du *locus typicus* de cette espèce dans le lac de Garde, en Italie.

Mots-clés : Amphipoda / Talitridae / puce de plage / espèces non indigènes / code-barres ADN

1 Introduction

The Oder is the second biggest river in Poland and thirtieth in Europe with a length of 854 km and the drainage area of 1,18,861 km². The river flows into the Baltic Sea and is the most significant waterway in Poland, navigable on most of its length. The Oder is fed, among others, by such rivers as Warta, Nysa Łużycka, Bóbr and connected through artificial channels to Havel, Spree, Vistula and Kłodnica rivers, which supports the migration of non-indigenous species through the so-called Central Invasion Corridor (CIC) *sensu* Bij de Vaate *et al.* (2002). Additionally, the Szczecin Lagoon, being a part of the

Oder deltaic system (Osaduczuk, 2004), with a large traffic port playing an important role in marine and inland shipping, is considered to be an essential gateway for the introduction of invasive species (Gruszka, 1999; Galil *et al.*, 2008; Gruszka *et al.*, 2013; Messner and Zettler, 2018). The number of alien macroinvertebrate species in the Oder River is still growing. Altogether, at least 33 non-indigenous species have been so far identified in the Oder and in the Szczecin Lagoon (Gruszka, 1999; Szlauer-Lukaszewska and Grabowski, 2012; Jabłońska *et al.*, 2015; Pabis *et al.*, 2017; Szlauer-Lukaszewska *et al.*, 2017; Jabłońska *et al.*, 2018; Cebulka and Krodziewska, 2019). Among them, nine amphipod species were identified: *Chelicorophium curvispinum* (Sars G.O., 1894), *Dikerogammarus villosus* (Sowinsky, 1894), *D. haemobaphes* (Eichwald, 1841), *Echinogammarus trichiatum* (Martynov, 1932),

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Fig. 1. Distribution of *Cryptorchestia garbinii* in Europe and sequences analyzed in this study. Yellow triangles — new records from the upper Oder in Krapkowitz and Zdzieszowice, yellow circles — other own records of the species with DNA barcodes; red circles — sequences obtained from the BOLD database, black dots — previously known records of the species (combined literature and personal communications data — see details in Tab. S1).

Melita nitida Smith, 1873, *Obesogammarus crassus* (Sars G.O., 1894), *Pontogammarus robustoides* (Sars, 1894), *Gammarus tigrinus* Sexton, 1939 and *Cryptorchestia garbinii* (*Orchestia cavimana*) Ruffo, Tarocco and Latella, 2014 (Konopacka *et al.*, 2009; Rachalewski *et al.*, 2013; Zettler and Zettler, 2017).

Lowry and Fanini (2013) erected the genus *Cryptorchestia* to accommodate semi-terrestrial and terrestrial species, associated with fresh and brackish waters, set apart from the typically marine, supralittoral beach hoppers of the genus *Orchestia* Leach, 1814 (see Davolos *et al.*, 2018a and literature therein). Currently, *Cryptorchestia cavimana* (Heller, 1865) is considered as endemic to Cyprus (Ruffo *et al.*, 2014; Davolos *et al.*, 2017). In contrast, *Cryptorchestia garbinii* (encompassing records of *O. cavimana* outside Cyprus), has a wide European distribution including sea coasts, deltas and estuaries of the Baltic, Black, Marmara, Mediterranean, and North seas (Herkül *et al.*, 2006; Konopacka *et al.*, 2009; Davolos *et al.*, 2018a, Tab. S1) (Fig. 1). *Cryptorchestia garbinii* is continually spreading in the European inland freshwater ecosystems, such as large rivers (e.g., Dnieper, Danube, Thames, Rhine) (Rehage, 1987; Martens *et al.*, 1999; Ruffo *et al.*, 2014) and lakes (e.g., Albano, Balaton, Doirani, Garda, Ohrid,

Prespa) (Carausu *et al.*, 1955; Karaman, 1993; Juhász *et al.*, 2006; Ruffo *et al.*, 2014; Davolos *et al.*, 2018a).

In Poland, the first observations come from the brackish waters of the Szczecin Lagoon, the islands of Wolin and Uznam, the Vistula Lagoon, the Martwa Wisła (Jażdżewski and Konopacka, 1995) and the Puck Bay (Spicer and Janas, 2006). The first record from inland waters (as an *O. cavimana*) was provided by Konopacka *et al.* (2009) from the lowest part of the Vistula River, 3 km upstream from its mouth (Fig. 1). No further localities from the Polish inland waters have been registered since then. Even Tykarska *et al.* (2019), in their work about the distribution of talitrids at the Polish Baltic coast, regarded this species as restricted only to the Vistula mouth.

Our study aims to document the southward spread of *C. garbinii* to the upper Oder river as well as to provide first molecular confirmation for the species identity in Poland. Taxonomic consistency is crucial for the studied species as its assignments to *O. cavimana* are still ongoing. Even four years after the taxonomic revision of *Orchestia* and redescription of *C. garbinii* and *C. cavimana* (Lowry and Fanini, 2013; Ruffo *et al.*, 2014), *C. garbinii* is still commonly reported as *O. cavimana* (Davalos *et al.*, 2018b), generating more confusions

Fig. 2. Sampling localities on the Oder River. A Krapkowice, B Zdzieszowice.

(e.g., Ramm and Scholtz, 2017; Jelassi *et al.*, 2018). Molecular identification based on the portion of cytochrome oxidase (COI) gene used as a DNA barcode as well as comparison with other European sequences of *C. garbinii* (including *locus typicus*) can be sufficient to reveal species assignment and potential cryptic diversity. Such a method has already been successfully implemented in studies upon various invasive invertebrates, including amphipods (e.g., Rewicz *et al.*, 2015, 2016; 2019; Hupało and Grabowski, 2018; Mauvisseau *et al.*, 2019).

2 Materials and methods

The individuals of *Cryptorchestia garbinii*, were collected on the groin near Krapkowice (11th of October, 2018) and Zdzieszowice (17th of October, 2019) (Fig. 2). The samples were collected by hand and with a benthic hand net from plant debris and grassroots along the water's edge and from among the stones and instantly preserved in 96% ethanol. Additional specimens that originated from previous sampling campaigns:

Poland 2009, 2016 as well as Italy 2011, 2016 were used (Tab. 1, Fig. 1). All the material was identified to the species level under the Nikon SMZ-800 stereomicroscope, based on available literature (i.e., Spicer and Janas, 2006; Ruffo *et al.*, 2014) and has been stored in the permanent collection of the Department of Invertebrate Zoology and Hydrobiology, University of Lodz, Poland.

The total DNA was extracted from the fifth pereopod or pleopods of the dissected individuals. DNA extraction, PCR reaction, enzymatic purification, and sequencing was proceeded following Rewicz *et al.* (2019). To verify the identification of obtained sequences, we used the BLAST tool (Altschul *et al.*, 1990). Then, the sequences were edited and aligned using Geneious 10.2.6 software package (Kearse *et al.*, 2012), and deposited in GenBank under accession numbers MK721831–MK721847; MN701467 – MN701476. The COI haplotypes were identified using the DnaSP v5 software (Librado and Rozas, 2009). All 22 sequences were deposited in the online database of the Barcode of Life Data Systems (BOLD) Systems (Ratnasingham and Hebert, 2007). We obtained the Barcode Index Numbers (BIN) which are indexing DNA sequences based on the genetic distance approach (Ratnasingham and Hebert, 2013). Additional COI sequences of *C. garbinii* (eight individuals), as well as the three outgroup sequences, were acquired from GenBank and BOLD (see Tab. 1). Sequences were finally trimmed to 500 bp. Phylogenetic tree was constructed in MEGA 6 (Tamura *et al.*, 2013) using the neighbor-joining method (Saitou and Nei, 1987) based on the K2p distance (Kimura, 1980) with a bootstrap test performed on 1000 replicates (Fig. 3). Relevant voucher information is accessible through the public dataset “DS-CGARPOL” (DOI: <http://dx.doi.org/10.5883/DS-CGARPOL>) in the Barcode of Life Data Systems (BOLD; <http://v4.boldsystems.org>).

3 Results

DNA barcoding confirmed the identity of all the 22 individuals identified as *C. garbinii* (Fig. 3). We have found only two haplotypes in the analyzed dataset. Almost all individuals from Great Britain, Italy, Germany, and Poland represented one haplotype. Only in the locality from Lake Garda (*locus typicus*), we found another unique haplotype, differing by a single mutation from the common one (Fig. 3). All the sequences grouped under one BIN (BOLD: AAD8710) which is up to now the only BIN known from the BOLD publically available data and is widespread in Western and Central Europe.

4 Discussion

The known distribution of *C. garbinii* stretches to the Balkans and the Ponto-Caspian region on the south-eastern part of Europe and Great Britain, France and Italy on the western part (Ruffo *et al.*, 2014, own unpublished data). We can observe an ongoing spatial expansion of *C. garbinii* in Europe, as new records have been reported in recent years from northern Germany, Netherlands, Belgium, Poland, Baltic States, Ukraine, Balkan Peninsula and from Turkey (Herkül *et al.*, 2006;

Table 1. Material of *Cryptorchestia garbinii*, *Cryptorchestia cavimana* and two outgroup species used in our study. N – number of analyzed specimens.

No	GenBank acc.	BOLD process ID	Locality	Latitude	Longitude	N	Reference
<i>Cryptorchestia garbinii</i> Ruffo, Tarocco, & Latella, 2014							
1.	MN701468, MN701472–MN701475	WW234-08 – WW238-08	United Kingdom, Medway estuary	51.33	–0.46	5	BOLD public database
2.	EF989708	GBCMA1151-08	Germany, Tegeler Lake	52.35	13.16	1	Browne et al., 2007
3.	JX094884, JX094873	GBCMA6264-13, GBCMA6275-13	Italy, Albano Lake	41.74	12.67	2	Wildish et al., 2012
4.	MK721834	AMPPL005-19	Italy, Lake Iseo	45.67	10.06	1	This study
5.	MK721836–MK721837, MK721840, MK721842–MK721843, MK721847	AMPPL006-19 – AMPPL011-19	Italy, Lake Garda	45.44	10.68	6	This study
6.	MK721838, MK721845–MK721846	AMPPL002-19 – AMPPL004-19	Italy, Lake Caldonazzo	46.03	11.24	3	This study
7.	MK721832–MK721833, MK721835, MK721838–MK721839, MK721841, MK721844	AMPPL012-19 – AMPPL017-19	Poland, Vistula estuary	54.33	18.93	6	This study
8.	MK721831	AMPPL001-19	Poland, Oder, Krapkowiec	50.48	17.97	1	This study
9.	MN701467, MN701469 – MN701471, MN701476	AMPPL123-19 – AMPPL127-19	Poland, Oder, Zdzeszowice	50.41	18.17	5	This study
<i>Cryptorchestia cavimana</i> (Heller, 1865)							
	JX094880	GBCMA6268-13	Cyprus	34.95	32.85	1	Wildish et al., 2012
<i>Speziorchestia guancha</i> (Stock & Boxshall, 1989)							
	JX094882	GBCMA6266-13	Spain, Tenerife	28.28	–16.86	1	Wildish et al., 2012
<i>Platorchestia platensis</i> (Krøyer, 1845)							
	MF544024	PPLA009-17	Sweden, Vägeröd	58.24	11.49	1	Hupalo and Grabowski, 2018

[Konopacka et al., 2009](#); [Rewicz et al., 2016](#); [GBIF, 2019](#); [Davalos et al., 2018a](#); Fig. 1, Tab. S1). Revealing the presence of this talitrid in the upper Oder confirms its ongoing expansion. The question arises of what the source for this new population is. *Cryptorchestia garbinii* was recorded in the Szczecin Lagoon, Poland, more than 20 years ago by [Jażdżewski and Konopacka \(1995\)](#) and more recently in Tegeler See north-west from Berlin, Germany ([Wolff and Scholtz, 2006](#)). Tegeler See has a direct connection to the Oder river through Mittelland Canal, a well-known part of the CIC ([Bij de Vaate et al., 2002](#)). Numerous Ponto-Caspian species, including amphipods, reached Western Europe through this watercourse ([Grabowski et al., 2007](#); [Leuven et al., 2009](#)). Oppositely, the killer shrimp, *Dikerogammarus villosus*, used CIC to invade Germany and Oder river eastward from western Germany ([Rewicz et al., 2015](#)). Hence, both potential sources – Szczecin Lagoon and Tegeler See seem equally plausible.

The obtained DNA barcode sequences unequivocally support the identification of *C. garbinii* in Poland (Fig. 3). The molecular diversity of this species in Central and Western Europe is quite interesting. Only two haplotypes have been identified in an area of more than 750 000 km², stretching N-S about 1360 km and W-E about 1300 km (Fig. 1). One of them is present all over the studied range, while the second haplotype occurs only in Lake Garda, which is a *locus typicus* of this species. Such low haplotype diversity may be, on the one hand, a result of fast colonization process, on another, a result of under-sampling. [Davalos et al. \(2017, 2018a\)](#) conducted studies upon phylogeny of the genus *Cryptorchestia* and found different haplotypes occurring in Lake Ohrid, Northern Macedonia, and in Turkey. However, since they used nuclear Histone 3, and the mitochondrial COI 3P markers, we cannot

compare our data. Interestingly, *C. garbinii* and two other *Cryptorchestia* species, *C. cavimana*, and *C. ruffoi*, which are endemic to the east Mediterranean islands of Cyprus and Rhodes, respectively, form a monophyletic clade ([Davalos et al., 2018a](#)). That may suggest a potentially different place of origin and diversification of this species, than Western Europe (*locus typicus*). Studies upon another amphipod, *Gammarus roeselii* Gervais, 1835, pointed out the Balkan Peninsula as the most probable origin of the species ([Grabowski et al., 2017](#)). This region is regarded as an essential faunal biodiversity hot-spots in Europe (e.g., [Wysocka et al., 2013, 2014](#); [Sworobowicz et al., 2015](#); [Mamos et al., 2016](#); [Jabłońska et al., 2020](#)). The Pontic region was, on the other hand, the place of origin of *Gammarus varsoviensis* Jażdżewski, 1975 ([Grabowski et al., 2012](#)). Both amphipod species were described hundreds of kilometers westward (vicinity of Paris, France, and Warsaw, Poland, respectively) from their factual place of origin. In the case of both species, we can observe much higher molecular diversity in the real place of origin than in the recently colonized area. We can expect a similar situation in the case of *C. garbinii*, and further phylogeographic studies including individuals from the Ponto-Caspian and Balkan regions, are undeniably needed to solve this problem.

In conclusion, we provide the first molecular evidence of the presence of *Cryptorchestia garbinii* in Poland and insight into the molecular diversity of the species in Central and Western Europe. This species is in the spatial expansion stage in Europe, and its population structure, as well as wide-scale phylogeography (especially in the Balkans and in the Ponto-Caspian region), are still insufficiently known and require thorough molecular research.

Fig. 3. Neighbor-joining tree based on COI K-2p distance for sequences of *Cryptorchestia garbinii*, *C. cavimana*, and two other talitrid outgroup species. Sequences from the Oder river are in bold. Each sequence name consists of two parts separated by a lower dash: (1) BOLD Process ID, (2) species name.

Supplementary Material

Supplementary table.

The Supplementary Material is available at <https://www.kmae-journal.org/10.1051/kmae/2020009/olm>.

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