Springing up like mushrooms: established populations of invasive red swamp crayfish in Slovakia

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Research Article

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Abstract

The invasive red swamp crayfish *Procambarus clarkii* is recorded for the first time in Slovakia from two natural localities fed by local thermal springs. The first locality is near Turčianske Teplice in Central Slovakia, just downstream of a commercial culturing facility for aquatic ornamentals, while the other is located approximately 150 km southwards, close to Komárno. Both *P. clarkii* populations are well-established, with numerous individuals of both sexes, size classes, and ovigerous females. Although none out of 32 screened adult crayfish of both populations tested positive for *Aphanomyces astaci*, the causative agent of the crayfish plague, its presence cannot be entirely excluded. So far, *P. clarkii* is confined to small stream stretches in both sites, however, its independent expansion into nearby watercourses can be expected, potentially further facilitated through human translocations. Given the supposedly limited territory conquered, it is strongly advised to implement urgent eradication and control measures. As thermal springs and thermally polluted waters are known hotspots of non-native and potentially invasive species; monitoring of these sites is advised.

Introduction

Freshwater biodiversity is globally threatened by the introduction of non-native species and their associated pathogens (Dudgeon et al., 2006; Carpenter et al., 2011). The introduction of non-native species, particularly those with invasive potential, is one of the main threats to the world's biodiversity in the Anthropocene (Turvey and Crees, 2019; Pyšek et al., 2020). Freshwater ecosystems are prone to species introductions which often result in biodiversity decline, profound alteration of the energy flow and the physical environment (Gallardo et al., 2015; Havel et al., 2015; Lipták et al., 2019; Haubrock et al., 2020). In freshwater ecosystems, non-native engineering species, such as crayfish, are particularly impactful (Reynolds et al., 2013; Kouba et al., 2016).

Crayfish are culturally and economically important animals, which have been long utilised in aquaculture (Ocialdegui et al., 2020a; Haubrock et al., 2021) and, more recently, in the global pet trade business due to their aesthetic appeal (Faulkes, 2015; Patoka et al., 2016; Chucholl and Wendler, 2017). However, in many instances, non-native crayfish accidentally escape or are deliberately introduced into new natural environments, where they establish viable populations, spread, and often become problematic (Holdich et al., 2009; Kouba et al., 2014). In the past, non-native and potentially invasive crayfish species were introduced to European inland waters for food purposes, but nowadays, European introductions are mainly linked to the pet trade (Weiperth et al., 2020; Bláha et al., 2022), and Slovakia is not an exception (Lipták et al., 2016, 2017).

Many European native crayfish species have become endangered or locally extinct due to interactions with their non-native counterparts and a weak immune defense against the crayfish plague pathogen, *Aphanomyces astaci*, which they transmit (Richman et al., 2015; Svoboda et al., 2016; Wiśniewski et al., 2020; Mojžišová et al., 2022). In Europe, one of the most widespread invasive crayfish species with remarkable ecological impacts is the red swamp crayfish *Procambarus clarkii* (Souty-Grosset et al., 2016;
Oficialdegui et al., 2020a). This North American crayfish species, primarily associated with warm waters in the southern part of the continent, can also find suitable habitats in thermal waters in central Europe, overcoming more easily low winter temperatures (Veselý et al., 2015; Haubrock et al., 2019). In this paper, we present the first reports of *P. clarkii* in thermal waters in Slovakia at two sites approximately 150 km apart. We describe the specific sites where it has been found and discuss possible introduction pathways, its likely further expansion, and the potential risks this invasion may entail if urgent management measures are not implemented.

**Methods**

**Description of the sites**

The first site, named Čepčínsky Brook, is located near the town of Turčianske Teplice in central Slovakia (Fig. 1), famous for its springs with warm water and thermal spas. The warm water-carrying thermal brook is seven kilometers long and springs at an elevation of 517 meters a.s.l. The Čepčínsky Brook flows into the Turiec River, a tributary of the Váh River, which has a basin area of 934 m² and receives water from more than 70 streams. The temperature of the Čepčínsky Brook (measured on May 5, 2023) was estimated to have 28.6°C and decreased in the downstream direction. Inversely, pH increased from 7.2 in the uppermost part to 7.8 some 300 meters downstream the uppermost measurement. The site was inspected at three points (Point 1: 48°51'42.0"N, 18°50'18.8"E, Point 2: 48°52'04.6"N, 18°50'07.1"E and Point 3: 48°52'13.4"N, 18°49'17.7"E). On the ground of the spring stands a large facility (48°51'42.8"N, 18°50'17.9"E), dedicated to the cultivation and trade of aquatic ornamentals, especially warm-water fish.

The second site is also a thermal brook situated between the town of Komárno and Kolárovo in the southern part of Slovakia (Fig. 2). The discovered crayfish locality (47°51’27.3”N, 18°00’19.6”E) is located at an elevation of 108 meters a.s.l. in an agricultural landscape consisting of crop fields interspersed by irrigation canals. The brook is fed by a nearby natural thermal spring and flows into the Stará Částa stream. This stream then freely discharges into the Kolárovo-Kameničná canal, which is closed at its downstream end by a pumping station (47°50’57.4”N, 18°02’02.0”E). The pumping station is responsible for expelling excess water from the canal to the Váh River. The Kolárovo-Kameničná canal, like many others, is artificially connected to the Váh River and used to irrigate the surrounding agricultural land. The temperature of the Brook near Komárno on May 25, 2023 was measured to be 30.4°C with a pH of 7.9.

**Crayfish sighting and monitoring**

Following the sighting of unspecified crayfish by a local citizen in 2019 and based on the later monitoring from the staff of the National Park Veľká Fatra conducted on May 31, 2022, that confirmed the presence of the crayfish, another barrage of investigations were conducted at the Čepčínsky Brook to collect and analyze the crayfish individuals in detail. Sampling took place on April 22, May 8, July 19, and August 28 and 29, 2023, and was stretched along three different sampling points indicated in Fig. 1. On all occasions, the site was visited during the daytime, potential hiding places of the crayfish were inspected,
and the crayfish were collected by a hand-held aquarist net. Minikin data loggers (Environmental measuring systems, Brno, Czech Republic) for the continuous monitoring of temperature were installed at the three sampling points at Čepčínsky Brook (Fig. 1) on May 8, 2023 and temperatures were registered hourly until June 25, 2023.

The second site (Fig. 2) was inspected for the first time on May 25, 2023 during an ichthyological survey aiming at the detection of potential non-native fish species. Following the confirmation of crayfish presence (24 crayfish were captured, including 13 adult males, 10 adult females, and one juvenile crayfish), a second visit was organized on July 18, 2023. During the second visit, the site was inspected at two points during the daytime, and crayfish were collected by a hand-held aquarist net.

All captured crayfish individuals were morphologically identified based on the available literature (i.e., Souty-Grosset et al., 2006). Crayfish individuals captured were measured (total carapace length) using a caliper, weighed, and sexed. In addition, the soft abdominal cuticle and muscle, one uropod, an eye stalk, and a walking leg joint were preserved in 96% ethanol for subsequent molecular analyses.

**Molecular analyses**

DNA used for crayfish identification (23 individuals) was isolated from the ethanol-preserved muscle tissue, while that used for *A. astaci* screening (32 individuals) was isolated from mixed-tissue samples using a modified salt precipitation protocol (Grabner et al., 2015). Molecular identification of crayfish was obtained with the universal eukaryotic primers pair LCO1490/HCO2198 (5'-GGTCAACAAATCATAAAGATTTGG-3' and 5'-TAAACTTTCAGGGTGACCAAAAAATCA-3') (Folmer et al. 1994) targeting the cytochrome c oxidase subunit I (COI). For the molecular detection of *A. astaci*, we used the primer pair 42/640 (5'-GCTTGTGCTGAGGATGTTCT-3' and 5'-CTATCCGACTCCGCATTCTG-3') targeting the internal transcribed spacer (ITS) regions and the 5.8 RNA gene of *A. astaci* (Oidman et al., 2006). All PCR reactions consisted of 20 µL composed of 10 µL of 2 × AccuStart II PCR ToughMix (QuantaBio), 0.5 µM of each primer, and one µL of DNA template. PCR conditions for the primer pair LCO1490/HCO2198 were as follows: 1 minute initial denaturation at 94°C, followed by five cycles of 1 minute at 94°C, 90 seconds at 45°C, and 90 seconds at 68°C, followed by further 40 cycles of 1 minute at 94°C, 90 seconds at 50°C, and 1 minute at 68°C, with a final extension of 5 minutes at 72°C. PCR conditions for the primer pair 42/640 were: 5 minute of initial denaturation at 96°C, followed by 45 cycles of 1 min at 96°C, 1 min at 59°C, 1 min at 72°C, followed by a final extension step of 10 min at 72°C. PCR products were sent to Microsynth Seqlab (Germany) for Sanger sequencing.

Obtained sequences were quality-checked and edited using Geneious v2023.1.2 (Biomatters) and aligned with the MAFFT v7.490 algorithm with standard settings (Katoh et al., 2019). For the initial identification of hosts and pathogens, obtained sequences were compared against records contained in GenBank using megablast (Morgulis et al., 2008). Then, sequences belonging to *P. clarkii* were compared with those of Oficiadegui et al. (2019) to identify the corresponding haplotype. Since the 600 bp fragment of mtDNA was identical for all crayfish in both sites, we used only one sequence (i.e., haplotype) per site to construct a Maximum likelihood phylogenetic tree with bootstrap support values (1000 replicates) in IQ-
Tree 2.2.0 (Minh et al., 2020). The TIM + F + G4 substitution model was selected based on Bayesian information criterion scores. The native noble crayfish *Astacus astacus* (Genbank accession number MW726635) was used as an outgroup.

**Results**

At the first site (Čepčínsky Brook), all the crayfish were observed upstream in the section containing the warmer water (i.e., sampling points 1 and 2), while we did not find any of them in the colder water occurring downstream at sampling point 3 (see Fig. 1 for details).

The second site (Brook near Komárno) was investigated at two points as well, with the presence of the crayfish confirmed only at the thermally influenced section (sampling point 1). Point 2, located behind the pumping station, was also inspected for crayfish presence, with negative results (see Fig. 2 for details).

Altogether 88 crayfish individuals were collected from the two locations (Table 1). Of these, 47 were females, and 41 were males. Overall, 54 individuals were extracted from Čepčínsky Brook (28 males and 26 females) and 34 from Brook near Komárno (13 males and 21 females). Relationship between the carapace length and the body weight of crayfish (males and females separately) for both sites is represented in Fig. 3. A group of animals weighing 10 to 15 g was common at both sites, with a few exceeding 20 g.

<table>
<thead>
<tr>
<th>Sampling site</th>
<th>No.</th>
<th>Sex</th>
<th>CL (mm) ± SD</th>
<th>Weight (g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Čepčínsky Brook</td>
<td>28</td>
<td>Male</td>
<td>31.1 ± 7.2</td>
<td>9.6 ± 5.0</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>Female</td>
<td>31.5 ± 6.9</td>
<td>11.0 ± 4.3</td>
</tr>
<tr>
<td>Brook near Komárno</td>
<td>13</td>
<td>Male</td>
<td>33.8 ± 5.4</td>
<td>10.5 ± 5.1</td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>Female</td>
<td>36.3 ± 5.0</td>
<td>10.7 ± 4.8</td>
</tr>
</tbody>
</table>

During the field trip on August 28 and 29, 2023, three females were captured holding juveniles. One female was inspected carrying 209 juvenile individuals attached to its abdomen. Three depigmented and completely white crayfish individuals were also observed and extracted from the site during the last visit.

The results regarding the continuous temperature monitoring conducted on the first site (at three sampling points) are presented in Fig. 4.

DNA barcodes confirmed that the collected individuals (Genbank accession number OR462185 and OR462186) were *P. clarkii* with 100% similarity to haplotype 4 (Genbank accession number MK026674) *sensu* Oficialdegui et al. (2019), a central haplotype with a worldwide distribution (Fig. 5). On the other hand, none of the individuals was PCR positive for *A. astaci*. 

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**Table 1**

Numbers, sex, and biometry of captured *P. clarkii* individuals in both Slovak localities (April 22 April, May 8, and July 18–19, 2023).
Discussion

The North American *P. clarkii*, reported in this study, is the fourth invasive crayfish species recorded in Slovakia, after the discovery of viable populations of marbled crayfish *P. virginalis* in 2010 (Janský and Mutkovič, 2010) and previously established spiny-cheek crayfish *Faxonius limosus* and signal crayfish *Pacifastacus leniusculus* (Kouba et al., 2014). Thus, invasive crayfish species now outnumber their three native counterparts, namely the *A. astacus*, narrow-clawed crayfish *Pontastacus leptodactylus*, and stone crayfish *Austropotamobius torrentium* (Lipták, 2013; Lipták et al., 2016, 2017). The presence of the above-mentioned invasive crayfish species will likely further exacerbate the decline of native crayfish, e.g. the narrow-clawed crayfish, whose populations are still presumably widespread in southern parts of Slovakia, which better suit the temperature requirements of the *P. clarkii* (Kouba et al., 2014; Lipták, 2014).

Due to its use for aquaculture purposes and attractive visual appearance (ranging from reddish coloration to various color morphs in cultured individuals), *P. clarkii* has been introduced into over 40 different countries around the world, becoming one of the most successful crayfish invaders worldwide (Oficialdegui et al., 2020a), and particularly in the European continent (Kouba et al., 2014). In Europe, this species was first introduced in south-western Spain and spread over multiple human-induced secondary introductions northwards (Oficialdegui et al., 2019). The species has a complex distribution history in Europe, likely involving multiple introduction events from various sources over the past decades (Oficialdegui et al., 2020b). *Procambarus clarkii* has been widely available in the pet trade in many European countries (Chucholl, 2013; Patoka et al., 2015; Weiperth et al., 2020; Lipták et al., 2023), including Slovakia (Lipták and Vitázková, 2015), consequently being source of releases into the wild (Maciaszek et al., 2019; Battisti and Scalici, 2020; Weiperth et al., 2020). Unfortunately, legal actions against the unlawful release of non-native crayfish are rarely implemented; thus, illicit releases and/or escapes are a common phenomenon (Patoka et al., 2018; Yuliana et al., 2021).

Our molecular analyses did not enable us to trace the introduction routes of these individuals, as all sequenced crayfish shared the most common haplotype, which hindered our ability to refine information regarding their possible origin. The presence of a single haplotype, namely haplotype 4 *sensu* Oficialdegui et al. (2019), can suggest a common origin for both sites, which might be related to the animals available on the Czech or Hungarian pet trade and/or translocation from other European countries. Similarly, independent introduction events cannot be ruled out given that this haplotype is commonly found in the vast majority of both its native and non-native distributions. In Europe, this haplotype was broadly found over the Iberian Peninsula, South France, and Italy, less so in the central and western parts of Europe, where haplotype 11 was more prevalent (Oficialdegui et al., 2019). Although an extensive screening of pet traded and feral *P. clarkii* populations in central and eastern Europe is ongoing (Oficialdegui *et al.*, in prep), the presence of the haplotype 4 in Slovakia is somewhat unexpected. Natural barriers such as mountain ranges and lack of connectivity with the Iberian, French, and Italian populations suggest human-induced translocation or escapes from culturing facilities or hobbyists. Accordingly, *P. clarkii* found in Čepčínsky Brook likely escaped from a nearby aquaculture facility that is dedicated to farming and selling aquatic ornamentals. However, we can not entirely rule out that the
presence of the crayfish in the Čepčínsky Brook may be linked to individual actions of crayfish enthusiasts. On the other hand, the crayfish individuals found in the Brook near Komárno may be the result of an illegal release(s) of \textit{P. clarkii} by hobbyists.

The \textit{P. clarkii} specimens at both localities can be considered to have well-established populations. This is supported by the presence of numerous mature and juvenile individuals as well as by the presence of ovigerous females at the Čepčínsky Brook, combined with previous evidence/observations of their occurrence gathered by local citizens. Since both populations maybe present in the respective areas for some time (at least from 2019 for the Čepčínsky Brook), \textit{P. clarkii} might have been already translocated by humans to other water bodies with similar habitats across the territory. In addition to secondary introductions facilitated by humans and natural spread primarily downstream, an expansion of \textit{P. clarkii} can also be facilitated by other animals such as birds, by attaching to their feet and feathers (Anastácio et al., 2014) as well as overland movements (Thomas et al., 2019).

The introduction of this species in other sites may occur due to often limited knowledge of the crayfish fauna and the common belief among the general public that all crayfish species are protected by law. Also it is thought that crayfish are generally perceived as indicators of good water quality, hard for the general public to identify at the species level (Lipták et al., 2023a), and perceived as aesthetically attractive (Lipták et al., 2023b), thus considered them suitable candidates to be introduced into freshwater environments. The spread of \textit{P. clarkii} toward the Váh River, the longest and arguably most important river in Slovakia (besides the Danube River), is highly concerning as native crayfish populations of \textit{A. astacus} inhabit its tributaries (Stloukal and Harváneková, 2005). Furthermore, reaching the Váh River would also provide a gateway to the Danube River and other native crayfish populations along its basin, similarly as is the case with the ongoing invasion of the \textit{P. virginalis} (Lipták et al., 2016, 2017).

Invasive species themselves have the potential to outcompete native crayfish counterparts and cause irreversible environmental damage (McCarthy et al., 2006; Scordo et al., 2023). Furthermore, associated pathogens, such as \textit{A. astaci}, might have further negative consequences when introduced alongside their hosts (Svoboda et al., 2014; Ungureanu et al., 2020). North American crayfish species, such as \textit{P. clarkii}, can carry the crayfish plague, a pathogen usually lethal to all non-American crayfish species (Svoboda et al., 2016; Putra et al., 2018). This pathogen might decimate local populations of native crayfish stocks (Mrugał et al., 2017; Mojžišová et al., 2020). Although none of the 32 screened \textit{P. clarkii} individuals resulted positive for the oomycete \textit{A. astaci}, the causative agent of the crayfish plague, we cannot exclude its presence from the investigated populations. Accordingly, a low \textit{A. astaci} prevalence is not uncommon in European \textit{P. clarkii} populations (Tilmans et al., 2014; Sieber et al., 2022). So far, \textit{P. clarkii} seems to be confined to small, restricted areas (i.e., brooks and canals); however, spores of \textit{A. astaci} might disperse downstream or be transported by animals feeding on fish and crayfish, potentially spreading in the broader region (Kozubíková et al., 2009).

The current finding also highlights the role of naturally thermal and thermally polluted water in the establishment of invasive species in temperate-cold environments and the need for their close
monitoring. These particular habitats are known hotspots of exotic diversity in Europe, where warm-adapted pet trade-linked biota, including their parasites, find a suitable environment (Emde et al. 2016; Maciaszek et al., 2019; Maciaszek et al., 2019; Weiperth et al., 2020; Bláha et al., 2022). This might facilitate the adaptation of warm-adapted species (Vodovsky et al., 2016) and the associated pathogens to the colder environment of the surroundings (Schneider et al., 2022). A warming climate might also play a significant role and facilitate the increase of water temperature, widening the species portfolio that could develop and exert their invasive potential (Kouba et al., 2021; Bohatá and Patoka, 2023).

*Procambarus clarkii* is identified as an invasive non-native species of concern in the European Union (EU 2014, 2016). Consequently, the release or translocation of this species is strictly forbidden and should not be undertaken under any circumstances. The sightings of this species as well as those suspected of being non-native by the general public should be immediately reported to the appropriate institution (e.g., Slovak Nature Conservancy in case of Slovakia) or experts in the field to raise the alarm and assess it. Education of the general public (Lipták et al., 2023a), and regular surveys of potential hotspots are highly recommended. Furthermore, there is an imperative need to prevent the escape of non-native species into the natural environment from facilities that may harbor them. This is because eradication strategies of invasive non-native populations are always highly demanding in aquatic ecosystems, time- and resource-consuming, often with an undesired final outcome (Krieg et al., 2020). Thus, prevention actions, when accurately done, remain the best way to stop and limit the spread of non-native species.

The recent finding, coupled with the restricted size of the brook and the relative isolation of the *P. clarkii* populations, renders the sites suitable for the eradication of this invasive, potentially plague-carrying crayfish species. Alongside the described monitoring, the Veľká Fatra National Park Administration proceeded with regular removal of *P. clarkii* at the Čepčínsky Brook, by mid-September accounting 3 800 individuals already removed, but many still present on the site. Thus, a more drastic and large-scale intervention is needed, ideally with local citizens' and stakeholders' support.

**Conclusions**

In conclusion, the introduction and establishment of *P. clarkii* in Slovakia stands as another instance of policy implementation and regulation failure within the European Union, and irresponsibility of the general public including stakeholders, as the species has been banned in the European Union since 2016 (EU, 2016). Considering the possible negative impact of *P. clarkii* on the native biota and biodiversity, the nearby presence of a national park at the Čepčínsky Brook, and the rich faunal and flora elements around the Vár River and nearby water-bodies as is the case for the Brook near Komárno, we strongly advise monitoring of the species to control (and possibly eradicate) both early-stage populations, addressing current policy implementation and regulation issues.

**Declarations**
Conflict of interest

The authors declare no competing interests.

Acknowledgements

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References


**Figures**

![Figure 1](image)

**Figure 1**

Details of the Čepčínsky Brook site. A) indicates the position of the study area at the national scale (background map downloaded from mapy.cz online portal). B) study area where the red circle indicates the location of the commercial culturing facility, the orange dots (sampling points 1 and 2) indicate places where the *P. clarkii* is currently present, the yellow dot (3) shows the section where the crayfish is still absent. Dotted lines with arrows represent the downstream route of the likely range expansion. C) Habitat in which the *P. clarkii* was found and collected. SK – Slovak Republic, CZ – Czech Republic, PL – Poland, AT – Austria, UA – Ukraine.
Figure 2

Details of the Brook near Komárno site. A) indicates the position of the study area at the national scale (background map downloaded from mapy.cz online portal). B) study area where the orange dot (sampling point 1) indicates the place where *P. clarkii* was discovered, and the yellow dot (point 2) shows the second sampling point, where *P. clarkii* was still absent; dotted lines with arrows represent the likely routes of the species range expansion. C) Habitat in which the *P. clarkii* were collected. SK – Slovak Republic, CZ – Czech Republic, PL – Poland, AT – Austria, UA – Ukraine.
Figure 3

Relationship between carapace length (mm) and weight (g) in male and female *P. clarkii* individuals obtained from the first site - Čepčínsky Brook and the second site - Brook near Komárno in Slovakia.
Figure 4

Continuous temperature monitoring at the Čepčínsky Brook. Temperature monitoring points correspond with the sampling points (see Fig. 1).
Figure 5

Maximum likelihood phylogenetic tree including *P. clarkii* found in this study, and three other invasive crayfish found in Slovakia, namely *P. virginalis*, *Faxonius limosus*, and *Pacifastacus leniusculus*. The native *Astacus astacus* was used as an outgroup. The tree was constructed using an IQ-Tree 2.2.0 and the TIM3 + F + G4 substitution model. The gray dots represent bootstrap branch support values (1000 replicates) above 90%.