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Short communication

The first record of the marbled crayfish adds further threats to fresh waters in Italy

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Abstract

The red swamp crayfish, *Procambarus clarkii*, is the most abundant invasive crustacean decapod in Italy. Evidence is however emerging for the presence of other Cambaridae that are erroneously assigned to the *P. clarkii* taxon. The marbled crayfish, belonging to a still uncertain species of the genus *Procambarus*, has been found for the first time in Italy in the Canale Maestro della Chiana (Tuscany, Central Italy), where it lives in sympatry with a large *P. clarkii* population. Although a single specimen was found, this record is particularly relevant due to the parthenogenetic reproductive habit of the marbled crayfish. However, molecular analyses based on *COI* barcoding did not reveal any differentiation within the *P. clarkii* population and excluded any form of hybridization between the two species. We will shortly discuss new pathways of invasive species and the threats posed by parthenogenetic species, even though they seem to be still sporadic.

Key words: barcoding, conservation, marbled crayfish, parthenogenesis, nonindigenous species

In the last decade, the human-mediated introduction of animal species into Italian inland waters has generated dramatic effects on native freshwater biota (Gherardi et al. 2008). Biological invasions are considered to be one of the "big five" issues in conservation biology worldwide (Sala et al. 2000). This is particularly true in Italy where most of the introduced species rapidly form self-sustaining populations in the colonized habitats and pose major threats to the invaded communities (Kolar and Lodge 2001; Gherardi et al. 2008).

Crayfish, with a long history of intentional or accidental introductions (Lodge et al. 2000), have attracted the attention of both scientists and the general public because of the negative impact they inflict on the biomass and species richness of freshwater communities (Nyström 2002; Geiger et al. 2005; Rodríguez et al. 2005). Most European countries have today at least one nonindigenous crayfish, still localized around the point of introduction or having spread across the invaded area (Henttonen and Huner 1999; Souty-Grosset et al. 2006).

Procambarus is one of the most frequently introduced crayfish genera, with the red swamp crayfish *Procambarus clarkii* (Girard, 1852) becoming a panglobal species due to the successful human spreading from its native range (north-eastern Mexico and south-central USA) to all over the world (Huner 2002). Recently, a new *Procambarus* species has been described by Scholtz et al. (2003) as 'marmorkrebs' or 'marbled' crayfish from an uncertain geographical origin and species identity. In their publication on Nature, the authors even raised the hypothesis that *Procambarus* sp. is a transgenic species created by laboratories in

Hong Kong. From a biological viewpoint, the marbled crayfish seemed to be a unique case of parthenogenetic species among decapods (Vogt et al. 2004; Seitz et al. 2005; Alwes and Scholtz 2006), although evidence of parthenogenesis was revealed only in captivity. Recently, Yue et al. (2008) reported on the occurrence of four natural clones in *Procambarus clarkii* from China, suggesting that parthenogenesis may be more widespread in crayfish.

Martin et al. (2007) demostrated with the analysis of six microsatellite loci that the 'marmorkrebs' indeed produces genetically uniform clones. These results and the absence of meioses in previous histological studies of the ovaries led the Authors to conclude the Marmorkrebs propagates apomictically. Nevertheless, Vogt et al. (2008) showed that batch-mates of this clonal crayfish, which were shown to be isogenic by microsatellite analysis, exhibited surprisingly broad ranges of variation in coloration, growth, life-span, reproduction, behaviour and number of sense organs, even when reared under identical conditions. Vogt (2008) carried out a complete and detailed review of the marbled crayfish discovery and research history, biology, culture and suitability as a laboratory model for various research disciplines.

Before the first records of free-living 'populations' in Europe (Scholtz et al. 2003) and Madagascar (Jones et al. 2008), this species has been known only from aquaria in Germany and Austria since the mid 1990s. In Europe, natural populations seem to be restricted to two sites in Germany and one in the Netherlands (Souty-Grosset et al. 2006).

In April 2008, a 'marmorkreb' individual was recorded in the Canale Maestro della Chiana, 43°16'50.64"N; 11°50'11.98"E (Tuscany, Central Italy) as part of a well acclimatized *P. clarkii* population. This crayfish clearly differed from the 'classic' phenotype of *P. clarkii* specimens inhabiting Italian inland waters (Figure 1). After the discovery of this specimen, other sampling campaigns were conducted to ascertain the density of marbled crayfish in the *P. clarkii* population and to classify cryptic specimens with ambiguous external morphology by the means of DNA barcoding.

A total of 20 crayfish of uncertain morphology were selected among more than 100 crayfish collected by electrofishing and traps. Sampling was carried out on May 8 and July 29, 2008 in 2000 m^2 (channel width: 2 m). Crayfish were

Figure 1. First record of marbled crayfish *Procambarus* sp. collected in Central Italy during spring 2008 (top). Comparison with *Procambarus clarkii* (bottom) (Photographs by Marzia Guffanti and Armando Piccinini)

sexed, body length was measured, and a muscular tissue sample was collected from each specimen to perform barcoding analysis based on *COI* mtDNA haplotyping using the primers HCO 2198 and LCO 1490 (Folmer et al. 1994; Trontelj et al. 2005; Dawnay et al. 2007). We followed the procedure described by Chiesa (2008): a reaction volume of 25µl containing 1U di Biotherm Red DNA Polymerase (Fisher Molecular Biology), Mg^{2+} 2.5mM, 10 pmol of forward and reverse primers, 1× reaction buffer and dNTPs 0.2mM was used, by applying 40 cycles of 30s at 95°C, 45 s at 45°C, and 1 min at 72°C, after an initial 10 min denaturation step at 95°C. PCR products were purified by eluition from a 2.5% agarose gel, then precipitated with 3 volumes of 100% ethanol, and washed with 70% ethanol. They were sequenced using FW or RV *COI* primers by CEQTM DTCS-Quick Start Kit (Beckman Coulter) on "CEQ™ 8000 DNA Analysis System" (Beckman Coulter). The amplified fragments were all the same length,

about 650 bp. They were compared with sequences available in genomic databases using Blast and multiple alignments of sense and antisense sequences were conducted using such softwares as Clustal X (Thompson et al. 1997) and Sequencer 4.2 (Gene Code Corporation), verifyng the correctness of the aligment at the nucleotide level.

Monitoring did not reveal the occurrence of other marbled crayfish and molecular analyses that compared the sequences obtained and those from GenBank for a 450 bp fragment of *COI* mitochondrial gene assigned all the investigated samples to the *P. clarkii* taxon (Figure 2). Among the 20 analyzed specimens, we found one polymorphic site corresponding to the transition

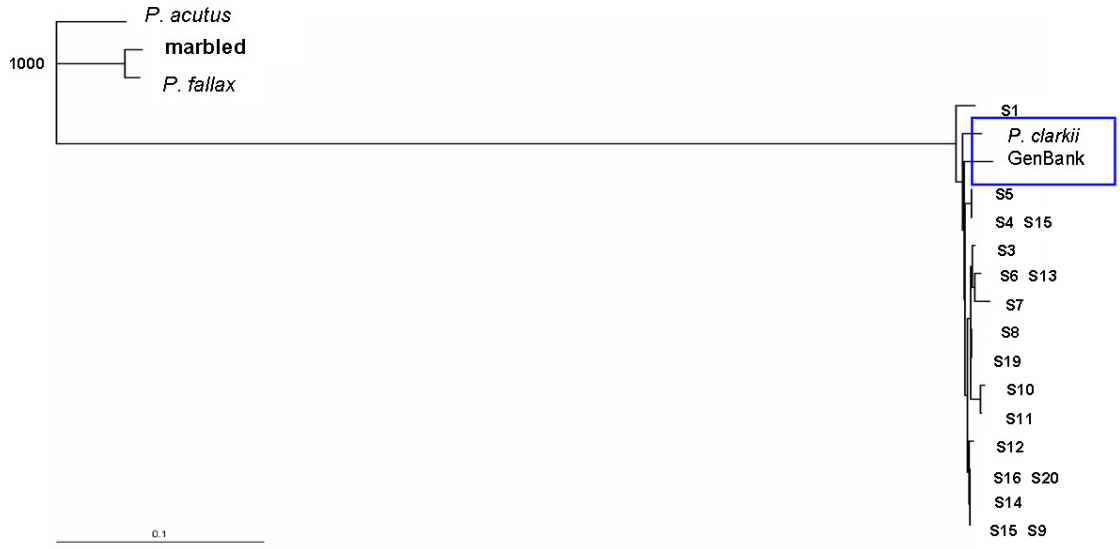

Figure 2. Phylogenetic tree based on *COI* mtDNA showing clustering of 20 experimental samples with a *P. clarkii* sequence (Accession no. DQ889125) obtained from GenBank. Association between *P. acutus*, *P. fallax* and marbled crayfish is referred to GenBank sequences (Accession No. AF474366, AY151524 and AY151523)

A-G over a total of 650 nucleotides sequenced. This limited amount of genetic variability confirms the origin of the Canale della Chiana population from a typical "founder effect". The genetic differentiation with respect to the *P. clarkii* sequences obtained from GenBank was assigned to 4 polymorphic sites (3 transitions and 1 transversion).

By comparison, a large amount of genetic variability was found in the unique marbled crayfish sequence deposited in GenBank. Since a large genetic variation seems unusual for a parthenogenetic species, additional sequences of 'marmorkrebs' will be necessary to perform a correct barcoding strategy. Indeed, the debate around the taxonomic identity of the marbled crayfish is still open: former studies indicated its closest relationships with *Procambarus fallax* while more recent ones with *Procambarus alleni* (Souty-Grosset et al. 2006).

In conclusion, our genetic analysis confirmed that the marbled crayfish is still sporadic in Central Italy. However, considering the parthenogenetic reproductive behavior of this species, future monitoring will be required. Although a single specimen, this record brings additional concern on the threats posed by invasive crayfish species to European freshwater communities. Indeed, marbled crayfish can be easily purchased through the eBay web-site, which rises additional concern on the new pathways of introduction of nonindigenous species through globalization and e-commerce.

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