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Research Article

Molecular identification of an alien temnocephalan crayfish parasite in Italian freshwaters

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Abstract

Although the symbionts of non-vertebrate metazoans have received scarce attention, scientific interest has recently increased as these symbionts can spread diseases and alter the diversity of the ecosystem where hosts are introduced. We report here a symbiont newly described by molecular markers and observed on the yabby *Cherax destructor* Clark, 1936, collected in Italian freshwaters. Preliminary morphological observations were performed and a species identification was proposed using molecular techniques. In particular, a 644-bp long *28S* rDNA fragment was successfully sequenced and could be aligned for the entire sample set. The minimum spanning network identified two haplotypes clustered into a single haplogroup (H1) together with *Temnosewellia minor* Haswell, 1888 reference sequences. Considering the introduction of temnocephalans, this represents the third discovery of alien crayfish symbionts in Italian freshwaters, but the first obtained by molecular data. Outside Europe, there are only few records of alien temnocephalans worldwide, introduced with the crayfishes from Australia. The occurrence of this new temnocephalid species and the great number of potential hosts species inhabiting Italian freshwaters suggest that the Italian peninsula might have many undescribed temnocephalid populations/species.

Key words: Temnocephalida, alien species, symbionts, molecular identification, *28S* rDNA, Italy

Introduction

Alien invasive species represent one of the most serious threats to biodiversity at different taxonomic levels (Mack et al. 2000) including freshwater ecosystems (Rodríguez et al. 2005; Gherardi 2007a).

Invasions by non-native crayfish have attracted the attention of scientists and policy makers (Gherardi and Holdich 1999; Lodge et al. 2000) and many European projects have aimed to mitigate their impact. Native taxa can be damaged by alien species, because of their tolerance to extreme environmental conditions, rapid spread and dispersal, and capability to transmit parasites (Edgerton 2002; Souty-Grosset et al. 2006; Gherardi 2007b; Longshaw 2011).

Multiple phyla of alien freshwater invertebrate symbionts have been recorded in association with the introduction of alien crayfish (Yamaguchi 1933; Tamura et al. 1985; Smith and Kamiya 2001;

Ohtaka et al. 2005; Longshaw et al. 2012). An example is the high impact of the crayfish plague on European indigenous crayfish populations, whose socio-economic relevance has led to extensive research focused on crayfish pathology (see Alderman et al. 1987; Diéguez-Uribeondo 2009; Diéguez-Uribeondo and Söderhäll 1993; Edgerton 2002).

Despite this, alien symbionts are often ignored (see Dörr et al. 2011; 2012a, b) unless they spread diseases or alter the biodiversity of the ecosystem where their hosts were introduced (Niwa and Ohtaka 2006). From this viewpoint, Italy seems particularly affected amongst European countries, since alien crayfish have spread quickly and established self-sustaining invasive populations (Scalici and Gherardi 2007; Gherardi et al. 2008a; Scalici et al. 2010). Different species such as *Astacus leptodactylus* Eschscholtz, 1823, *Orconectes limosus* (Rafinesque, 1817),

Pacifastacus leniusculus (Dana, 1852), the marbled crayfish *Procambarus fallax* (Hagen, 1870) *f. virginalis*, and the abundant *Procambarus clarkii* (Girard, 1852) (Gherardi et al. 2008b; Scalici et al. 2009b; Nonnis Marzano et al. 2009) are frequently recorded over most Italian freshwater basins. The establishment of alien species may cause changes in the composition of native communities (Keith and Allardi 1997) significantly altering competition for trophic and spatial resources (Lévêque 1997), and carrying pests and pathogens (Blanc 1997; Lodge et al. 2000).

The genus *Cherax* was recently added to the alien species database for Italian inland waters, due to the records of *Cherax quadricarinatus* (Von Martens, 1868) although confined to farms – Aquiloni et al. (2010) and *Cherax destructor* Clark, 1936 subspecies *destructor*, which has been found in the Natural Reserve of "Laghi di Ninfa" (Central Italy) (Scalici et al. 2009a).

A few platyhelminth-like fauna were observed on the cuticle of both abdomen and chelae of *C. d. destructor* (Scalici et al. 2009a). Previous papers (Gasparo et al. 1984; Quaglio et al. 1999) described alien temnocephalans in Italy, namely *Temnocephala minor* Haswell, 1888 synonym of *Temnosewellia minor* Haswell, 1888- although they were not characterized by molecular techniques. We herein reported the presence in Central Italy of an alien symbiont species, introduced by exploiting alien vectors. In particular, the aim of this study was twofold: (1) to identify the symbiotic species by using a preliminary morphological investigation followed by a detailed genetic approach, and (2) to provide a baseline for further molecular comparisons with other populations and/or species.

Methods

A total of 57 ectoparasites were collected from the crayfish *C. d. destructor,* introduced in the Natural Reserve of 'Laghi di Ninfa', Central Italy. Once captured, ectoparasites individuals were preserved in cold (8°C) absolute ethanol and then stored at -20°C at the laboratory until further processing.

Following macroscopic analysis using a binocular stereoscope, 37 platyhelminths were subjected to detailed microscopic analyses using scanning electron microscope (SEM). After the ethanol dehydration, they were critical-point dried, gold-sputtered, mounted on stubs, and then examined using a Philips XL30 SEM.

We then extracted and purified high molecular weight genomic DNAs, using Wizard genomic DNA Purification kit (Promega) according to the manufacturer's protocol. DNA quality was visually inspected by 1% agarose gel electrophoresis in TAE buffer and by spectrophotometry at 260–280 nm. The extraction procedure typically yielded not less than 30 ng/µl of HMW (high molecular weight) DNA. A 633bp fragment of *28S* rDNA was amplified using the specific primers *MKL-D3A.2* 5'- CCCGAAAGATGGTGAACTAT- 3' and *MKL-D6B* 5'- GGAACCCTTCTCCACTTC AGT - 3' (Litvaitis et al. 1996). These primers are specific for platyhelminth species and have been previously used in other studies on platyhelminth phylogeny (Litvaitis et al. 1996; Litvaitis and Rohde 1999). A reaction volume of 50 µl containing 1U GoTaq Polymerase (Promega), Mg^{2+} 1.5 mM and dNTPs 0.2 mM, and 10 pmol of each primer was used. PCR – touch down profile was set as follows: 2 cycles of 15 s at 94 °C, 15 s at 52–48–46 °C, and 30 s at 70°C; then 30 cycles of 15 s at 94°C, 15 s at 50°C, and 30 s at 70°C, after an initial 1 min denaturation step at 94°C and a final extension at 70°C for 10 min. PCR products were purified by Wizard Gel and PCR Clean Up System (Promega). Forward and Reverse Sanger sequences were performed at Macrogen Inc (http://www.macro gen.com). They were compared with sequences available in genomic databases using Blast, and multiple alignments of sense and antisense sequences were conducted using Clustal X (Thompson et al. 1997) and Sequencer 4.2 (Gene Code Corporation), verifying the alignment correctness also at the amino acid level.

The identification of variable and parsimony informative sites, the pairwise genetic distances and the nucleotide base composition were calculated with MEGA 5.1 (Tamura et al. 2011).

Along the *28S* chromatograms only one double peak was found, underlining the presence of heterozygote individuals. Since only this double peak was present, sequences were resolved manually. TCS 1.21 (Clement et al. 2000) was used to evaluate statistical parsimony with a 95% confidence using additional sequences representative of Temnocephalida and already registered in GenBank: *Temnosewellia minor* (AY157164 and AF022864), *Didymorchis sp.* (AY157163).

Neutrality tests investigated the divergence of the data set from the neutrality model. They are useful to evaluate the population history, such as the presence of founder effects or balanced selection. Among neutrality tests, Tajima's D

Table 1. *28S* rDNAsequences of *Temnosewellia minor* and other Rhabdocoela species already registered in GenBank included in the dataset. Accession numbers and taxonomic classifications are reported.

GenBank Accession Number	Species
AY157163	*Didymorchis* sp.
KC529601	*Gieysztoria acariaia* (Marcus, 1946)
KC529597	*Gieysztoria ashokae* (Van Steenkiste et al., 2012)
KC529606	*Gieysztoria beltrani* (Gieysztor, 1931)
KC529602	*Gieysztoria pavimentata* (Beklemischev, 1926)
KC529611	*Gieysztoria rubra* (Fuhrmann, 1894)
KC529567	*Halammovortex* sp.
KC529568	*Jensenia angulata* (Jensen, 1879)
AF022864	*Temnosewellia minor* Haswell, 1888
AY157164	*Temnosewellia minor* Haswell, 1888

statistic (1989) is probably the most widely used test of neutrality and reflects the shape of the genealogy, whereas Nei and Li's statistics are based on the fact that singletons, i.e. polymorphisms that affect just one individual in a sample, play a relevant role on different population histories. DnaSP 5.10.01 (Librado and Rozas 2009) was used to perform the Tajima's (1989), Nei's and Li's neutrality tests on the entire sample set, including or not AY157164 and AF022864. To estimate the evolutionary divergence between sequences, the number of base differences per site among sequences was evaluated in MEGA 5.1.

The phylogenetic reconstruction included 10 additional sequences of *Rhabdocoela* species (Table 1), further than *Halammovortex* sp. (KC529567) and *Jensenia angulata* (Jensen, 1879) (KC529568), considered closely related to Temnocephalidae (Van Steenkiste et al. 2013).

Prior to construction of the evolutionary tree, it is important to carry out statistical selection of best-fit models of nucleotide substitution. The model that best fitted the data obtained was checked through J Model test (Darriba et al. 2012). Then, neighbour joining (NJ) phenograms were constructed using the Jukes Cantor (JC) model of DNA evolution. A bootstrap resampling procedure was performed on the entire data set with 1000 replicates. A Maximum Likelihood (ML) tree was constructed through PHYML (Guindon and Gascuel 2003), based on the JC model, with a gamma distribution shape parameter of 4.

Results

Specimens showed a concave body shape with five tentacles on the anterior portion and a sucker on the posterior one (Figure 1). The five anterior tentacles were prehensile and very extensible.
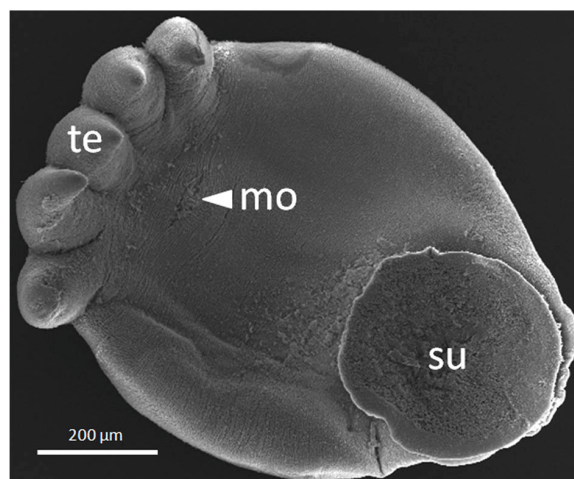

Figure 1. Scanning electron micrographs of the ventral body side, tentacles (te), mouth opening (mo), and sucker (su) of the investigated ectoparasites observed on the crayfish exoskeleton (magnification 107x).

The body length was about 1.1 mm (0.8–1.7) without tentacles, and the width 0.6 mm (0.4–1.0). Light black pigment seemed to be confined to a single pair of eyes located dorsally on the anterior body portion. The posterior adhesive disc diameter was 0.411 mm (307–552 μm). The morphology enabled their identification as temnocephalans.

We then performed molecular analyses focused on sequences of *Rhabdocoela*, particularly on those considered as closely related to Temnocephalidae (see Methods).

Sequences of *28S* rDNA fragments were successfully obtained and could be aligned unambiguously for the entire sample set for 633 bp. The 5'-3' extremes were eliminated to delete bases with a low sequencing quality. Only one mutation was found across samples (G540T).
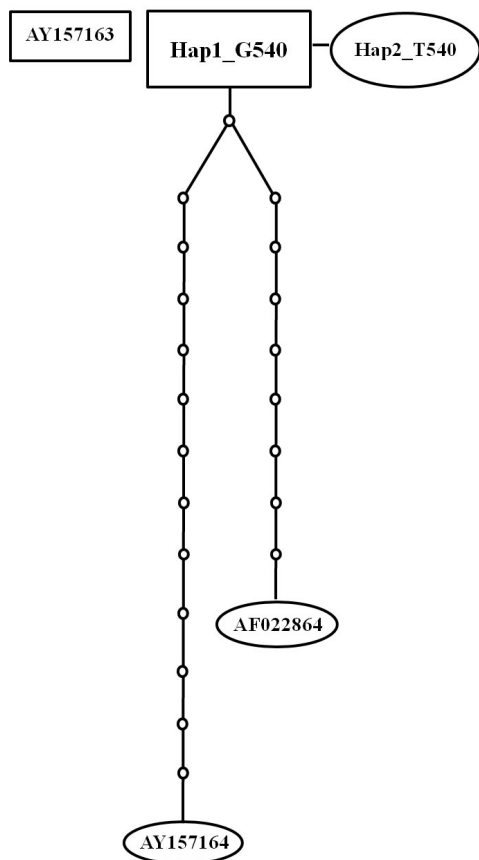
211

Figure 2. Minimum spanning tree of the haplotypes found in Italy which clustered in haplogroup 1 (H1). The most representative haplotype of H1 is reported in a square. Other haplotypes are reported in ovals.

Globally, 49% of samples were heterozygotes G540T, 9% were 540T and 42% were 540G. TCS 1.21 identified two haplotypes for Italian samples (Hap1, Hap2; Figure 2) (GenBank Accession Numbers KC110765-66) and underlined that these two haplotypes clustered into a single haplogroup (H1) that included also the two sequences belonging to *Temnosewellia minor* (AY157164 and AF022864), while *Didymorchis* sp. clustered in a separated haplogroup.

Neutrality tests performed for our nucleotide sequences underlined the presence of 1 segregating site, the neutrality test statistics ranged from -0.48 (Fu and Li's D) to 1.54 (Tajima's D). The same test performed on our sequences together with AF022864 and/or AY157164 underlined the presence of 7–14 segregating sites and markedly negative neutrality tests (Table 2).

The nucleotide sequence composition across Italian haplotypes was moderately A+T rich (51.0%), coincident with those obtained for the same fragment for Temnocephalida, for example for *Temnosewellia minor* (AY157164, AF022864) (both 51.0%) or *Didymorchis* sp. (AY157163) (50.5%). Most of the other platyhelminths showed lower (42–50) or higher (52–55) A+T percentages. Higher values of A+T% emerged from the observation of the two taxa closest to Temnocephalidae: 52% for *Halammovortex* sp. (KC529567) and 53% for *Jensenia angulata* (KC529568).

Bootstrap ML (Figure 3a) and NJ phenograms (Figure 3b) underlined a structure in which AY157164, AF022864 and Hap1-Hap2 haplotypes formed a single branch, reflecting the structure suggested by TCS (Figure 2). This branch is strictly related to that of *Didhymorchis* sp. and also to those of *Halammovortex* sp. (KC529567) and *Jensenia angulata* (KC529568) which, as already mentioned, are two taxa closely related to Temnocephalidae. The branching of Temnocephalidae sequences from other platyhelminths was supported by 93% and 96% bootstrap values, respectively in ML and NJ.

Discussion

Our study enabled the identification of an alien temnocephalid from Italian inland waters. In particular, our morphological and molecular investigations recognized the most probable species as *Temnosewellia minor*, even though additional temnocephalid species molecular sequences are necessary to confirm this identification.

Temnosewellia minor is a freshwater ectosymbiont belonging to the taxon Temnocephalida recognized as rhabdocoel turbellarians by Benham (1901). They are mainly associated with parastacid crayfish in Australia (Cannon 1991; Sewell et al. 2006), although they are able to inhabit other invertebrate hosts, such as freshwater crabs and shrimps, mainly in the southern hemisphere (Gelder 1999). A detailed description of the keys to the genera and a complete checklist of species of Australian temnocephalans are reported in Sewell (2013).

As for distribution, *T. minor* can be considered as native to New South Wales, Victoria, and southern Queensland, in Australia (for details, see references reported at: http://turbellaria.umaine.edu/turb3.php?action=11&code=6752&syn=2).

Morphological classification and statistical analysis of molecular data indicated our samples as temnocephalans, with *Temnosewellia minor* the most probable species. The A+T fraction was

Table 2. Number of segregating sites and statistical value for the three neutrality tests.

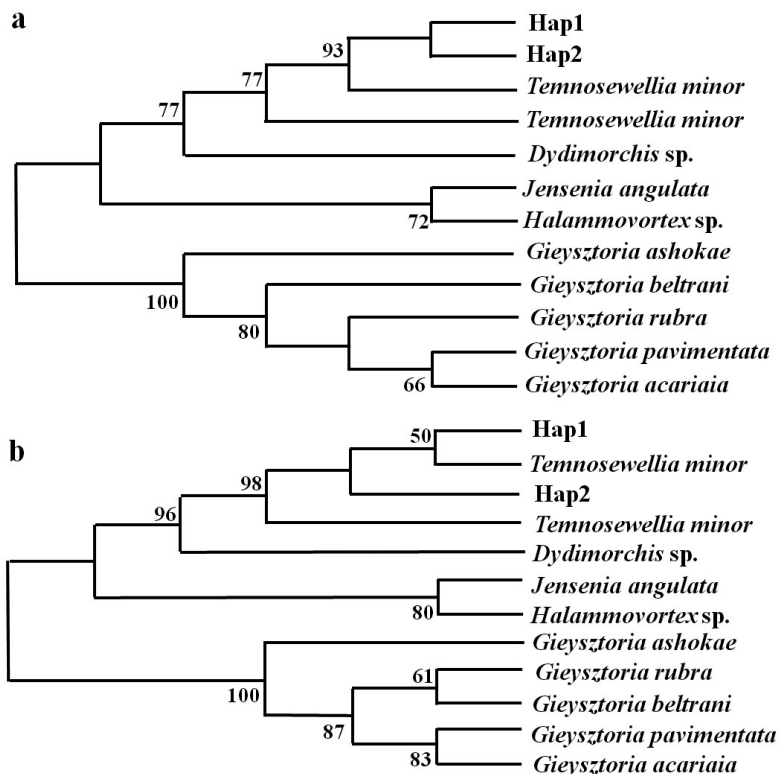|	our sequences	with AF022864 and AY157164	with AF022864	with AY157164
Segregating sites	1	20	7	14
Tajima's D	1.54	-2.26	-1.33	-1.99
Fu and Li's D	0.48	-7.00	-4.16	-6.02
Fu and Li's F	0.93	-6.22	-3.79	-5.43

Figure 3. Relationships of taxa inferred using the Maximum likelihood (ML) method (a) and the Neighbour joining (NJ) method (b). The confidence probability, expressed as percentages, that the interior branch length is greater than 0, as estimated using the bootstrap test (1000 replicates), is shown next to the branches. These trees are drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree.

comparable with those obtained in the other species of the Temnocephalida group. Yet, with the exception of *Diascorhynchus rubrus* Boaden, 1963, it differed from other platyhelminths by showing either lower or higher values. Moreover, even *Temnosewellia minor* showed a certain level of differentiation with respect to our sequences (Figure 2). Such differentiation is within the range of intra-specific variation. In fact, the occurrence of AY157164 and AF022864 in the same haplogroup of our sequences strongly suggested that all these sequences belong to the same species. This conclusion is supported by the fact that TCS analysis is usually very powerful in the distinction of species, even in the case of species complex (Lucentini et al. 2011).

Two pieces of evidence suggest the existence of a founder effect in the Laghi di Ninfa population: (i) the moderate level of genetic diversity within the population characterized by only two different haplotypes clustering within a single haplogroup (Figure 2), and (ii) the high differentiation of our sequences from AY157164 and AF022864. The occurrence of a slight founder effect is also suggested by the positive values of neutrality tests performed on this population, strongly changed in negative values when calculated considering AY157164 and AF022864 or just one of them (Maruyama and Fuerst 1985a,b).

The clustering of sequences was supported by both NJ and ML analyses; these trees underlined

a structure in which AY157164, AF022864 and Hap1-Hap2 haplogroups formed a unique branch. This branch was strictly related to other species of Temnocephalidae; *Didymorchis* sp AY157163, KC529567 *Halammovortex* sp. and KC529568 *Jensenia angulata,* two taxa close to Temnocephalidae (Van Steenkiste et al. 2013). The branching of Temnocephalidae sequences from other platyhelminths was supported by very high bootstrap values both in NJ and ML (>93%). Furthermore, it is important to note that the concordance between ML and NJ suggested that taxon sampling had not affected the main phylogenetic inference in the NJ tree, the small sample size notwithstanding.

Despite the great diversity of potential symbionts of freshwater decapods (Gelder et al. 1999), there are few records of alien temnocephalans (*Temnocephala minor*) on European (Matjasic 1963) and particularly in Italian (Gasparo et al. 1984; Quaglio et al. 1999) inland waters. Longshaw et al. (2012) reported unidentified temnocephalids in the gills of 10% of the *Cherax peknyi* (Lukhaup and Herbert, 2008) seized from Indonesia at the BIP at Manchester Airport. Moreover, *C. quadricarinatus* seized at the same site from a shipment from Singapore were found infected with temnocephalids (Longshaw et al. 2012). It was also reported that *T. minor* on *Cherax* spp. was imported into Turkey (Xylander 1997). Worldwide records are also few. Descriptions refer to *Temnosewellia minor* (Haswell, 1887), introduced with the brown crayfish *Cherax tenuimanus* (Smith, 1912) from Western Australia into Japanese freshwaters (Gelder 1999), *Diceratocephala boschmai* Baer, 1953 found on the body surface and branchial chamber of Australian marron crayfish in a Uruguayan farm (Volonterio 2009), and recently on *Cherax* spp. farmed in Thailand (Ngamniyom et al. 2014). Moreover, translocations of temnocephalids were recorded in Japan (Oki et al. 1995) and South Africa (Mitchell and Kock 1988; Avenant-Oldewage 1993), where they have been found with imported *C. tenuimanus* specimens.

These results highlight the importance of biosecurity and control of symbionts on transported animals and plants to decrease the spread of parasites and potential pathogens. The confirmed occurrence of this temnocephalid species and the great number of potential host species inhabiting Italian freshwaters suggest that the Italian peninsula might host many undescribed temnocephalid populations/species.

This paper underlines the importance and usefulness of molecular barcoding for the rapid identification of alien species in freshwater environments, which can be often difficult without an expert in classic taxonomy and systematics. Genetic markers may be helpful to identify the source population from which an alien species has come (Frankham et al. 2009; Geller et al. 2010) and its population dynamics along with acclimatization and spreading. Therefore, this molecular approach is a worthwhile tool for the control of symbionts and parasites of transported animals to avoid or limit pathogens spreading to threatened species (*sensu* Cunningham et al. 2003).

Limitations of the study include the low number of samples and the paucity of comparative sequences available in Genbank, particularly of Temnocephalidae and specifically the genus *Temnosewellia*. These problems are hard to resolve, this being the third record for temnocephalans in Italian freshwaters (see Gasparo et al. 1984; Quaglio et al. 1999), but the first that combines morphology with molecular data. Additional studies ought to be carried out to improve the parasite and pathogen identification by means of molecular techniques, in order to update molecular data.

In conclusion, in this paper we propose two original sequences obtained from a European population of alien temnocephalans, presumably *Temnosewellia minor,* as a contribution both to the future identification of the species in other invaded communities and to further species comparison.

Acknowledgements

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