Establishment of *Chydorus sphaericus* (O.F. Müller, 1785) (Crustacea: Cladocera) in Australia: consequences of mass fish stocking from Northern Europe?

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ABSTRACT

There are 11 species of genus *Chydorus* Leach, 1816 (Cladocera: Chydoridae) in Australia, including populations of *Chydorus sphaericus* (O.F. Müller, 1785) morphologically undistinguishable from European ones. Our genetic study of *C. sphaericus* from two large artificial water bodies in the Adelaide region of South Australia led us to conclude the taxon was introduced to Australia from Europe by human activity, at least in the two studied water bodies. To provide a comprehensive sister-taxon coverage and survey of intraspecific variation, our data were complemented by sequences on *Chydorus sphaericus* group from GenBank and Barcode of Life Data Systems website. We speculate that it was related to a mass stocking of introduced species of fishes from Europe. To confirm whether *C. sphaericus* is an invasive species due to human-mediated introductions, or whether it is a native Australian taxon, further extensive molecular studies (involving nuclear genes) and detailed morphological comparisons are needed. This is a first report on the invasion of a non-daphnid species of Cladocera to Australia. Its significance for Australian ecosystems needs special future studies.

Key words: *C. sphaericus*, Australia, Northern Europe, South Australia.

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INTRODUCTION

One of the main goals of modern biology is to identify and differentiate species in different groups of organisms. *Cladocera* (Crustacea: Branchiopoda) is a well-known superorder of freshwater animals present in all continents, including Antarctica, with well-known differences in identification and discrimination of species (Forró et al., 2008). *Chydoridae* Dybowski & Grochowski, 1894 emend. Frey, 1967, is the largest family of order *Anomopoda* Sars, 1865 and *Cladocera in toto*. The former contains more than half of all described species of *Cladocera*. *Chydoridae* have attracted the attention of hydrobiologists since the pioneering publications on *Cladocera* (Müller, 1776, 1785).

*Chydoridae* were the primary organisms of Frey’s (1985, 1987) studies which led to the well-known and widely accepted conclusion that cladoceran species display a non-cosmopolitism in their distribution. Subsequently, continental endemism and regionalism were found to be common patterns for other *Cladocera* families (Smirnov, 1976; Haney and Taylor, 2003; Adamowicz et al., 2004; Korovechny, 2004; Bekker et al., 2012) and orders (Korovechny, 2004; Xu et al., 2009; Millette et al., 2011).

In the past two decades, hydrobiologists have examined *Chydoridae* more critically, taking into consideration morphological characters such as head pores, mandibular articulation, trunk limb setation and by comparing populations from different continents with those from type localities (Frey, 1993; Kotov, 2009; Van Damme et al., 2011; Sinev and Kotov, 2012; Van Damme and Sinev, 2013). These observations have resulted in a significant increase in the number of known species from different continents. The first publications on phylogenetics (Sacherová and Hebert, 2003) and barcoding (Elias-Gutierrez et al., 2008; Jeffery et al., 2011) of *Chydoridae* have appeared recently, although most recent geneticists work with a single genus *Daphnia* Müller, 1785.

Among *Chydoridae*, there are many well-recognised, small and relatively well-studied genera, and several large genera with a confused taxonomy, such as *Alona* Baird, 1843 (subfamily *Aloninae*), *Pleuroxus* Baird, 1843 or *Alonella* Sars, 1862 (subfamily *Chydorinae*) (Smirnov 1971, 1996). Probably the most common *Chydoridae* genus, which is present in a majority of tropical, temperate and even polar water bodies, is *Chydorus* Leach, 1816 (subfamily *Chydorinae*). Species of this genus are more frequently recorded in hydrobiological publications as compared with other *Chydoridae*, because the former usually occur in plankton – the primary object of hydrobiological studies. Smirnov (1996) listed 30 valid species in the world fauna, but this number was significantly underestimated, and some new species were subsequently added, i.e., by Smirnov and Sheveleva (2010). The genus contains several taxa now regarded as cosmopolitan or tropicalopolitan, which seem to be complexes of congener species with a relatively wide or very narrow distribution.
Four species of *Chydorus* have been recorded from Australia by Smirnov and Timms (1983). Two other taxa, *C. barroisi* (Richard, 1894) and *C. hybridus* Daday (1905), were transferred to *Ephemeropterus* Frey (1982). When Smirnov & Timms submitted their publication, they did not know about Frey’s paper (1982). Most recently, Shiel and Dickson (1995) and Smirnov (1995) list 11 valid Australian species: *C. eurynotus* Sars, 1901; *C. clelandi* Henry, 1919; *C. herrmanni* Brehm, 1933; *C. kalliptigos* Brehm, 1933; *C. obscurirostris* Frey, 1987; *C. opacus* Frey, 1987; *C. ovalis* Kurz, 1874; *C. parvus* Daday, 1898; *C. pubescens* Sars, 1901; *C. reticulatus* Daday, 1898; *C. sphaericus* (Müller, 1785). Some of these taxa definitively form groups of congeners and need to be revised worldwide (Smirnov, 1996).

*Chydorus sphaericus*-like populations are common in Australia (Smirnov and Timms, 1983). Smirnov (1971) and Frey (1980, 1985, 1987) studied *Chydorus sphaericus* s.lat. in detail. This taxon was regarded as cosmopolitan for a long time, although similar forms were described from different continents, sometimes based on very dubious diagnostic characters. For example, a special name has been suggested for Australian *sphaericus*-like populations, *C. leonardi* King, 1853, but any differences between this and *C. sphaericus* s.str. are unknown. Frey (1980), Shiel and Dickson (1995) and Smirnov (1995) concluded that *C. sphaericus* in Australia is a species complex, none of which is actually *C. sphaericus* s.str. - the latter is present in Eurasia only. Smirnov (1996) found some populations in Australia that were undistinguishable from European populations and concluded that the distribution of this taxon is Holarctic and probably worldwide. Belyaeva and Taylor (2009) genetically analysed the Holarctic populations and recognised at least six well-differentiated clades in the *C. sphaericus* group, some of them locally distributed. Later Jeffery et al. (2011) found even more taxa from this complex in Arctic Canada.

The objective of the present study was to consider all available data, morphological and molecular (ribosomal and nuclear genes), of the *C. sphaericus* to clarify the taxonomical position of Australian *C. sphaericus*.

**METHODS**

**Studied water bodies**

South Para reservoir (completed in 1958) is situated approximately 60 km north of Adelaide (Fig. 1). It has a surface area of 4 km², and a storage capacity of approximately 45,330 ML with a maximum depth of 42 m at full supply level. South Para reservoir receives water from its catchment, which covers an area of 326 km² and occasionally supplemented from River Murray through the Mannum pipeline.

Myponga reservoir (completed in 1962) is situated approximately 70 km south of Adelaide. It has a surface area of 2.8 km², and a storage capacity of approximately 26,800 ML with a maximum depth of 42 m at full supply level (Brookes et al., 2005). It receives water solely from its catchment, which covers an area of 124 km². It is intensively managed using artificial aeration and destratification from two surface mechanical mixers and multi-diffuser bubble-plume aerators to control cyanobacterial growth (Lewis et al., 2001).

**Zooplankton sampling**

In February 2010, abundant ovigerous females of *C. sphaericus* were collected in two reservoirs, using a conical plankton net with a mesh size of 35 µm. The plankton net was towed behind a boat for 5-7 min at a speed of about 2 m s⁻¹ in mid open water (Suthers and Rissik, 2009). Specimens were fixed and preserved in the field with denatured ethanol to obtain a final concentration of alcohol of 70%, and then stored in 200 mL Cospak PET bottles.

**Morphological identification**

Specimens were selected from preserved samples under a binocular stereoscopic microscope, and studied under an optical microscope in a drop of glycerol. Ten parthenogenetic females from each locality were dissected under a stereoscopic microscope for the study of appendages and postabdomen, under a high-power Olympus BX51 microscope, to check the identity of Australian populations of *C. sphaericus*, with those from Europe (Frey, 1980, 1985; Alonso, 1996; Belyaeva and Taylor, 2009). Digital photographs were taken using Olympus BX51 microscope under high resolution using the polarizing photography, and composite line drawings were made from these photographs for different parts of the specimens. The inbuilt imaging software Image J® was used for measurements.

**Molecular methods**

Ten specimens of ovigerous females of *C. sphaericus* were selected from material collected from each reservoir, to examine their genetic variability. Total genomic DNA was extracted for each specimen using QIAMP DNA extraction kits as described in the manufacturer’s protocol. Polymerase chain reaction (PCR) was used to amplify an approximately 680 bp fragment of the COI gene with Folmer primer pair (LCO 1490 and HCO 2198) (Folmer et al., 1994). Each 50 µL PCR reaction consisted of 5 µL of genomic DNA template, 3 µL of 50 mM MgCl₂, 5 µL of 10X Buffer, 1.5 µL of each primer, 1 µL of 10 µM dNTP’s, 0.24 µL of Taq platinum polymerase and 32.76 µL of DNA free Milli-Q water. PCR
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profile consisted of an initial hot start (2 min 30 sec at 94°C) for 1 min followed by: 5 cycles each of 94°C for 35 s, 48°C for 40 s and 72°C for 1 min; followed by 35 cycles each of 94°C for 30 s, 56°C for 40 s and 72°C for 1 min, finishing with a step of 72°C for 10 min. PCR products were run in 2% agarose gels containing 10 µL of SYBR® Safe DNA gel stain (Invitrogen Inc., Carlsbad, CA, USA) for 2 to 4 h at 80 to 100 V and visualized using UV-transillumination. The amplified bands were sharp and clean, which were cut and purified from the agarose gel using QIAquick® Gel Extraction Kit.

The sequencing reaction for both forward and reverse directions involved: 0.5 µL of primer, 1 µL of Big Dye Terminator v 3.1, 3.5 µL of sequencing buffer and 1 to 3 µL of purified PCR product. Concentration of PCR product was as follows: 1 µL of the purified PCR product for strong band with DNA concentration >8 ng µL⁻¹, 2 µL for moderate band with DNA concentration between 3 to 5 ng µL⁻¹ and 3 µL for weak band with DNA concentration <3 ng µL⁻¹, with the total volume made up to 25 µL using DNA free Milli-Q water. Sequencing thermal cycle consisted of 1 cycle of 1 min at 95°C, followed by 25 cycles of 95°C for 15 s, 50°C for 10 s and 60°C for 4 min with final overnight incubation at 25°C. Sequencing product was then purified using MilliporeTM384 - SEQ Filter plates using 1X Tris Buffer. The purified PCR products along with primers (LCO1490 and HCO2198) were sent to the Australian Genome Research Facility Ltd., Australia and Macrogen Inc., South Korea, for sequencing on the AB 3730xl Platform sequencer. Both forward and reverse genomic strands were sequenced to confirm the accuracy of each haplotype sequence. DNA sequences were edited using Bioedit (ver. 7.0.0) and aligned using Clustal W (Thompson et al., 1994), using Gap open penalty set to 100, so that gaps became less frequent. Estimates of se-

Fig. 1. South Para and Myponga reservoirs with sampling sites. Legends: filled circles and stars represents permanent pelagic sampling sites. Image taken from Google maps on the 4th April 2011.
sequence divergence were calculated for COI gene using the Kimura 2-parameter (K2P) distance model (Kimura, 1980) and a simplified tree was constructed using the Mega 5 software (Tamura et al., 2011). Branch support values were estimated using 1000 bootstrap replicates. All other parameters were set to their default values.

**GenBank dataset**

To provide a comprehensive sister-taxon coverage and survey of intraspecific variation, our data were complemented by sequences on *Chydorus sphaericus*-group from GenBank and Barcode of Life Data Systems websites, with the total number of sequences=139, number of haplotypes=79, as available on 20 October 2012.

GenBank sequence ID names were formatted with accession numbers provided from GenBank for downloaded sequences. Haplotypes were identified using Collapse 1.2 (Posada and Buckley, 2004). Barcode sequences generated from this study and those extracted from GenBank were aligned using Clustal W application implemented in software Bioedit Ver.7.0.0. (Hall, 1999) using Gap open penalty set to 100, so that gaps become less frequent.

**Phylogenetic analysis**

The best fit models of nucleotide substitution were selected using the Model Generator (Keane et al., 2006) and the Maximum Likelihood (ML) tree was constructed using PhyML ver. 3.0 (Guindon and Gascuel, 2003). Following parameter settings were used for phylogenetic analysis: input sequences in interleaved format; best model of the nucleotide substitution was selected from Model Generator; transition/transversion ratio and proportion of invariant nucleotide sites estimated by PhyML; a BIONJ tree was initially used; tree topology search option was set to Best of Nearest Neighbour Interchange (NNI) and Subtree Pruning and Regrafting (SPR) with random starting tree set at 5; non-parametric bootstrap analysis was set to YES with 1000 replicates. Within and among clade, distances were calculated and NJ phylogenetic analyses were carried out in MEGA 5.0 using model and gamma rates distribution with the shape parameter estimated by JModelTest and with pairwise deletion of gaps.

Bayesian analyses were performed by using Mr. Bayes v. 3.0 (Huelsenbeck et al., 2001). Number of substitution types was set to 6 and the rates were set to gamma with a proportion of invariant sites. All priors were left to default to allow estimation of parameters from data. Four independent Markov Chain Monte Carlo analyses were run simultaneously for 2 million generations and sampled every 500 generations. The first 25% generations were discarded as the burn-in and a 50% majority rule consensus tree was calculated from remaining trees.

**RESULTS**

**Morphological diagnosis of the *C. sphaericus* species complex**

*Chydorus* specimens from two South Australian reservoirs (Fig. 2) clearly belongs to *C. sphaericus* group, which is characterized by the following parthenogenetic female characters: i) sub-globular body; ii) pointed rostrum; iii) tri- angular labral keel with a sharply pointed tip; iv) a small flap on inner face of valve near its anterior margin; v) no denticles in postero-ventral portion of valve; vi) postab- domen relatively narrow, with well-expressed preanal angle; vii) teeth on postanal margin of postabdomen small; viii) antenna 1 with aesthetascs only in terminal position, and with a sensory seta in its middle; ix) antenna II with setae: 0-0-3/0-1-3; x) inner distal lobe of limb 1 with a small seta and two large setae of which one is additionally chitinised. Unfortunately, morphological characters of parthenogenetic females alone are insufficient to determine exact species within the *sphaericus*-group (Belyaeva and Taylor, 2009).

**Mitochondrial gene tree**

A 510 bp fragment of the COI gene was successfully amplified for *Chydorus* sp. (number of haplotypes=2). The intraspecific divergence was 0% (raw and ML-corrected divergences), whereas interspecific variation was 3% (raw and ML-corrected divergences) when compared with *C. sphaericus* from Germany (EU719127.1 and EU719129.1) (Fig. 3). The Australian haplotypes belong to *Chydorus sphaericus* s.str. and are definitively grouped with a small sub-clade Clade A of Belyaeva and Taylor (2009), containing haplotypes from Iceland and Greenland.

**DISCUSSION AND CONCLUSIONS**

*Chydorus sphaericus* from South Australia shows a close genetic similarity with *C. sphaericus* from Europe, and the former is especially close to populations from Iceland and Greenland. Genetic similarity between South Australian and Holarctic haplotypes indicates the former are not continental endemics and their transfer from the original distributional range to Australia was very recent. Such cases are mainly explained by human activity.

The expansion of geographical distribution of species via anthropogenic factors is common and has been reported previously for many planktonic crustaceans such as copepods (Gutierrez-Aguirre and Suárez-Morales, 2000; Suárez-Morales et al., 2005; Duggan et al., 2006; Briski et al., 2011b; Sukhikh et al., 2013). Cladocerans are among the most famous invaders of water bodies due to their destructive effect on native ecosystems. For example, invasive predatory onychopods have significantly reduced zooplankton species richness in the Great Lakes of North America (Yan et al., 2002), Baltic Sea
Fig. 2. Parthenogenetic female of Chydorus sphaericus from Myponga reservoir: A) General view; B) Head; C) Anterior portion of valve, inner view; D) Postero-ventral portion of valve, inner view; E-F) Postabdomen, lateral view; G) Antenna II; H) Limb I. Scale bars: 0.1 mm. Numbers correspond to diagnostic characters of C. sphaericus group listed in the text.
The most famous cladoceran invaders are species of *Daphnia*. Different directions of invasions are revealed. European *Daphnia pulex* has been transferred in ballast tanks of transoceanic ships to North American Great Lakes (Briski et al., 2011). In contrast, a massive stock of *Micropterus salmoides* (largemouth bass) from USA to East Africa was accompanied by the appearance of an American asexual *pulex*-like lineage, which now has successively replaced native species of *Daphnia* in many African water bodies (Mergen et al., 2005), except for on high mountains, where some native endemic species survived (Kotov and Taylor, 2010).

Perhaps the most successive invader is *D. lumholtzi* Sars, 1885, which was introduced together with African blue tilapia or/and Nile perch to southern USA (Sorensen and Sterner, 1992), and which has now colonized all of the USA (Benson et al., 2013), Mexico (Elías-Gutiérrez et al., 2008), Brazil (Zanata et al., 2003) and Argentina (Kotov and Taylor, 2014).

Several exotic species of *Daphnia* have already appeared in Australia: the Palearctic *D. obtusa* Kurz, 1874 (Benzie and Hodges, 1996), *D. dentifera* Forbes, 1893 (Duggan et al., 2006) from North America and East Asia and aforementioned North American asexual *D. pulex* lineage (Duggan et al., 2012). The latter authors speculated that the appearance of *D. pulex* in New Zealand was related to mass stocking to this country of several species of fishes (brown trout, rainbow trout and land-locked salmon) from North America.

In the 19th-20th century Australia was a target for mass stocking of several introduced species of fishes from Europe, mainly brown trout, Atlantic salmon, European carp, European perch from the United Kingdom (Fletcher, 1986; Morgan et al., 2004). Each of these fish species has been introduced several times to different parts of the continent, and then Australian populations have often been used for artificial dispersion across Australia. Among non-native fishes, European carp (*Cyprinus carpio*) has the strongest impact on native ecosystems (Koehn et al., 2000). Several genetic strains of European carp from different regions of Europe (England and Prussia) and later European carp from tropical Asia were introduced (Koehn et al., 2000). We do not have enough data to associate the appearance of *Chydorus sphaericus* in Australia with a certain campaign to stock European fish, but it is highly likely that in the course of such introductions, the ephippia from northern Europe were occasionally brought to Australia.

**CONCLUSIONS**

At present, we cannot say that our assumption for the two populations from South Australia of European *C. sphaericus* s.str. can be applied to the whole of Australia. It is possible that other populations of *Chydorus* in Australian water bodies might belong to other taxa, or could even be endemic to Australia. But there is also a chance that in the course of numerous transportations and simultaneous introduction of fishes within Australia, this continent was widely infected by *C. sphaericus*. To confirm whether *C. sphaericus* is an invasive species due to human-mediated introductions, or whether it is a native Australian taxon, further extensive molecular studies (involving nuclear genes) and detailed morphological comparisons are needed. This is a first report on the invasion of a non-daphniid species of Cladocera in Australia.

The use of molecular methods in studies of invasive species has added a new dimension to the traditional morphological approach. The former allows us to understand some valuable traits of the invasive process, such as an exact geographic region of origin of an invasive species. Use of genetic tools, such as DNA barcoding, could be important for the identification of organisms, in addition to traditional morphological descriptors (Costa et al., 2007; Ferri et al., 2009). The success in using sequences from COI gene region to distinguish phylogroups (species, intra-specific groups and haplotypes) and to reveal hidden species diversity in the Cladocera (Elías-Gutiérrez et al., 2008; Belyaeva and Taylor, 2009) has been remarkable. But this method, however, is only effective for those species which have been studied extensively in the past, with well-documented morphology, reproduction, ecology and geographical distribution (Sites and Marshall, 2004). Nevertheless, combining morphology and molecular data has been found to be more successful for species recognition, identification and refinement (Ferri et al., 2009).

Some invaders are very destructive for ecosystems like *D. lumholtzi* in the Americas (Sorensen and Sterner, 1992) or carp in Australia (Koehn et al., 2000). At this moment we do not have any ideas on significance of the introduction of *Chydorus sphaericus* for Australian ecosystems, this question requires special studies.

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Fig. 3. Maximum likelihood analysis of COI gene for the *Chydorus sphaericus* complex. Numbers above branches are Maximum likelihood (100 replicates) and underlined numbers are from Bayesian analysis. Asterisk represents sequences from the current study.
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