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Research Article

Mitochondrial DNA indicates different North American east coast origins for New Zealand and German invasions of *Skistodiaptomus pallidus* (Copepoda: Calanoida)

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Abstract

The freshwater calanoid copepod *Skistodiaptomus pallidus* (Herrick, 1879), native to the Mississippi basin of North America, has recently established non-indigenous populations in New Zealand, Germany and Mexico. We used the mitochondrial cytochrome *c* oxidase subunit I (COI) gene to more precisely identify the origins of *S. pallidus* populations within New Zealand and Germany. The *S. pallidus* COI sequences suggested that both New Zealand and German populations were most similar to those from the most easterly regions of the USA (e.g., New York, Virginia and Georgia). However, several haplotypes were found to be divergent between the New Zealand and German populations, indicating the exact sources of the introductions were likely different for each country. German sequences possessed two of the major haplotypes known from the east coast of USA, while New Zealand had one, indicating a shipping related vector of introduction with greater propagule supplies to Germany is plausible. Although both German and New Zealand populations contained haplotypes identical to common east coast North American sequence records, both non-indigenous populations had haplotypes not yet recorded in the USA. Further sampling of the native range will be required to determine the exact origin of the non-indigenous *S. pallidus* populations and may also help to identify more precisely the vectors and pathways of the translocations.

Key words: aquaculture, *Ctenopharyngodon idella*, exotic species, calanoid copepods, invasion vectors

Introduction

Long-distance translocations of aquatic invertebrate taxa, as a result of human-related activities, are well documented globally (Ricciardi and MacIsaac 2000; Havel and Hebert 1993). Freshwater invertebrates can be transported as active and dormant life-stages by a variety of vectors, including in association with species utilised in aquaculture or the aquarium trade and in the ballast of shipping vessels (Duggan et al. 2005; Ferrari and Rossetti 2006; Briski et al. 2012). The consequent transportation of non-native freshwater taxa across biogeographical barriers is resulting in the global homogenisation of species with potential consequences to diversity and ecosystem function (Olden et al. 2004; Briski et al. 2012). Accurate and early identification of non-native taxa, their vector for movement and invasion history, are key steps in developing management practises to reduce both invasion rates and potential impacts on recipient aquatic systems (Holeck et al. 2004; Comtet et al. 2015).

DNA sequences are increasingly co-utilised with morphological features as an accurate means of identifying taxa and are applicable to all life-cycle stages. Further, slight variations in DNA sequences can aid in the discrimination of the biogeographical origins of invasive species (Havel et al. 2000; Hebert et al. 2003; Briski et al. 2011). For example, Makino et al. (2010) used mitochondrial cytochrome *c* oxidase subunit I (COI) gene sequences to identify regional

haplotypes of the Japanese calanoid copepod *Sinodiaptomus valkanovi* Kiefer, 1938. This allowed an assessment of the invasion corridor through which the species made a "jump" from its native range surrounding the Seto Inland Sea to sites in north-east Japan, and a subsequent and more recent long-distance invasion into New Zealand (Duggan et al. 2006; Makino et al. 2010).

The calanoid copepod *Skistodiaptomus pallidus* (Herrick, 1879), native to temperate North America, is emerging as an intercontinental invader, having recently been found to have established populations in the geographically disjunct locations of New Zealand, Germany and Mexico (Duggan et al. 2006; Brandorff 2011; Suárez-Morales and Arroyo-Bustos 2012). First recorded outside of its North American range in New Zealand in 2000, *S. pallidus* was initially found in constructed ponds at the Auckland Regional Botanic Gardens, although how long ago the species had established there is not known (Duggan et al. 2006). This was followed by its detection among live food for fish sold from an aquarium store in 2004, raising the possibility that *S. pallidus* was being dispersed via the aquarium trade (Duggan et al. 2006). Records were further expanded during a systematic survey by Banks and Duggan (2009), who showed *S. pallidus* had successfully colonised constructed ponds elsewhere in New Zealand. The first record of *S. pallidus* establishing in a natural lake was observed coincident with an intentional translocation of grass carp (*Ctenopharyngodon idella* Valenciennes, 1844) from aquaculture facilities into Lake Kereta, Auckland, for aquatic weed control (Duggan et al. 2014; Hofstra 2014). Further populations have subsequently been found in other lakes that have been subject to grass carp releases (Duggan et al. 2014; Hofstra 2014; ICD, SNB, unpubl. data). The link between aquaculture and the spread of *S. pallidus* in New Zealand has been further reinforced by the detection of *S. pallidus* and other non-native taxa within aquaculture facilities used in the cultivation of grass carp stocks; these ponds were also determined to be the source of the individuals sold as live food in aquarium stores (Duggan and Pullan 2017). While grass carp translocations have been identified as the major vector of spread within New Zealand, some sites of establishment have not been subject to grass carp introductions. As such, it is not clear whether all known populations are derived from a single importation event, or spread by a single vector.

The first discovery of *S. pallidus* in Europe was made in 2010, in samples collected from the moat of Bremen (Stadtgraben) adjoining the Weser River, northern Germany (Brandorff 2011). A second population was found a few months later, in a pond in the area of the Juliusplate on the floodplain of the Weser River, although it is unknown how long the populations had been established at either site (Brandorff 2011). Characteristics of the sites were similar in that they were both shallow eutrophic constructed water bodies, indirectly linked with the Weser River − a waterway that had been subject to considerable shipping activity from diverse international locations during the twentieth century (Brandorff 2011). The introduction by transportation in a shipping related vector such as ballast water or residual sediments was considered most likely (Brandorff 2011).

The distribution of populations of *S. pallidus* has expanded within North America, including into the Lake Tahoe Keys, California, well outside of the north-central and Mississippi basin where it is considered native (Byron and Saunders 1981; Torke 2001; Reid and Hudson 2008). Additionally, a more recent intracontinental range expansion has occurred with *S. pallidus* recorded in the Jose Lopez Portillo Reservoir in Sinaloa, Mexico, possibly in association with aquaculture within the reservoir or via bird migration from the north (Suárez-Morales and Arroyo-Bustos 2012). Overall, combining the new records in New Zealand and Germany, and its expanding distribution in North America, *S. pallidus* can be considered an emerging freshwater invader of international significance.

The genetic diversity of *S. pallidus* has received some attention, with mitochondrial genes utilised in the study of populations across the species' North American range (Thum and Harrison 2009). Thum and Derry (2008) studied 13 regional *S. pallidus* populations in order to determine whether geographically isolated copepod populations had colonised different regions of North America from glacial refugia following the end of the last glaciation (Stemberger 1995). Mitochondrial DNA data indicated that there were four highly divergent clades from different geographic regions and that dispersal from those regions had been limited by natural drainage boundaries for a considerable time (Thum and Derry 2008). Thum and Harrison (2009) found that ITS data supported three of the clades identified using COI and cytochrome *b* mtDNA data, which differentiated sites in Michigan (Clade C), Illinois (Clade B) and states on the east coast, including Connecticut, New York and Georgia (Clade A). Overall, the results of these studies indicate that *S. pallidus* represents a cryptic species complex across its native range, comprising distinct species in different geographic regions.

Our aim was to investigate the origins of the two intercontinental invasions by *S. pallidus*, into New Zealand and Germany. To do so, we sequenced individuals from these countries at the COI gene locus

Table 1. Sources of *Skistodiaptomus pallidus* material examined in this study. Specimen codes starting with "NZPL" were obtained from the Barcode of Life Datasystems (BOLD) database.

Locality	Specimen code	Geo-reference		Collection date	N
New Zealand					
Albany Pond	NZPL711, 713	36°43′33.53″S	174°42′28.56″E	Aug 2011	2
Auckland Botanic Gardens	NZPL059	37°00′39.33″S	174°54′23.89″E	Apr 2007	1
Chelsea Pond 4	N12	36°49′12.00″S	174°43′29.00″E	Nov 2015	1
Hamilton (Aquarium Store)	NZPL058	37°46′20.00″S	175°17′00.60″E	May 2007	1
Lochview	N01, N02, N03	37°11′13.41″S	174°54′09.86″E	Nov 2015	3
Maygrove	N13, N14, N16	36°35′21.97″S	174°41′04.11″E	Nov 2015	3
Montgomerie North	N05	36°59′05.93″S	174°46′46.03″E	Nov 2015	1
Lake Rotomanu	N27, N28, N29	39°02′32.00″S	174°06′54.20″E	Dec 2015	3
Tahuna Torea	N07, N08, N09	36°52′20.49″S	174°52′55.41″E	Nov 2015	3
Te Aroha	N30, N31, N32	37°31′45.00″S	175°42′40.20″E	May 2015	3
Germany					
Moats of Bremen	G18-G22, G25, G26	53°04′30.50″N	8°48′48.33″E	Nov 2015	7

and compared these sequences with those available from native populations in North America. We hypothesized that: 1) due to the different suggested vectors for introduction into Germany and New Zealand, each area would contain unique haplotypes arising from separate origins; 2) specimens collected from New Zealand would have strong genetic similarity based on the expectation that the propagule size introduced into New Zealand in association with fish farming was likely to be small compared to vectors such as shipping; and 3) there would be greater diversity in the specimens sampled from Germany than New Zealand, based on the expectation that an introduction via a shipping related vector would potentially introduce a greater number of propagules or result in repeated introductions.

Methods

Sampling

In order to estimate haplotype diversity within New Zealand populations, ten sites were selected over a broad geographic range from the known distribution of *S. pallidus* within the North Island (Table 1; Figure 1). Sampling was carried out during November and December 2015 at six New Zealand sites known to have received authorised grass carp releases.

Sequences from three additional sites from the Barcode of Life Data Systems (BOLD; www.boldsystems.org) were included in analyses. The BOLD records included one store-bought individual purchased in Hamilton in May 2007, among live food sold for aquarium fish, which originated from an aquaculture facility in Te Aroha (Figure 1); additional specimens of this population were sourced directly from the aquaculture facility in May 2015. The Auckland Botanic Gardens and Albany Pond were sites that had not received any authorised grass carp releases. Zooplankton were sampled by making multiple horizontal net hauls from the water body shoreline with a plankton net (40 µm mesh-size, at a haul speed of approximately 1 $m.s^{-1}$) until a concentrated zooplankton sample was obtained. Samples were transferred to containers and preserved with ethanol (90% final concentration) immediately following collection and cool-stored in a lidded bin to minimise light exposure. The German samples were collected from the moat of Bremen in early November 2015 using the method described above, and individuals sent to New Zealand for genetic analysis. Once in the laboratory, all samples were transferred to dark-storage at 4 °C. Specimens were identified morphologically using standard taxonomic keys (e.g., Dussart and Defaye 1995) prior to genetic analyses.

DNA extraction and amplification

Extraction of total genomic DNA was undertaken for each whole individual specimen using the Red Extract n Amp (Sigma-Aldrich) kit following the manufacturer's instructions using: 10 µL extract solution (ex) and 2.5 µL tissue preparation solution (TP) per reaction tube. Tubes were then placed into a darkened area to incubate at room temperature for three hours. Following incubation, tubes were heated to 95 °C for 3 minutes in an Eppendorf thermocycler before 10 µL of neutralising solution was added to each tube. Tubes were then vortexed before being stored at 4° C. We selected the mitochondrial cytochrome *c* oxidase subunit I (COI) gene locus for study as comparative sequences from across the native range obtained by Thum and Derry (2008) and Thum and Harrison (2009) were available from GenBank

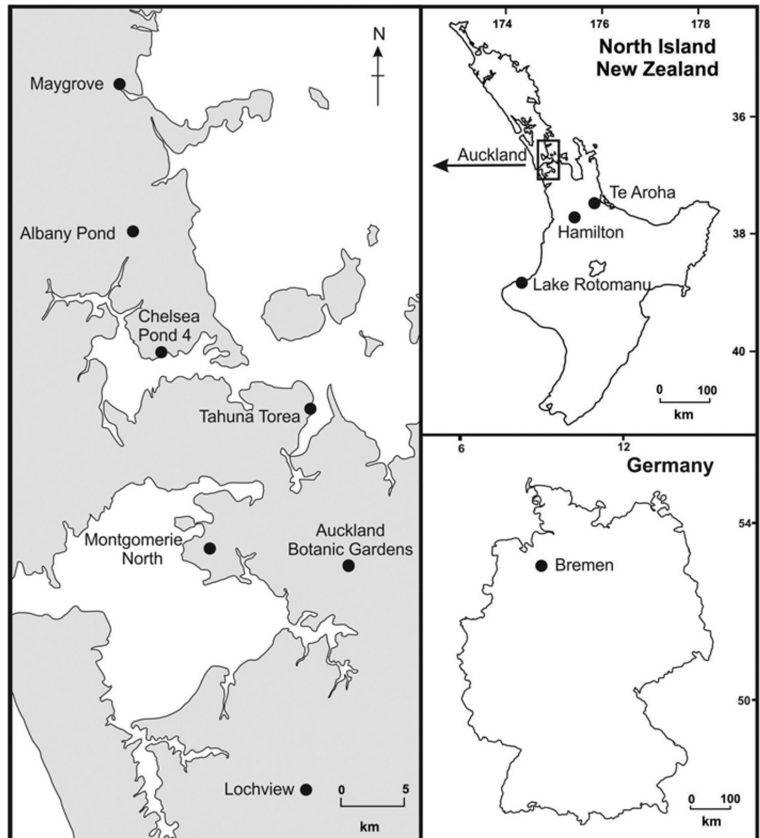

Figure 1. Locations of ponds where *Skistodiaptomus pallidus* were collected for this study.

(Benson et al. 2000). Previous analyses of cytochrome *b* and ITS genes have been congruent with those using COI haplotypes (Thum and Derry 2008; Thum and Harrison 2009), suggesting that COI provides an accurate indication of genomic differences.

A 658 bp fragment of the COI gene was PCR amplified from each extraction at the University of Waikato using either the universal primers HCO2198 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and LCO1490 (5'-GGTCAACAAATCATAAAGA TATTGG-3') (Folmer et al. 1994) or the paired primers Lep F1 (5'-ATTCAACCAATCATA AAGATATTGG-3') and Lep R1 (5'-TAAACTTCT GGATGTCCAAAAAATCA-3') (Hebert et al. 2004). The PCR reactions of 15 µL were made up of 7.5 µL of PCR Master Mix solution (iNtRON Biotechnology Inc., Korea), 0.3 µM of each primer (forward and reverse), 5.7 µL of deionised (Milli-Q) water and 1 µL of sample extract. Thermo-cycling consisted of an initial denaturing period of 1 minute at 94 °C followed by 5 cycles of 94 °C for 1 minute, 45 °C for 1.5 minutes and 72°C for 1 minute. This was followed by denaturation and polymerase amplification through 35 cycles of 94°C for 1 minute, 51°C for 1.5 minutes and 72 °C for 1 minute. The final extension period occurred at 72 °C for 5 minutes. A subsample (3 µL) of each PCR product was visualised under UV light in a MultiImage light cabinet (Alpha Innotech, Protein Simple, CA USA) following electrophoresis at 44 volts for 45 minutes in 1% agarose gel containing 5 µL/100mL RedSafe Nucleic Acid Staining Solution (20,000x) (iNtRON Biotechnology Inc., Korea). Successfully amplified products were cleaned using a master mix containing 0.1 µL Exonuclease I (EXO) (10 U/µL) and 0.1 µL Shrimp Alkaline Phosphate (SAP) (1 U/µL) solution (Illustra Global Science) and 5 µL PCR product following manufacturers' instructions. Purified products were sequenced in both directions using the same primers as per PCR on an ABI3130xL sequencer at the University of Waikato DNA Sequencing Facility.

Genetic analyses

Taxonomic verification of *S. pallidus* sequences produced in this study was carried out using the GenBank nucleotide BLASTn algorithm with default parameters, which confirmed matches with Thum and

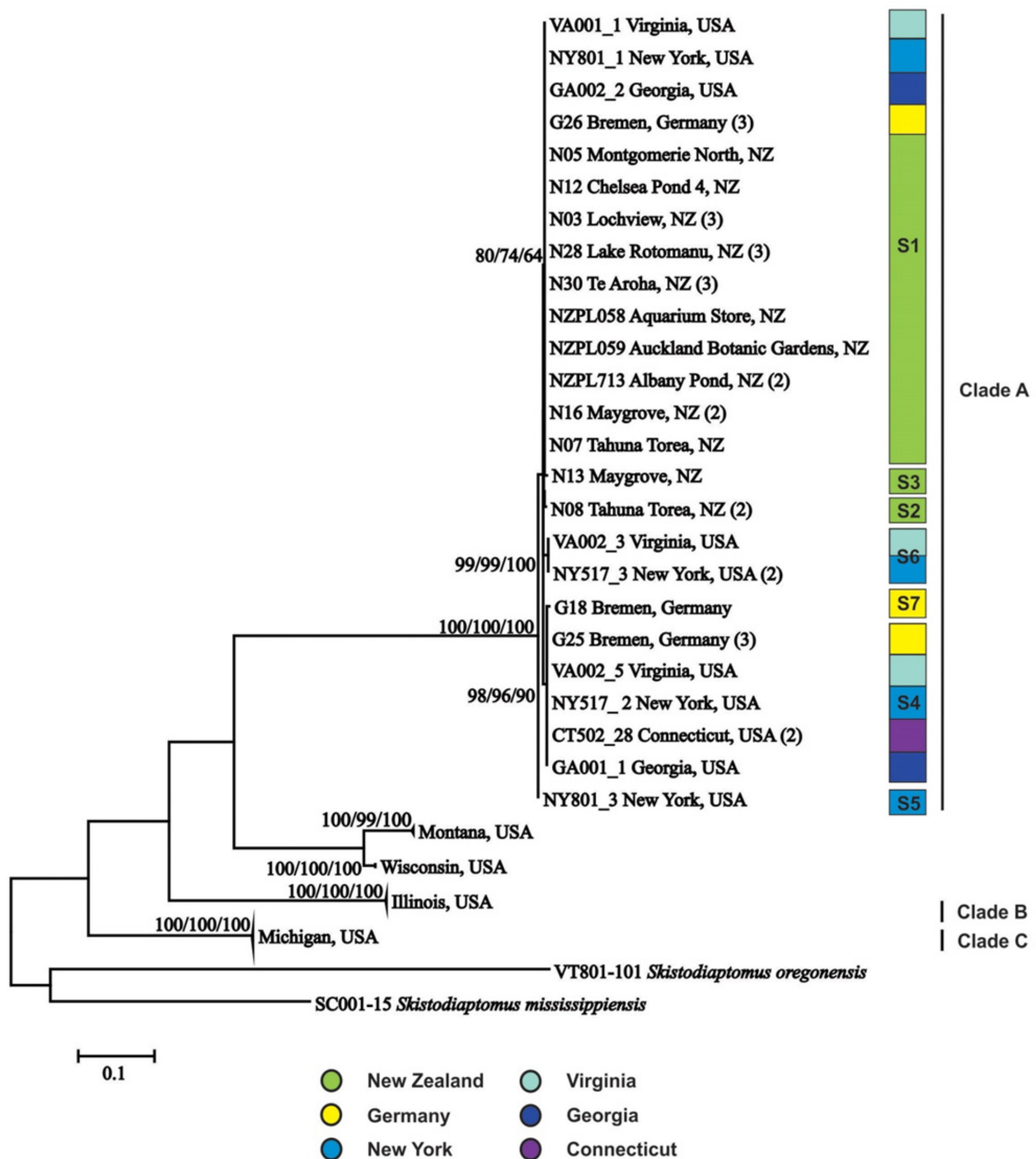

Figure 2. Maximum Likelihood tree estimated from mitochondrial COI gene sequences for *Skistodiaptomus pallidus* individuals collected in Germany, New Zealand (NZ) and the United States (USA). Bootstrap support values (1000 replicates) greater than 75% are shown at nodes for ML/MP/NJ analyses. Assigned haplotypes S1–S7 in Clade A, and the number of individuals with identical sequences (in parentheses) are shown for each collection location. Triangles represent collapsed cluster diversity. Three divergent Clades (A–C) as defined by Thum and Harrison (2009) are shown; Montana and Wisconsin sequences were not assigned to clades by these authors, and are therefore unlabelled.

Derry (2008) sequences (Benson et al. 2000). We attempted to sequence three *S. pallidus* individuals from each of the six New Zealand sites, which successfully produced 17 COI sequences of >640 nucleotides. Ten individuals were sequenced from the German site producing seven COI sequences of >640 nucleotides. We obtained a further four New Zealand sequences (Albany, Pond n = 2, Auckland Botanic Gardens n=1 and Hamilton Aquarium Store n = 1), from BOLD (Table 1).

Primer regions were trimmed and sequences were aligned using multiple sequence comparison by log-expectation (MUSCLE) as implemented in Geneious v6.1.2 (Drummond et al. 2010). The confirmed absence of stop codons indicated that mitochondrial DNA had been amplified. There were no insertions

or deletions, and the alignment was straightforward. Haplotypes were verified by visual inspection of the trace files. Sequences were aligned with a further 27 North American *S. pallidus* COI sequences downloaded from GenBank: accession numbers EU825105.1–EU825131.1 (Thum and Derry 2008; Supplementary material Table S2). The alignment was further trimmed to 631 bp to be compatible with the sequence lengths of the USA populations.

To test assumptions of homogeneity for tree-based analyses, chi-square (X^2) tests as implemented in PAUP*4.0 (Swofford 2001) were used to determine base pair frequency among sites and at first, second and third codon positions. *Skistodiaptomus oregonensis* and *S. mississippiensis* were used as outgroups (accession numbers EU582590.1 and EU582579.1; Thum and Harrison 2009). Neighbour Joining (NJ), Maximum Parsimony (MP) and Maximum Likelihood (ML) trees were produced using Molecular Evolutionary Genetic Analysis (MEGA) v5.2.2 (Tamura et al. 2011). Bootstrap values of 75% and above support for branches (1000 replicates) were included in the final tree (Felsenstein 1985). The optimal model of nucleotide substitution for the ML tree was the General Reversible Model (GTR+I+G) as determined using jModelTest v2.1.1 (Darriba et al. 2012). For NJ and MP tree construction the Jules-Cantor model of nucleotide substitution was used with gamma distribution. To assess intraspecific divergence, a pairwise genetic distance matrix of evolutionary divergence was produced in MEGA. To visualise haplotype relationships, identified haplotypes were allocated an identifier (Table S2) and a haplotype network was produced for haplotypes within Clade A (i.e., east coast populations) using TCS v1.21 (Clement et al. 2000). All sequences and supporting information were deposited in the BOLD database under the project NZPLC (Freshwater Calanoids of New Zealand and Australia).

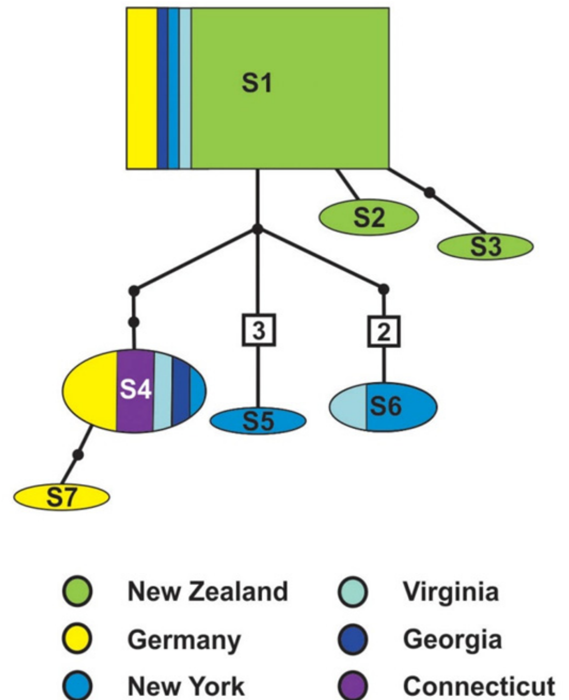

Figure 3. Network of seven unique *Skistodiaptomus pallidus* haplotypes (labelled S1–S7) from Clade A (*sensu* Thum and Harrison 2009), with colour coding indicating the collection location for each. The boxed numbers indicate the number of mutational steps that separate haplotypes and black dots represent single mutational steps.

Results

A total of 57 individuals were included in our final alignment (7 German, 21 New Zealand, 27 public USA, 2 outgroup) of 631 nucleotide positions. The Maximum Likelihood tree indicated that sequences were grouped into four clades and 15 unique *S. pallidus* haplotypes were identified (identified as S1 to S15; Figure 2). Of these, the New Zealand specimens consisted of one major haplotype (S1) present at all sites, with two additional haplotypes from ponds at Maygrove (S2) and Tahuna Torea (S3) (Figure 2, Table S1). New Zealand (S1) sequences formed a group with specimens sampled from eastern USA sites in Virginia, New York and Georgia (VA001, NY801, GA002). The German samples consisted of three haplotypes, the first (S1) shared with New Zealand, Virginia, New York and Georgia and the second (S4) shared with sequences obtained from sites in Virginia, New York, Georgia and Connecticut (VA002, NY517, GA001 and CT502) (Figure 2). The third haplotype from Germany (S7) was not recorded elsewhere.

Of the 631 nucleotide positions, 453 were constant, 176 were parsimony informative and 2 were variable but parsimony uninformative. Composition across all nucleotide positions was biased towards A-T (A = 25.76%; T = 36.58%; G = 21.58%; C = 16.08%). Base frequencies were homogeneous across sequences where all sites were analysed (X^2_{162} = 23.28, p = 1.000). Base frequencies across variable sites (X^2_{162} = 99.82, p = 0.999) and uninformative sites (X^2_{162} = 103.11, p = 1.000) were also homogeneous. Base frequencies were homogeneous for first (X^2_{162} = 3.53, p = 1.000), second (X^2_{162} = 0.06, p = 1.000) and third codon positions, with greatest variation in third codon positions (X^2_{162} = 94.90, p = 1.000). The ML analyses, as

estimated using the GTR+I+G model (–lnL=2655.89), were concordant with NJ and MP results and indicated that the sequences from both New Zealand and Germany were positioned in Clade A (i.e., with east coast USA populations), as defined by Thum and Harrison (2009) (Figure 3).

Discussion

Analysis of the *S. pallidus* COI sequences placed the New Zealand and German specimens within the monophyletic group identified as Clade A by Thum and Harrison (2009), with strong bootstrap support. In the native range, Clade A is made up of specimens collected from the most easterly regions of the USA, which includes New York, Connecticut, Virginia and Georgia (Thum and Harrison 2009). Although Clade A haplotypes were common to waterbodies spread over the eastern USA, they were highly divergent from those collected in Michigan, Illinois (Clades B and C), Wisconsin and Montana. There were two widespread haplotypes within Clade A in the USA, and while New Zealand specimens included one of these (S1), German specimens included both (S1 and S4). The presence of both major haplotypes in the German population is an indication that the propagule supply may have been larger than that into New Zealand, and is consistent with the speculated large or multiple introductions from shipping in Germany. Additionally, with New Zealand and Germany each having unique minor haplotypes not yet detected by the limited intensity of sampling of the native range outlined in Thum and Derry (2008), it is likely that German and New Zealand populations originated from different sources from within the eastern United States.

Brandorff (2011) indicated that the most likely vector for *S. pallidus* introduction into the German sites was via ballast-related transport from North America, as there had been a large volume of shipping into the port of Bremen during the 20th century. The transportation and introduction of non-native taxa is well documented in studies related to the Laurentian Great Lakes, with numerous inter-continental invasions attributed to shipping related vectors, although documented introductions are typically into the lakes, not from them (Duggan et al. 2005; Drake and Lodge 2007). Movement of organisms between the Weser River and the partitioned moat could occur due to a pumping system that moves water into the moat from where it can flow back into the river (Brandorff 2011). However, while *S. pallidus* has been found in an increasing number of sites adjoining the river, it has not been detected within it (GOB, unpubl. data).

Despite the advent of ballast water exchange, shipping vectors are still potentially very active (e.g. Chain et al. 2016). In contrast to shipping in Germany, international shipping in New Zealand is limited to marine environments and hence, this mode of introduction is unlikely. The range of *S. pallidus* includes natural and constructed water bodies within the Lake Ontario drainage, in close proximity to the Great Lakes, although the New York sequences used for comparison in this study were from sites over 100 km from major ports adjoining Lakes Erie and Ontario (Mills et al. 1993; Thum and Stemberger 2006). Further, *S. pallidus* specimens have been periodically sampled from Lakes Ontario, Erie and St Clair (Mills et al. 1993). While Mills et al. (1993) argued these likely washed in with drainage from surrounding areas, Reid and Hudson (2008) have argued that *S. pallidus* should not be considered non-native to the Laurentian Great Lakes due to its distribution in surrounding systems. Nevertheless, their presence in the lakes suggests that transport by a shipping vector could occur from these locations through ballast related transport (Mills et al. 1993). For example, between 1981 and 2000, Lakes Ontario and Erie accommodated the first port of call in the Laurentian Great Lakes for 72% of transoceanic vessels declaring no ballast on board, and after cargo was unloaded, ballast was typically taken on board (Holeck et al. 2004). Other vectors of introduction into Germany, such as the movement of aquarium or ornamental taxa, are also possible but less likely (Duggan 2010).

The genetic similarity among a variety of New Zealand populations indicates *S. pallidus* may have been introduced there from a limited or single introduction event, with later spread to other New Zealand sites, including in association with translocations of grass carp. While there is no obvious vector of introduction for *S. pallidus* into New Zealand, within New Zealand a vector related to the transportation of fish is suggested by the strong association of *S. pallidus* with aquaculture facilities used in the domestic culturing and translocation of grass carp (Duggan et al. 2014; Duggan and Pullan 2017). The similarity in sequences between ponds that have, and have not, received grass carp introductions in the current study suggests all known populations may have arrived in a single introduction event and that later spread to non-carp ponds may be via spread from natural vectors (e.g., intestinal or external transport on birds) from already invaded ponds.

The geographic range of Clade A haplotypes in its native range, from Georgia to New York, and their proximity to major population centres in the eastern United States, indicates the similarity between the German and New Zealand *S. pallidus*

haplotypes is probably coincidental and may reflect the association between human activities and invasions (Carlton 1996). The similarity in German and New Zealand populations, being dominated by Clade A haplotypes, is consistent with genetic theory around founder events, in that haplotypes more frequently found in the source population may be more likely to be represented in the founder populations, particularly where introduction size is small. Nevertheless, haplotype diversity for the COI locus within each clade is probably underestimated in the native range due to the limited data currently available, which is typically derived from two to five specimens per site, and represented by single haplotypes from each site (Thum and Derry 2008). Consequently, these data cannot yet provide a specific location for the origin of haplotypes found in New Zealand and Germany, which might otherwise better inform the probable transportation vectors. The use of further markers and more intensive sampling of sites from the eastern United States will provide a more detailed indication of the distribution of haplotypes and intra-specific diversity in *S. pallidus*. Further, sequencing of individuals from the non-indigenous Mexican *S. pallidus* population will allow for its comparison with the inter-continental invasions, which is important given the common suggested vector of aquaculture between New Zealand and Mexico.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Pairwise sequence divergence (uncorrected *P*-distance) for a 631bp mtDNA COI fragment for 15 haplotypes of *Skistodiaptomus pallidus* sampled from New Zealand, Germany and the United States.

Table S2. Field code, haplotype codes and accession numbers for sequences from the United States with clades defined by Thum and Harrison (2009).

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