

Short Communication

Identifying invertebrate invasions using morphological and molecular analyses: North American *Daphnia 'pulex'* in New Zealand fresh waters

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Abstract

We used a DNA barcoding approach to identify specimens of the *Daphnia pulex* complex occurring in New Zealand lakes, documenting the establishment of non-indigenous North American *Daphnia 'pulex'*. Morphological delineation of species in this complex is problematic due to a lack of good morphological traits to distinguish the species, as there is a relatively high degree of morphological stasis within the group through evolutionary time. Accordingly, genetic analyses were used to determine the specific identity and likely geographic origin of this species. Morphologically, individuals most closely resembled *Daphnia pulicaria* or *Daphnia pulex* sensu lato, which cannot be separated morphologically. Furthermore, each of these taxa comprises separate species in North America and Europe, despite carrying the same names. We identified individuals using a 658 bp nucleotide portion of the mitochondrial cytochrome c oxidase subunit I gene (COI) as North American *Daphnia 'pulex'*, being distinct from European *Daphnia pulex* sensu stricto and *D. pulicaria* from Europe or North America. Cellulose allozyme electrophoresis was used to confirm that individuals were not hybrids with *D. pulicaria*. North American *Daphnia 'pulex'* in New Zealand were first recorded in New Zealand from South Island lakes that are popular for overseas recreational fishers, indicating a possible source of introduction for this species (e.g. on/in fishing gear). Our study provides an additional example of how genetic techniques can be used for the accurate identification of non-indigenous taxa, particularly when morphological species determination is not possible. The growth of global databases such as GenBank and Barcode of Life Datasystems (BOLD) will further enhance this identification capacity.

Key words: biological invasions; biosecurity; cladocera, DNA barcoding

Introduction

The widespread establishment of non-indigenous species is progressively homogenising the world's floras and faunas. For example, the number of invertebrate invaders recognised in fresh waters in New Zealand has increased markedly in recent years, comprising species from diverse biogeographical realms (Duggan 2002; Duggan et al. 2006; Collier et al. 2011). Whereas some of these newly recognised invaders, such as the snail *Melanoides tuberculata*, are likely to have been established for some time (Duggan 2002), others such as the cladoceran *Daphnia galeata* and the calanoid copepod *Skistodiaptomus pallidus* represent recent invasions (Duggan et al. 2006), indicating that both the detection and invasion rates are increasing.

Here, we report on a new record of a *Daphnia* species belonging to the *D. pulex* species complex in New Zealand. The identification of species in this complex is difficult. Determination of species based on morphological methods is problematic due to a lack of reliable morphological traits as a result of morphological stasis combined with a poor taxonomy (Colbourne and Hebert 1996; Mergeay et al. 2008). Whereas the *D. pulex* complex was considered to comprise a small number of species that could be differentiated morphologically, a number of new species have been identified with the aid of genetic techniques (e.g., Hebert and Finston 1996; Kofínek and Hebert 1996). Although a number of the species within the *pulex* complex can be separated by morphological means, based on distinctive features that were identified following genetic

analyses, definitive identification of some members still requires genetic analysis (Benzie 2005). For example, the species *Daphnia pulex* and *D. pulicaria*, within the *D. pulex* complex, are currently impossible to distinguish confidently using morphological characters, but are genetically distinct (although see Mergeay 2008 and Cristescu 2012). Both Hebert (1995) and Benzie (2005) note that whereas there are some morphological features that are indicative of identity, definitive taxonomic assignment of these species depends on genetic determination. Furthermore, both *Daphnia pulex* and *D. pulicaria* sensu lato were thought to be widely distributed in the Northern Hemisphere, but have now been found to be genetically distinct (yet morphologically indistinguishable) between Europe and North America. Accordingly, the *D. pulex* complex is comprised of different species on each continent, although the same names persist in each respective location (Colbourne et al. 1998; Mergeay et al. 2008). An additional complicating factor is that, similar to several other *Daphnia* species, North American *Daphnia 'pulex'* and *D. pulicaria* are able to form viable hybrid populations, requiring allozyme analysis to confidently determine their status (e.g., Hebert et al. 1989). For example, based on DNA sequencing a North American *Daphnia 'pulex'* invasion was recognised in African lakes (Mergeay et al. 2005), although this species was later found by allozyme analysis to be a hybrid North American *Daphnia 'pulex'* × *D. pulicaria* (Mergeay et al. 2006).

DNA barcoding and other genetic techniques are now commonly used to confirm the morphological identifications of aquatic invaders, including in New Zealand (e.g., Stevens et al. 2002; Makino et al. 2010; Collier et al. 2011). Here we provide an example that required genetic analyses to identify a non-indigenous species that is a member of a species complex, and to determine its geographic origin.

Methods

To date we have observed the non-indigenous *Daphnia* species from a number of South Island lakes, where it is widespread (Table 1). The earliest samples we have of this species were collected from the Central Otago lakes, in March 2005. Morphologically, the *Daphnia* species conforms to *D. pulex* or *D. pulicaria* sensu lato, within the *D. pulex* complex, based on the keys of Hebert (1995) and Benzie (2005), and thus

requires genetic analyses for species level determination. The identity of the *Daphnia* species was resolved by direct sequencing of the polymerase chain reaction product from a 658 bp nucleotide portion of the mitochondrial cytochrome *c* oxidase subunit 1 gene (COI) from seven individuals from four lakes (three specimens from Lake Benmore, two from Lake Wanaka, and one each from Lake Hayes and Lake Heron) using the methods of Penton et al. (2004). The COI sequences we obtained were aligned with *Daphnia pulicaria* and *D. pulex* COI sequences downloaded from Genbank using the default values in Clustal X (Thompson et al. 1997). Alignment was straightforward as there were no insertions or deletions. *Daphnia neoobtusata* was used as an outgroup to root the phylogeny. Phylogenetic trees were estimated using the maximum likelihood GTR + G model as recommended by Jmodeltest (Posada 2008) and 1000 bootstrap replicates (Felsenstein 1985) were generated to assess support for the nodes in Mega (Tamura et al. 2011). All sequences were deposited in the project "Daphnia pulex in New Zealand" (NZPLB) in the Barcode of Life Datasystem (BOLD) database (<http://www.barcodeoflife.org>) and cross referenced to GenBank (accession numbers HM622590 – HM622593, and JX150976 – JX150976). However, as mitochondrial genes are typically passed through the female lineage, and in North America the two species in question may hybridise as *D. 'pulex'* × *D. pulicaria* (i.e., with maternal *D. 'pulex'*), we tested for the presence of hybrids using an allozyme analysis. Specifically, we assessed using cellulose allozyme electrophoresis (Hebert and Beaton 1989), whether individuals were homozygous (non-hybrid) or heterozygous (hybrid) at the lactate dehydrogenase locus (LDH: EC 1.1.1.27), an established method for determining hybridisation in these species, as per Hebert et al. (1989). *Daphnia pulicaria* is the only species that North American maternal *D. 'pulex'* is known to form hybrids with under natural conditions, and thus only this one allozyme locus was required for analysis (e.g., Hebert et al. 1989; Taylor and Hebert 1993). Identification of these species using LDH is commonly undertaken in North America; however, this is only possible if known *D. 'pulex'* and *D. pulicaria* are analysed with the unknown population, where relative mobilities can be assessed (slow = *D. 'pulex'* and fast = *D. pulicaria*). Unfortunately, this is not possible for situations where both species are not present

Table 1. South Island water bodies where *Daphnia 'pulex'* has been sampled to date.

Water Body	Geo-reference	Date of first record and Collector
<i>Clutha River drainage system</i>		
Lake Hayes	-44° 58' 48.00", 168° 48' 36.00"	3 Mar 05 (Burns)
Lake Johnson	-45° 00' 08.34", 168° 43' 53.88"	4 Mar 05 (Burns)
Lake Moke	-45° 00' 10.52", 168° 33' 51.34"	4 Mar 05 (Burns)
Lake Wakatipu	-45° 03' 28.80", 168° 39' 08.99"	5 Mar 05 (Burns)
Lake Wanaka	-44° 31' 33.19", 169° 06' 55.06"	10 Nov 09 (Burns)
<i>Waitaki River drainage system</i>		
Lake Benmore	-44° 22' 33.22", 170° 12' 50.14"	8 Dec 08 (Robinson)
Lake Waitaki	-44° 40' 29.56", 170° 23' 59.41"	10 Dec 08 (Robinson)
Lake Ruataniwha	-44° 16' 46.86", 170° 04' 09.49"	3 Jan 08 (Robinson)
Tekapo Canal	-44° 04' 14.93", 170° 21' 34.19"	5 Nov 07 (Robinson)
Ohau Canal	-44° 15' 39.37", 169° 58' 44.74"	5 Nov 07 (Robinson)
<i>Canterbury rivers drainages</i>		
Lake Heron	-43° 28' 36.62", 171° 10' 30.84"	17 Mar 09 (Robinson)
Lake Pegasus	-43° 18' 32.19", 172° 41' 48.85"	25 Mar 11 (Robinson)
Lake Sarah	-43° 02' 50.56", 171° 46' 36.55"	17 Mar 09 (Robinson)
<i>Dunedin</i>		
Ross Creek Reservoir	-45° 50' 48.93", 170° 29' 55.38"	20 Nov 08 (Burns)
Rossville Reservoir	-45° 48' 17.37", 170° 36' 01.97"	20 Nov 08 (Burns)
Sullivan's Dam	-45° 48' 26.03", 170° 31' 22.69"	22 Dec 08 (Burns)
Tomahawk Lagoon #2	-45° 54' 05.44", 170° 33' 02.18"	24 Aug 10 (Burns)
<i>Southland</i>		
Munro's Dam	-46° 29' 28.00", 168° 34' 53.00"	1 Oct 11 (Burns)
Lake Manapouri	-45° 33' 33.32", 167° 36' 24.25"	12 Mar 10 (Robinson)

(i.e., New Zealand). At least five individuals for allozyme analysis were analysed on fresh individuals collected from each of Lake Heron and Lake Wanaka.

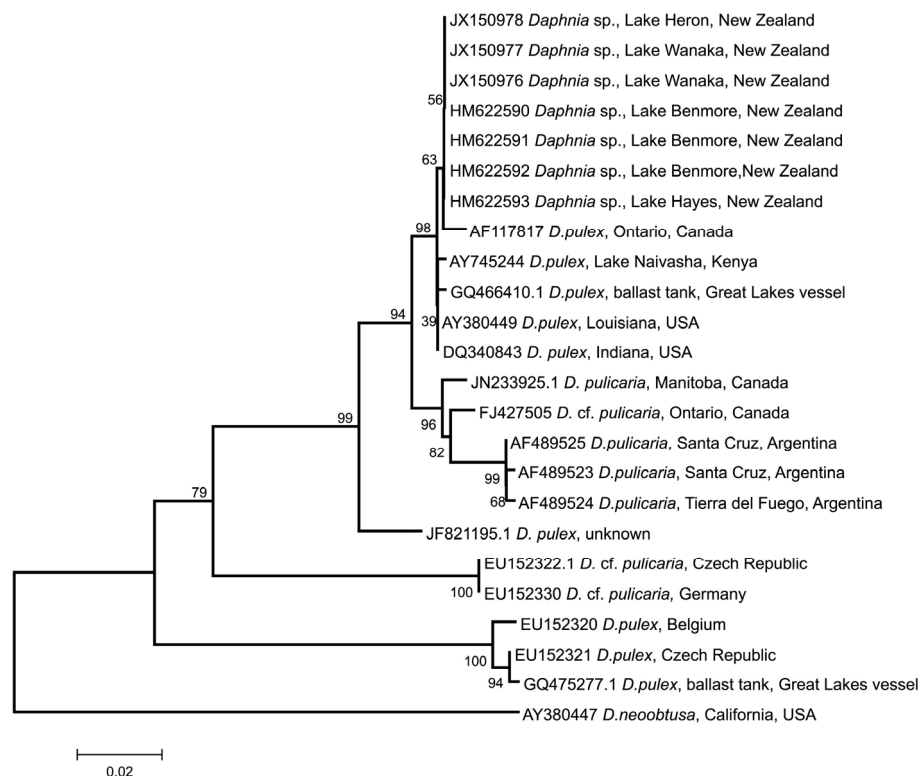
Results

Based on morphological features (shape of head and rostrum, position of eye, spinulation of carapace margins, shape of postabdomen, relative size and setation of postabdominal processes, number and shape of postabdominal spines, size and number of teeth on combs of the postabdominal claw), adult female *Daphnia* collected most closely resembled *Daphnia pulicaria* sensu lato and *Daphnia pulex* sensu lato, which cannot be separated morphologically (Hebert 1995; Benzie 2005). Morphologically, the specimens collected from the Central Otago and Waitaki lakes differ from the descriptions of *D. pulex* in Benzie (2005) in having a more elliptical than oval carapace in lateral view and a

tail spine longer than 0.3 carapace length, and a moderate-sized eye in a large optic vesicle that is set back from the frontal margin of the head. Specimens from Lake Wanaka kept in culture produced males and ephippia.

The phylogeny for our *Daphnia* specimens, estimated by maximum likelihood, indicate the New Zealand populations formed a monophyletic group with North American and African populations of *Daphnia 'pulex'* (Figure 1). Our sequences differed by 0.2 - 1.6% (1 to 10 nucleotides) from North American *D. 'pulex'* sequences in GenBank (for example, accession number DQ340843). Our sequences differed from GenBank sequences for *Daphnia pulicaria* by between 3% and 11% (between 16 to 62 nucleotides; GenBank accession numbers FJ427505 and EU152322). Our sequences differed from *D. pulex* sequences EU152320 and EU152321 in GenBank, described as *D. pulex* sensu stricto from Europe (Mergeay et al. 2008), by 11%, indicating that the South Island *Daphnia* originated from North America and not

Figure 1. Phylogeny hypothesis for *Daphnia pulex* and *pulicaria* sensu lato estimated using the maximum likelihood GTR + G model using COI sequences. Numbers to the left of nodes designate percentage bootstrap support for that node. The sequences from New Zealand *Daphnia pulex* specimens are at the top of the figure.



Europe. Allozyme analyses showed that individuals were homozygous for the LDH locus, confirming the designation of North American *D. pulex* and not the hybrid *D. pulex* × *D. pulicaria*.

Discussion

The phylogeny estimated for the *Daphnia* COI sequences placed our *Daphnia* with North American *D. pulex*, distinct from European *Daphnia pulex* sensu stricto and *D. pulicaria* from Europe or North America, with strong bootstrap support. Globally, non-indigenous populations of *Daphnia* species have been recorded from a number of locations outside their native ranges. Most notable is an African strain of *Daphnia lumholtzi* first recorded from a reservoir in Texas in 1991, which has subsequently spread widely in North America (Havel et al. 1995); this species was unmistakable, morphologically, from North American congeners due to the invaders' possession of a large distinct head spine. At the other extreme, North American *Daphnia pulex* × *D. pulicaria* have also invaded several African lakes, displacing

native strains of the morphologically indistinguishable *Daphnia pulex* (Mergeay et al. 2005; 2006). Our sequences closely grouped with this African invader (Figure 1), although allozyme analysis indicates the New Zealand invader is not a hybrid with *D. pulicaria*. Cultures maintained in the laboratory in our study produced both males and ephippia, suggesting the populations may reproduce by sexual reproduction (although see Hebert et al. 1989; Innes et al. 2000). To date, all nonindigenous populations of North American *Daphnia pulex* (including their *D. pulicaria* hybrids) recorded from Europe and Africa have been found to be obligate parthenogens (e.g., Mergeay et al. 2006; Mergeay pers. comm).

The African *D. pulex* × *D. pulicaria* invasion is likely to have occurred through the stocking of largemouth bass (*Micropterus salmoides*) from the USA (Mergeay et al. 2006), while the *Daphnia lumholtzi* invasion into North America was also likely to have occurred via fish stocking of Nile Tilapia from Africa (Havel et al. 2000). The transportation vector for North American *D. pulex* into New Zealand is not obvious, although the distribution of the first recorded

populations may provide some indication. Our earliest samples were collected from lakes in the Clutha River and Waitaki River drainages, including Lakes Benmore, Waitaki and Wakatipu, which contain well established populations of non-indigenous brown trout (*Salmo trutta*), rainbow trout (*Oncorhynchus mykiss*) and some contain land-locked salmon (*Oncorhynchus nerka*, *O. tshawytscha*). However, despite introductions of the latter three species being from North America, releases of these to New Zealand are historic (late 19th and 20th centuries), and any fish used in recent stockings of these lakes will have originated from hatcheries within New Zealand (McDowall 1990). Nevertheless, the presence of these salmonids makes these lakes extremely popular for recreational fishing, including for many international tourists, indicating a possible means of introduction in association with fishing and boating equipment. Fishing equipment, for example felt-soled waders, is a likely vector for another recent invader to New Zealand, a diatom, *Didymosphenia geminata*, which is currently restricted to South Island rivers where fishing is popular (Kilroy et al. 2008). Fouling of fishing lines by the small crustaceans *Bythotrephes longimanus* and *Cercopagis pengoi*, or their diapausing stages, is thought to have aided their spread through North American lakes (e.g., Jacobs and MacIsaac 2007). North American and European *Daphnia pulex* have been recorded from ballast tanks of transoceanic ships in the North American Great Lakes (Briski et al. 2010; Figure 1). However, such vessels do not enter freshwater ports in New Zealand, making this transportation vector unlikely in this instance. New Zealand has only one recognised native *Daphnia* species, the relatively uncommon *Daphnia carinata*, while cladoceran diversity overall is also low (Chapman and Green 1987). As such, a lack of strong niche overlap with existing species may have facilitated the establishment of North American *D. 'pulex'* in New Zealand (e.g., Dzialowski 2010).

The establishment of non-indigenous *Daphnia*, such as the North American *D. 'pulex'*, might have large effects on lake and pond biota. For example, Vanni (1986) found that *Daphnia 'pulex'* greatly reduced the abundances of both phytoplankton and other zooplankton (particularly rotifers) when it was introduced to enclosures in a North American pond. Similarly, a recent invasion of *Daphnia galeata* to New Zealand coincided with reductions in chlorophyll *a*

concentration, increased Secchi transparency and a reduction in rotifer abundances in Lake Puketerini (Weavers Lake) following its establishment (Balvert et al. 2009). Such changes are likely to influence higher trophic levels also.

The rate of invasion of freshwater invertebrates appears to be increasing, and the recent establishment of North American *Daphnia 'pulex'* in New Zealand is yet another example of this trend. Overall, our results provide further evidence of how genetic techniques can play a major role in the identification of new non-indigenous species, particularly if these new taxa belong to species complexes. In addition, we show that genetic techniques can also provide valuable information on donor (i.e., source) regions of new invaders, and may thus provide clues on invasion pathways, which may be used to reduce invasion rates. Such methods will become more applicable as global databases, such as GenBank and BOLD (Ratnasingham and Hebert 2007), grow in size. However, care must be taken for a lack of distinction between sibling species, or erroneous naming of taxa, in these databases. For example, in GenBank three distinct species are called simply "*Daphnia pulex*" (Figure 1). As eradication of aquatic invertebrate species is impossible once they are established, assessments of transportation vectors from significant donor regions identified by such means can be investigated to determine management strategies for reducing further introductions.

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