Research Article

The invasive parthenogenetic marbled crayfish *Procambarus virginalis* Lyko, 2017 gets foothold in Belgium

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Abstract

In 2020, four populations of the marbled crayfish *Procambarus virginalis*, which is included on the list of Invasive Alien Species of Union concern, were discovered in northern Belgium. These records represent the first established populations of this invasive parthenogenetic species in the Benelux. The marbled crayfish seems well-established at all sites where it was discovered. Genetic analysis confirmed the species' identity with the obtained COI Folmer fragments being 100 percent identical to reference sequences of *P. virginalis* from Germany, Italy, Sweden and the Czech Republic. We proposed a single diagnostic nucleotide for unambiguous character-based species identification between *P. virginalis* and *P. fallax*. The finding of this new species through opportunistic surveys instigated by citizen science reports indicates considerable knowledge gaps on crayfish distribution in Belgium. Considering the regulated status of most species in Belgium, we advocated the further set-up of dedicated crayfish surveillance using passive and active monitoring including environmental DNA detection.

Key words: invasive species, Cambaridae, non-native species, *Procambarus fallax* forma *virginalis*

Introduction

Invasive non-native crayfish are an emerging threat worldwide, and several of these species are known to have a negative impact on the water quality and the biodiversity of wetlands, lakes and rivers (Gherardi 2007). Because of their invasive character, the import, sale and rearing are prohibited in an increasing number of countries worldwide. In Europe, five non-native crayfish species are regulated by the EU IAS Regulation: *Faxonius limosus* (Rafinesque, 1817), *F. virilis* (Hagen, 1870), *Pacifastacus leniusculus* (Dana, 1852), *Procambarus clarkii* (Girard, 1852) and *P. virginalis* Lyko, 2017. Each of these species is included on the list of Invasive Alien Species of Union concern in an effort to constrain their further spread across Europe (European Union 2014). Nonetheless, all these species have already established viable populations in many European countries (Kouba et al.

2014; Tsiamis et al. 2017) and the effectiveness of these regulations are issue of debate (Patoka et al. 2018).

Until recently only four non-native crayfish species were known in Belgium (Boets et al. 2012), a number which was lower than in the neighbouring member states Germany, France and The Netherlands (Kouba et al. 2014). However, since 2019, a fifth species, *Procambarus acutus* (Girard, 1852) s.l., was found at four different sites across Flanders, Belgium (Scheers et al. 2020), and in spring 2020, indications were obtained of the presence of a sixth species, the marbled crayfish *Procambarus virginalis*, in the northern part of Belgium.

The latter is an enigmatic species, that recently gained considerable attention, both because of its cryptic origin and the fact that it is the only obligate parthenogenetic decapod known to date (Vogt et al. 2018; Hossain et al. 2018). As a consequence, in this species male individuals do not exist and populations consequently consist of only females. The species was first discovered in the German aquarium trade in 1995 (Scholtz et al. 2003), whereas to date no native populations are known (Lyko 2007; Vogt et al. 2018). Initially, the species was regarded as a parthenogenetic form of *Procambarus fallax* (Hagen, 1870) and tentatively described as *P. fallax* forma *virginalis* (Martin et al. 2010a). Later, the marbled crayfish *P. virginalis* was found to be distinct from *P. fallax* based on cytogenetic, genetic and phenotypic differences between both taxa (Vogt et al. 2015; Martin et al. 2016). Since then, the species was described as a valid species by Lyko (2017) and thoroughly studied by Vogt et al. (2018), Hossain et al. (2018) and by authors cited herein.

Anthropogenic releases into the wild have resulted in viable populations in various countries (Lyko 2017). At present, the marbled crayfish is known from Europe, Madagascar and Japan (Vogt et al. 2018) and the risk related to possible future introduction was also assessed for New Guinea (Yonvitner et al. 2020). In Europe, established populations are known from Croatia (Cvitanić 2017), Czech Republic (Patoka et al. 2016), Estonia (Ercoli et al. 2019), France (Collas 2019b), Germany (Martin et al. 2010b), Hungary (Lökkös et al. 2016), Italy (Vojkovská et al. 2014), Malta (Deidun et al. 2018), Romania (Pârvulescu et al. 2017), Slovakia (Janský and Mutkovič 2010) and Ukraine (Novitsky and Son 2016). Furthermore, there are records from The Netherlands and Sweden, but in these countries the species did not establish viable populations (Koese and Soes 2011; Soes 2016; Bohman et al. 2013).

The aim of this study was 1) to confirm the presence of the marbled crayfish in Belgium with both morphological and genetic methods; 2) to ascertain the current distribution of this species in Belgium and 3) to study the bionomics of the existing populations when discovered.

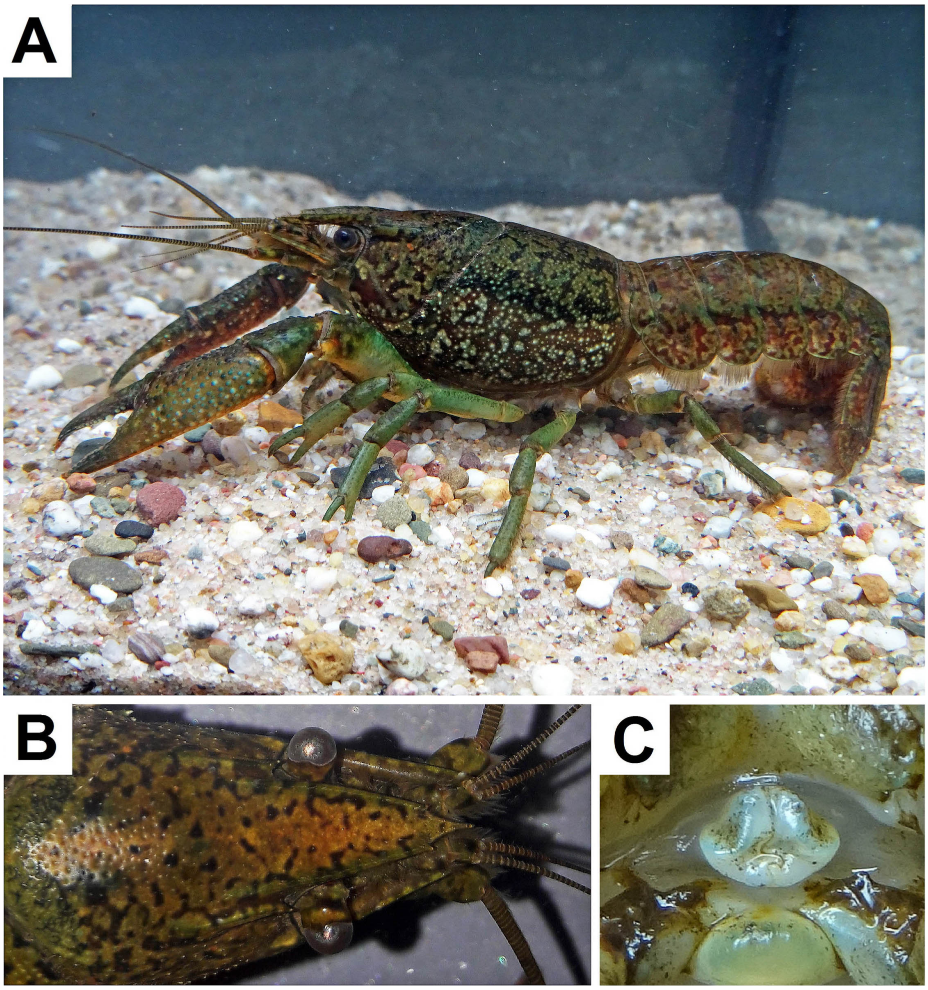

Figure 1. Marbled crayfish *Procambarus virginalis*: A. live specimen (left lateral view); B. rostrum (dorsal view); C. *annulus ventralis* (ventral view). Photographs by Thomas Abeel.

Materials and methods

Revision of records on citizen science platforms

To ascertain correct identification of all recorded non-native crayfish species in Belgium, we rigorously revised all Belgian records of both Astacidae and Cambaridae of which photographic evidence was available on the citizen science data platforms www.waarnemingen.be, www.inaturalist.org and www.rivierkreeften.be. The preliminary identification of the photographed specimens was based on external characteristics of this species as described by Lyko (2017) (Figure 1A–C).

Collecting on site

Based on the revised citizen science records, field surveys were carried out at sites where populations of the marbled crayfish were expected to occur. Sampling was performed by visual encounter surveys at sites A, B and D between the 27th of May and the 8th of July 2020. Surveys were conducted at night with the aid of flashlights. Site C was nearly completely dry at the time of sampling (3rd of June 2020) and therefore this site was only surveyed during the daytime by hand sampling. Observed crayfish were caught with a hand net, transported in plastic zip-lock bags and afterwards preserved in pure ethanol for later identification. Traps were not used during this study.

DNA sampling and analysis

Genomic DNA was extracted with the DNeasy Blood & Tissue Kit (Qiagen) following the manufacturer's protocol with 1-hour lysis and final elution in 140 µL AE buffer. We amplified the 658 bp barcoding fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) by polymerase chain reaction (PCR), using the degenerated Folmer primers dgLCO-1490 (GGT CAA CAA ATC ATA AAG AYA TYG G) and dgHCO-2198 (TAA ACT TCA GGG TGA CCA AAR AAY CA) (Meyer 2003). PCRs were performed in 26 µL of reaction mixture containing 6 µL of extracted DNA (10 ng/µL), 0.4 µM of each primer, 1x Taq buffer with KCl, 2 mM $MgCl_2$, 200 µM of each dNTPs and 0.8 U Taq polymerase (Thermo Fisher Scientific). Each PCR was composed of an initial denaturation at 94 °C for 2 min followed by 35 amplification cycles (denaturation at 94 °C for 30 s, annealing at 52 °C for 40 s and elongation at 72 °C for 1 min) and a final elongation step at 72 °C for 5 min. Amplified DNA was purified using the ExoSAP-IT method (Thermo Fisher Scientific), checked on a 1% agarose gel and left undiluted in case of weak amplification and diluted 2.5 times in case of strong amplification. Sequencing reactions with both primers were performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific) in a 10 µL volume containing 2 µl of purified DNA, 0.4 µM of dgLCO-1490 or dgHCO-2198 primer, 0.5x Ready Reaction mix and 0.5x Sequencing buffer. The cycling profile for both directions started with an initial denaturation at 96 °C for 1 min followed by 35 amplification cycles (10 s at 96 °C, 5 s at 50 °C and 4 min at 60 °C). After purification with the BigDye XTerminator Purification kit (Thermo Fisher Scientific) products were analyzed on an ABI 3500 genetic analyzer (Thermo Fisher Scientific). Sequences were checked for quality, aligned and a consensus sequence from both directions generated in Geneious v.10.2.6 (Biomatters Ltd.). Sequences were submitted at the European Nucleotide Archive (ENA) with accession numbers LR884225–LR884234 (http://www.ebi.ac.uk/ena/data/view/LR884225-LR884234).

The generated 658 bp COI sequences (Folmer region) were compared with reference *P. virginalis* sequences available from the International Nucleotide Sequence Database Collaboration (INSDC). Next, a Tamura-Nei neighbour-joining tree was constructed with both these newly generated Belgian sequences and reference sequences from *P. virginalis*, and some closely related crayfish species. Finally, a sequence of *P. leniusculus* was also incorporated and used as an outgroup to root the tree (Figure 4).

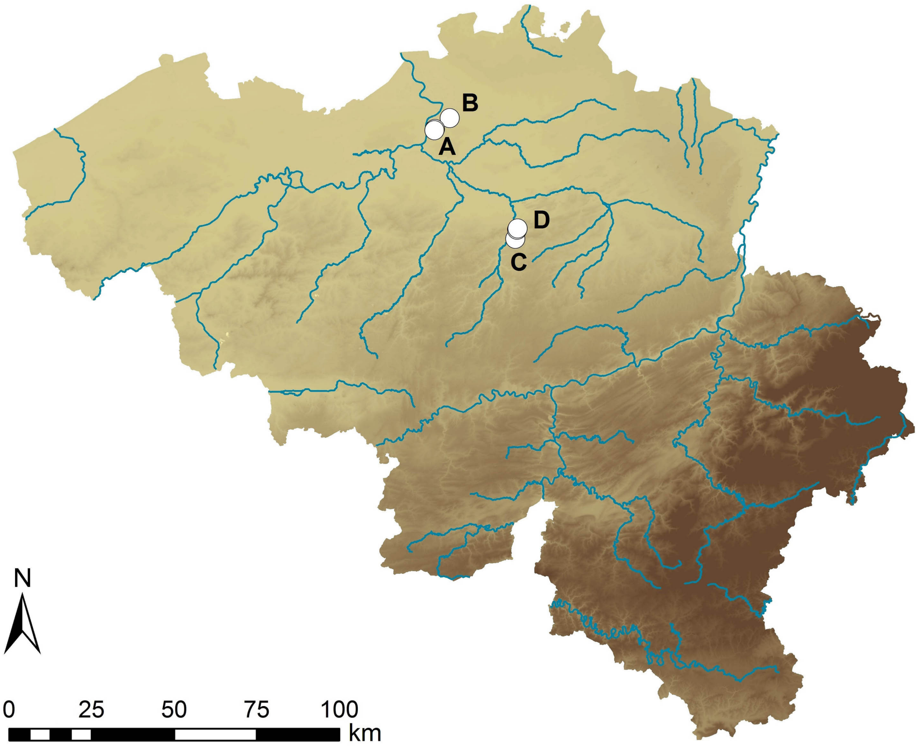

Figure 2. Distribution of the marbled crayfish in Belgium: A. Schoonselhof; B. Wolvenberg; C. Sports complex Heverlee; D. Provincial Domain Kessel-Lo.

Morphological characteristics

Morphological measurements were conducted in the laboratory on all collected specimens. For each specimen the total length (TL: length measured from tip of rostrum to end of telson), carapace length (CL: length measured from tip of rostrum to posterior edge of carapace) and carapace width (CW: measured at widest point) was determined using a caliper with an accuracy of 0.1mm.

Results

Historical and recent observations

A total of seven records (submitted between 2017 and 2020) of the marbled crayfish was discovered based on a revision of photographic evidence on the citizen science platform www.waarnemingen.be. The records originated from four different sites and were originally reported as another species. Six of these records were incorrectly identified as *F. limosus* and another one was identified as "crayfish indet." without further specification. The other online platforms, www.iNaturalist.org and www.rivierkreeften.be, did not reveal any records of this species. The presence of established populations of the marbled crayfish was indeed confirmed at each of these four sites by visual encounter surveys and hand collecting on site (Figure 2, Table 1). During these field surveys, 66 specimens were collected in total, none of which were carrying eggs at the time of collecting. No other crayfish species were encountered at the surveyed sites.

Table 1. Surveyed sites.

Site	Name	Coordinates	Type
A	Schoonselhof cemetery, Wilrijk, Antwerp	51°10′N; 4°22,04′E (ditch), 51°9,81′N; 4°21,94′E (moat)	eutrophic ditch and castle moat with little vegetation
B	Wolvenberg, Berchem, Antwerp	51°11,77′N; 4°25,88′E	large, eutrophic, shaded pond in forest
C	Sports complex Heverlee, Leuven, Flemish Brabant	50°52,04′N; 4°42,78′E	artificial ornamental basin
D	Provincial Domain Kessel-Lo, Leuven, Flemish Brabant	50°53,49′N; 4°43,26′E	large eutrophic pond

Figure 3. Collecting sites of the marbled crayfish in Belgium: A. Schoonselhof (ditch); B. Wolvenberg; C. Sports complex Heverlee; D. Provincial Domain Kessel-Lo. Photographs by Thomas Abeel.

Sites with marbled crayfish

Site A: Schoonselhof cemetery, Wilrijk, Antwerp

The Schoonselhof cemetery contains about 65 ponds and ditches, of which several are interconnected by means of culverts. Most of these eutrophic ponds and ditches contained dense aquatic vegetation consisting of *Elodea nuttallii* and *Potamogeton* spp. and riparian vegetation of *Typha latifolia* and *Phragmites australis*. In the center of the cemetery there is a castle moat, lacking any aquatic vegetation other than some non-native water lilies (*Nymphaea* sp.). On the citizen science platform www.waarnemingen.be there was a single record of the marbled crayfish for this location, mentioning an estimation of about 100 specimens (Loïc van Doorn, April 17th 2020). During our survey, the marbled crayfish was only encountered in one ditch (water surface area 390 m^2) (Figure 3A) and in the castle moat (water surface area 18,756 m^2) with respectively two and nine specimens.

Site B: Nature reserve Wolvenberg, Berchem, Antwerp

At the site Wolvenberg, an isolated nature reserve in a heavily urbanised area, a population of the marbled crayfish was found in a pond (Figure 3B).

The pond (water surface area 6,980 m²) is heavily shaded by trees and the substrate consists of a layer of decaying leaves. The vegetation consists of large patches of greater water-moss *Fontinalis antipyretica*. On www.waarnemingen.be there are two records of this species, both from 2019 (Eric Molenaar, January 27th and October 20th 2019). During a short survey 43 specimens were collected along a twenty meter transect.

Site C: Sports complex Heverlee, Leuven, Flemish Brabant

The site at the sports complex of Leuven consists of an artificial, concrete, shallow, ornamental basin with coarse gravel substrate (water surface area 972 m²). Vegetation was absent with the exception of some *T. latifolia* in one corner. On www.waarnemingen.be a single record of this species (Sebastiaan Verbeke, July 26th 2018) was found. This basin was nearly completely dry at the time of sampling, and has been so since summer 2018. All specimens were found among stones and plumbing inside the filter in about 5 cm of stagnant water (Figure 3C). Some individuals of non-native marsh frog *Pelophylax ridibundus* were also present in this water. In total, ten marbled crayfish, including juveniles, were collected at this site, with also some dead specimens present.

Site D: Provincial Domain Leopoldspark Kessel-Lo, Leuven, Flemish Brabant

This recreational domain is a public park with a couple of differently sized lakes. The riparian vegetation of these eutrophic lakes is mainly dominated by *Carex* species (*C. acutiformis*, *C. acuta*, *C. rostrata*) and *T. latifolia*, partly shaded by trees, whilst the aquatic vegetation is mostly patchy with *Potamogeton pusillus*, *Najas marina* and *Chara globularis*. Based on the records on the citizen science platform www.waarnemingen.be, the marbled crayfish was found in two large ponds with records from November 2017 to November 2019 (Fred Vanwezer, Tony Van Mellaert). Some of the pictures show predation on the crayfish by coot *Fulica atra* and mallard *Anas platyrhynchos*. During the visual encounter survey, the species was however only encountered in the third largest pond (water surface area 9,060 m²) (Figure 3D). In this pond, four individuals were observed and only two were collected.

Molecular identification

Molecular analyses of 12 specimens (4 individuals originating from sites A and B, and 2 individuals from sites C and D) yielded useful good quality sequences of the full 658 bp COI Folmer fragment from only 10 individuals (the DNA of one individual from site A and C could not successfully be amplified). Each of the obtained sequences (GenBank accession numbers LR884225–LR884234) appeared 100 percent identical to the known sequences of *Procambarus virginalis* available from the International Nucleotide Sequence

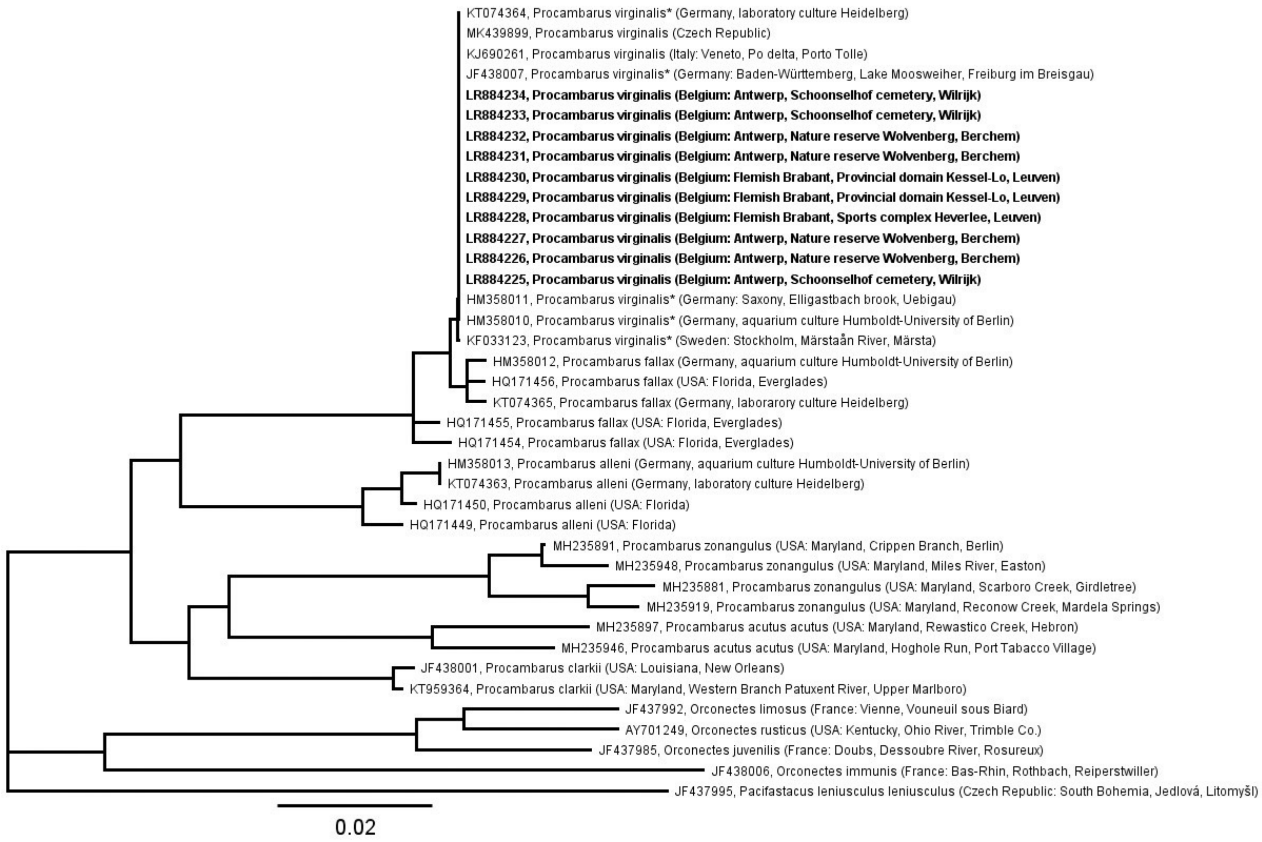

Figure 4. Tamura-Nei neighbour-joining tree showing the Belgian marbled crayfish (*Procambarus virginalis*) specimens (in bold) to be identical to known COI sequences from *P. virginalis* (* indicates original sequences of marbled crayfish still under the old species name *P. fallax* (forma *virginalis*) in INSDC) together with a selection of other closely related crayfish species and a specimen of *Pacifastacus leniusculus* as outgroup. The scale bar indicates substitutions per site. Sequences are indicated by their INSDC accession number, species and origin.

Table 2. Sequence differences at 10 positions of the 658 bp Folmer barcoding fragment on the COI gene. A single diagnostic nucleotide at position 397 for character-based species identification of *Procambarus virginalis* (397T) and *P. fallax* (397C) is indicated in bold. Number of identical INSDC accession numbers for each haplotype in parentheses.

658 bp COI sequence	69	166	202	253	349	**397**	400	500	565	581
HM358010, *P. virginalis* (17)	T	A	T	G	A	**T**	C	C	G	C
HM358012, *P. fallax* (2)	.	G	C	A	.	**C**	.	.	.	.
HQ171456, *P. fallax* (1)	.	G	.	A	G	**C**	.	.	.	.
HQ171455, *P. fallax* (2)	.	.	.	.	.	**C**	T	T	A	.
HQ171454, *P. fallax* (4)	G	.	.	.	.	**C**	.	T	A	T

Database Collaboration (INSDC) (i.e. acc. nos. HM358010, HM358011, JF438007, KT074364 from Germany, Martin et al. 2010b; Filipová et al. 2011; Vogt et al. 2015; KJ690261 from Italy, Vojkovská et al. 2014; KF033123 from Sweden, Bohman et al. 2013; and MK439899 from the Czech Republic, Patoka et al. 2016). Each of the analysed Belgian specimens clearly clustered within this group of *P. virginalis* reference sequences (Figure 4).

Within the COI Folmer fragment of *P. virginalis*, there exists one diagnostic nucleotide, T, at position 397, that consistently differs from all known *P. fallax* haplotypes, in which it is characterized by a nucleotide C at the same position (Table 2).

Table 3. Measurements in millimeter (minimum, maximum and average) of total body length (TL), carapace length (CL) and carapace width (CW) per site in mm (N = number of specimens).

	N	TL			CL			CW		
SITE		min	max	avg.	min	max	avg.	min	max	avg.
A	11	44.4	93.0	66.3	20.1	44.0	31.3	8.7	21.0	14.2
B	43	29.0	96.6	62.0	13.0	46.5	29.9	5.5	21.5	12.8
C	10	37.0	83.0	55.2	17.0	39.0	25.8	8.0	19.0	12.3
D	2	60.0	74.0	67.0	29.0	34.0	31.5	13.0	15.5	14.3
All sites	66	29.0	96.6	61.9	13.0	46.5	29.6	5.5	21.5	13.0

Morphological characteristics

All collected specimens were identified as *P. virginalis* based on the combination of following morphological characteristics: typically marbled pattern on carapace and dorsal side of the pleon, with dark dorsolateral fascia on carapace (Figure 1A), acuminate rostrum with small triangular acumen (Figure 1B), bell-shaped *annulus ventralis* (Figure 1C), size (TL average 61.9 mm and exceeding 90.0 mm in larger individuals (Table 3) and the absence of males. There existed substantial variation in the extent of the marmorated pattern on the carapace, both in size and density of the individual spots. One adult individual had the carapace completely devoid of any marbling.

The total body length of all collected specimens ranged from 29.0 to 96.6 mm with an average of 61.9 mm. The carapace length and width ranged from 13.0–46.5 (average = 29.6) and 5.5–21.5 (average 13.0) respectively (Table 3). The differences in total body length (TL), carapace length (CL) and carapace width (CW) between the populations are minor and rather insignificant (Table 3). With exception of site D in which only two specimens were collected, all populations included both relatively small and large specimens which indicates marbled crayfish were propagating at these sites.

Discussion and conclusion

With the discovery of four populations of the marbled crayfish in Belgium, there are currently six species of non-native invasive crayfish known from the country, all with established populations. This number is still rather low compared to neighboring countries, such as France, where currently nine non-native crayfish species are known (Collas 2019a). Germany and the Netherlands have respectively seven and six established non-native crayfish species (Kouba et al. 2014). Interestingly, the crayfish fauna in France, Germany, and the Netherlands does not concern the same list of species. All four Belgian marbled crayfish populations most likely originate from independent releases or dumping of specimens from private aquaria. Also escape from garden ponds cannot be excluded as this species is sometimes sold at garden centres (Patoka et al. 2017). This also indicates the species is probably not rare in the Belgian aquarium trade or at least that it was kept and traded in the recent past. Based on the photographs we revised on the citizen science platform www.waarnemingen.be, the marbled crayfish is at least already present since 2017, but was consistently misidentified as *Faxonius limosus*, a common species in Belgium. The latter thus illustrates that more consistent screening of records submitted to citizen science platforms may result in earlier detection of additional species and the detection of new populations of established non-native crayfish. The system www.waarnemingen.be is by far the most widely used recording platform in Belgium, mostly targeting naturalists and the general public (Swinnen et al. 2018). Although it represents a general recording platform for all species, it has a dedicated portal for performing early warning on a selection of invasive alien species, including the IAS of Union Concern *sensu* EU Regulation 1143/2014. Apart from facilitating rapid response and control actions, this portal offers identification sheets for established as well as new crayfish species and e-mail alert subscription to the user (Vanderhoeven et al. 2015).

The molecular observations are in line with the parthenogenetic reproduction mode of the exclusively female *P. virginalis* and earlier described genetic homogeneity and clonality (Vogt et al. 2015; Lyko 2017). Moreover, all marbled crayfish are identical to each other. The small percentage of differences with *P. fallax* for the 658 bp Folmer fragment of the COI gene falls within the intraspecific variability of the *P. fallax* cluster (Figure 4) (Vogt et al. 2018). Although such a distance based barcoding approach for species identification is not feasible for marbled crayfish, it can be expected that a character based barcoding approach can potentially help, due to the lack of intraspecific variability within the clonal *P. virginalis*. Indeed Lyko (2017) already documented the occurrence of six mitochondrial single nucleotide variants. In addition, Vogt et al. (2018) highlighted ten base positions at which *P. virginalis* and *P. fallax* show substantial differences in their nucleotides along the standard 658 bp COI Folmer fragment. With this study, we propose that position 397C>T can be used as a single diagnostic nucleotide for easy character-based species identification between *P. fallax* (397C) and *P. virginalis* (397T) (Table 2).

No berried (egg-carrying) females were encountered during this survey. Records of egg-carrying females from other countries indicate that the reproduction period varies among countries. In other wild European populations, females carrying eggs were recorded in Romania in April and May (Pârvulescu et al. 2017), in Germany from June to October (Chucholl and Pfeiffer 2010), in Croatia in June and September (Cvitanić 2017), in the Czech Republic in September (Patoka et al. 2016) and in Ukraine in October (Novitsky and Son 2016). These observations suggest that reproduction may thus occur from spring until autumn, which is most probably related to water temperature. As we visited the Belgian sites from the end of May to early July, it is plausible that the sampling took place just before the main reproductive period. The absence of berried females can however also be the result of lower activity of berried females which may retreat into burrows or other shelters for longer periods.

Martin et al. (2010b) question the invasive status and threat of the marbled crayfish in Europe based on its limited number of established populations. However, Chucholl et al. (2012) report the strong increase of this species in Europe, with established populations known from only two countries in 2012 to 12 countries in 2020. The success of the marbled crayfish as an invader has been attributed to several factors. First, Lyko (2017) stated that, due to their predicted ability to found large populations from single animals, marbled crayfish introductions have a significant potential to endanger indigenous crayfish species through competition or pathogen transmission. The marbled crayfish is a confirmed carrier of the oomycete *Aphanomyces astaci* Schikora (Keller et al. 2014), which causes the crayfish plague (Tilmans et al. 2014). Crayfish plague is the main cause of the decline of susceptible native crayfish in Europe because native crayfish species show very high mortality rates when exposed to this pathogen (Holdich et al. 2009). Second, the marbled crayfish is regarded relatively drought resistant (Kouba et al. 2016) and can complete terminal phases of embryogenesis, including hatching, as well as early post-embryonic development under high air humidity conditions only (Guo et al. 2019). Marbled crayfish are also known to construct burrows, between 5 and 16 cm deep, in which they retreat during droughts (Kouba 2016). Third, marbled crayfish are competitive, and maybe even dominant, over several other invasive crayfish (Chucholl and Pfeiffer 2010; Jimenez and Faulkes 2011; Hossain et al. 2019). Fourth, the species has a large ecological amplitude. In Madagascar, the rapidly expanding population of marbled crayfish has invaded a large variety of habitats and shows a high ecological plasticity, occurring both in lentic and lotic environments (Andriantsoa et al. 2019). Lastly, marbled crayfish are able to migrate over land (Chucholl et al. 2012).

Since August 2016, the marbled crayfish is included on the list of Invasive Alien Species of Union concern. European Member States are required to take preventive actions on pathways of unintentional introduction, set up surveillance, take measures for early detection and rapid eradication of newly discovered populations and manage this species if already widely spread on the territory. Despite the new EU Regulation, systematic, dedicated and active surveillance for alien crayfish is currently absent in Belgium. The findings of such new species, like marbled crayfish and *P. acutus* (Scheers et al. 2020), through opportunistic surveys instigated by citizen science reports, clearly indicates a considerable knowledge gap on the distribution of crayfish species. Without a proper view on distribution and abundance of the different species, a recommendation on management objectives at country level is, however, difficult to formulate. A crayfish surveillance system could be a combination of active surveillance consisting of systematic surveys by regional public authorities or scientific institutions, and passive surveillance using citizen science with local managers, anglers, naturalists and crayfish enthusiasts (e.g. hobbyists). Active surveillance can be concentrated on vulnerable protected areas, in areas adjacent to or within dispersal distance of known populations and pest-free areas at risk of invasion. This would also allow to raise awareness on non-native crayfish impacts on local ecosystems with these interest groups. In areas with a low-density of crayfish, surveillance can be supplemented with e-DNA samplings, which can simultaneously reveal the presence of crayfish plague (Rusch et al. 2020). Passive and active environmental DNA surveys can indeed be very helpful to detect invasive species early and rapidly acquire a landscape level image of the distribution of such invasive species (Simmons et al. 2016). Legally, mandatory notification of crayfish presence by land owners/managers could be enforced.

Adriaens et al. (2019) have formulated management strategies aimed at eradicating and limiting the spread of crayfish in Belgium. For marbled crayfish, which was at that time unknown to occur in the country, the recommendation was an eradication strategy as a guiding principle of the EU Regulation for species not yet present in Belgium. The new findings presented here might warrant revising these management objectives, as the species appears already established, and derogations are foreseen in the Regulation. However, the four known sites are all isolated water bodies which can still offer the prospect of rapid eradication. A site-based approach is needed to determine feasibility of eradication from every water body. At the site C, a concrete basin which can be drained easily, eradication is straightforward and should be executed as soon as possible. As the species is known to disperse on land, attention should be taken to prevent the dispersal during management interventions using fencing (Peay and Hiley 2001). The other locations (A, B, D) are bigger water bodies and the possible management options should be investigated for each site individually. Eradication of invasive non-native crayfish is notoriously difficult since the animals can retreat in deep burrows, where they can survive up to several months, and because of their ability to move over land (Gherardi et al. 2011). Successful eradications of invasive non-native crayfish are very rare and mostly concern chemical control using biocides such as Pyblast (Peay et al. 2006; Gherardi et al. 2011; Stebbing et al. 2014), a method which is prohibited in Belgium. Alternatively, an integrated pest management strategy can be used, i.e. applying intensive trapping and native predatory fish introductions as active removal or restoration methods (Stebbing et al. 2014; Souty-Grosset et al. 2016). At least on one site, predators of crayfish such as pike *Esox lucius* and waterfowl are already present. The use of the pathogen *A. astaci*, which causes the crayfish plague, has also been reported as an eradication method against *Cherax destructor* in Spain (Diéguez-Uribeondo and Muzquiz 2005). Crayfish species of North American origin are, however, resistant to the crayfish plague and serve as its vectors (Holdich et al. 2009). This method is therefore not suitable for the control of *P. virginalis*, which is itself a confirmed carrier of this pathogen (Keller et al. 2014; Mrugała et al. 2015). Moreover, in Belgium, relic populations of the native *Astacus astacus* occur in hydrologically isolated systems (Boets et al. 2012; Schrimpf et al. 2017) and the introduction of crayfish plague to these areas needs to be prevented at all cost. We recommend to closely monitor this species at the known sites and conduct further surveillance of the marbled crayfish in Belgium as a whole in order to detect new colonisations.

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