First Molecular Evidence for Underestimated Biodiversity of Rhachotropis (Crustacea, Amphipoda), with Description of a New Species

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Abstract

The crustacean genus Rhachotropis has a worldwide distribution and amongst the largest bathymetric range known from any amphipod genus. DNA barcoding of new material from around New Zealand and the Ross Sea indicated depth-related biogeographic patterns. New Zealand Rhachotropis do not form a monophyletic clade. Species from bathyal depths on the Chatham Rise, east of New Zealand, show lower sequence divergence to bathyal species from California and the Arctic than to abyssal New Zealand species. Species sampled in the Kermadec Trench, north of New Zealand below 5000 m, seem to be more closely related to Ross Sea abyssal species than to the New Zealand shelf species. The worldwide geographic and bathymetric distribution for all Rhachotropis species is presented here. Depth may have a greater influence on phylogeny than geographic distance. Molecular and morphological investigations of Rhachotropis specimens from the Chatham Rise, New Zealand revealed a new species to science which is described in detail, including scanning electron microscopy. This increases the number of described species of Rhachotropis to 60 worldwide.

Introduction

The amphipod genus Rhachotropis (Eusiridae) contains 59 known species with a worldwide distribution (Fig. 1) [1]. Rhachotropis species appear to have a patchy distribution although some species are locally very abundant [1,2], especially in benthic slope communities [3]. Species in general have a relatively high swimming capacity, indicative of a partial pelagic lifestyle [3]. Phylogenetic analyses based on morphological characters have been unsatisfying or not possible. The numerical analysis of 20 morphological characters and corresponding character states by Bousefield & Hendrycks [4] focused on gross external morphology rather than mouthparts and reproductive features that may actually prove more significant phylogenetically as the authors suggested. Even though Rhachotropis show an impressive horizontal and vertical distribution, the genus has not been studied with molecular phylogenetic tools. This is a first preliminary analysis of the mitochondrial cytochrome oxidase c subunit 1 (COI) sequences of Rhachotropis specimens collected from bathyal and abyssal depths around New Zealand and in the Ross Sea.

This paper describes one new species collected on the Chatham Rise, east of New Zealand, and increases the number of known and described Rhachotropis species to 60, and the New Zealand Rhachotropis to four species. At least two further species from New Zealand waters appear to be new, but in too poor condition as to be formally described.

Results

COI

Relationships for nine Rhachotropis specimens from New Zealand and the Ross Sea are shown in Fig. 2 and represent the topology inferred by both analyses. The trees were rooted with the Antarctic outgroup Eusirus sp., and include additional close matches for northern hemisphere Rhachotropis COI sequences held in GenBank: R. inflata, R. aculeata, R. inflata, R. helleri, and a putative new species from California R. sp 28 (Table 1). The DNA barcodes revealed six well supported clades of Rhachotropis specimens from New Zealand and the Ross Sea with a further four clades for the northern hemisphere species (Fig. 2). Three specimens from the Chatham
Rise, New Zealand, had identical COI sequences and were described as *R. chathamensis* Lorz, 2010. Two specimens from the Ross Sea had identical COI sequences and belong to *R. abyssalis* Lorz, 2010. A further three specimens from New Zealand had three unique COI sequences; one specimen which is described in this paper as *R. novazealandica* Lorz, 2012 (Fig. 2), while the other two specimens remain undescribed: R. sp. A and R. sp. B (Fig. 2). A fourth single specimen from the Ross Sea with a unique sequence was described as *R. rossi* Lorz, 2010 (Fig. 2).

Sequence divergence was zero within the *R. chathamensis* and *R. abyssalis* clades, and low within the 24 specimens of *R. aculeata* (0.0089), 9 specimens of *R. helleri* (0.0003), and 13 specimen of *R. inflata* (0.037). A single specimen identified as *Rhachotropis inflata* (Cornwallis Island) is separated distinctly from the remaining clade (separated by 3% sequence divergence). And one tentative species, *R*. sp 28 from California, is represented by one sequence retrieved from GenBank. Inter-clade sequence divergences ranged from 0.143–0.370 with an overall average divergence 0.284. The lowest divergence (0.143, Table 2) was between *R. novazealandica* spec. nov. from New Zealand and *R. sp. 28* from California, while the greatest divergence was between the two putative species *R*. sp. A and *R. sp. B* (0.370, Table 2) from New Zealand.

Morphological investigation revealed a species new to science which is described herein. Even though only a single damaged specimen exists, the COI sequence and detailed morphological descriptions will allow corroboration by future collections.

**Figure 1.** Global distribution map of the genus *Rhachotropis* with the species represented in the molecular part of this paper in triangles, the Southern Ocean species in squares and the remaining species (~40), including unidentified ones in small circles. doi:10.1371/journal.pone.0032365.g001

**Systematics**

Order AMPHIPODA Latreille, 1816
Suborder GAMMARIDEA Latreille, 1802
Family EUSIRIDAE Stebbing, 1888
Genus *Rhachotropis* S.I. Smith, 1883

*Rhachotropis novazealandica* spec. nov. Lorz, 2012

(Figs. 3, 4, 5, 6, 7)

**Material examined.** Holotype: NIWA 42864, female, 17 mm TAN0705/12, 13 Apr 2007, Box corer at 520 m, 44° 7.57 S, 174° 50.74E, R.V. Tangaroa, Collector: Ocean Survey 20/20 Chatham Rise, New Zealand.

**Etymology.** *Rhachotropis novazealandica* spec. nov. is named after New Zealand where the species was collected.

**Diagnosis.** Body delicate. Rostrum longer than head. Eyes absent. Head twice as long as pereonite 1, lateral lobes produced. Pereonites smooth. All pleonites bearing dorsal processes, pleonite 1 also bearing dorsolateral processes.

**Description.** Antenna 1 second article of peduncle with several plumose setae, article 2 slightly shorter than article 1, twice as long as article 3; flagellum broken after 10th-articulate. Antenna 2 peduncle article 3 longer than article 4, several plumose setae on third article; flagellum broken after 6th article.

Mandible with smooth incisor process well developed, lacinia mobilis denticulate, molar process conical. Left and right molars have several pores in the middle. The tip of the left mandibular palp...
Figure 2. Relationships of COI sequences from *Rhachotropis* specimens. BOLD Accession Numbers are given for each specimen along with locations. Numbers at nodes are ML bootstrap percentages (>80%) after 1000 replications, and Bayesian inference posterior probability values (>0.90); scale bar represents an interval of the TIM+I+G model. The tree topology represents the 50% majority rule consensus of all Bayesian trees and has been rooted with the Antarctic *Eusirus*. doi:10.1371/journal.pone.0032365.g002
| Species | BOLD | Sample ID | GenBank Acc# | Area | Lat  | Lon  | Depth |
|---------|------|-----------|--------------|------|------|------|-------|
| Rhachotropis abyssalis | AMPNZ095-09 | 60483 | GU804296 | Ross Sea | −76.19 | 176.30 | 447 |
| Rhachotropis abyssalis | AMPNZ094-09 | 60484 | GU80484 | Ross Sea | −76.19 | 176.30 | 447 |
| Rhachotropis aculeata | WWGSL070-08 | GSL31-39 | FJ81879 | St. Lawrence Gulf | 48.15 | −63.54 | |
| Rhachotropis aculeata | WW865-08 | GSL31-07 | FJ81880 | St. Lawrence Gulf | 47.90 | −65.35 | |
| Rhachotropis aculeata | WWGSL098-08 | TE-004T21-40-04 | FJ81881 | St. Lawrence Gulf | 48.39 | −59.55 | 150 |
| Rhachotropis aculeata | WW851-08 | TE-004T69-02 | FJ81882 | St. Lawrence Gulf | 50.82 | −58.59 | 233 |
| Rhachotropis aculeata | WW850-08 | TE-004T69-01 | FJ81883 | St. Lawrence Gulf | 50.82 | −58.59 | 233 |
| Rhachotropis aculeata | WW105-07 | RA03CN0906 | FJ81884 | St. Lawrence Gulf | 49.92 | −64.62 | |
| Rhachotropis aculeata | WW129-07 | RA02CN0306 | FJ81885 | St. Lawrence Gulf | 51.14 | −58.05 | |
| Rhachotropis aculeata | WW459-08 | BSM07T13-04 | FJ81886 | Cote-Nord | 50.25 | −66.70 | |
| Rhachotropis aculeata | WW458-08 | BSM07T13-03 | FJ81887 | Cote-Nord | 50.25 | −66.70 | |
| Rhachotropis aculeata | WW402-08 | 3L-WT772-S60-01 | FJ81888 | Grand Bank | 46.61 | −49.24 | 74 |
| Rhachotropis aculeata | CCNUN228-07 | NUN-0228 | JQ12476 | Somerset Island | 72.77 | −93.36 | |
| Rhachotropis aculeata | CCNUN149-07 | NUN-0149 | JQ12465 | Resolute | 74.68 | −94.86 | |
| Rhachotropis aculeata | CCNUN150-07 | NUN-0150 | JQ12468 | Resolute | 74.68 | −94.86 | |
| Rhachotropis aculeata | CCNUN151-07 | NUN-0151 | JQ12467 | Resolute | 74.68 | −94.86 | |
| Rhachotropis aculeata | CCNUN152-07 | NUN-0152 | JQ12466 | Resolute | 74.68 | −94.86 | |
| Rhachotropis aculeata | CCNUN178-07 | NUN-0178 | JQ12475 | Devon Island | 74.67 | −91.70 | |
| Rhachotropis aculeata | CCNUN005-07 | NUN-0005 | JQ12473 | Devon Island | 75.76 | −88.12 | |
| Rhachotropis aculeata | RBGC036-03 | MaEus000 | DQ889127 | Resolute | |
| Rhachotropis aculeata | WW023-07 | CA196 | JQ12474 | Beaufort Sea | 70.90 | −128.90 | |
| Rhachotropis aculeata | WW024-07 | CA197 | JQ12472 | Beaufort Sea | 70.90 | −128.90 | |
| Rhachotropis aculeata | GBCMA0080-06 | AY271853 | AY271853 | Resolute | |
| Rhachotropis chathamensis | AMPNZ101-09 | 42768.d | GU804298 | New Zealand | −43.80 | 175.32 | 418 |
| Rhachotropis chathamensis | AMPNZ100-09 | 42768.c | GU804299 | New Zealand | −43.80 | 175.32 | 418 |
| Rhachotropis chathamensis | AMPNZ098-09 | 42768.a | GU804300 | New Zealand | −43.80 | 175.32 | 418 |
| Rhachotropis helleri | CCNUN449-08 | NVAMP-0004 | JQ12483 | Resolute | 75.08 | −94.86 | |
| Rhachotropis helleri | CCNUN007-07 | NUN-0007 | JQ12484 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN008-07 | NUN-0008 | JQ12482 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN009-07 | NUN-0009 | JQ12481 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN010-07 | NUN-0010 | JQ12477 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN011-07 | NUN-0011 | JQ12480 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN012-07 | NUN-0012 | JQ12485 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN013-07 | NUN-0013 | JQ12479 | Resolute | 74.68 | −94.86 | |
| Rhachotropis helleri | CCNUN014-07 | NUN-0014 | JQ12478 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN620-08 | CCNUN620 | JQ12491 | Resolute | 75.08 | −94.86 | |
| Rhachotropis inflata | CCNUN621-08 | CCNUN621 | JQ12492 | Resolute | 75.08 | −94.86 | |
| Rhachotropis inflata | CCNUN622-08 | CCNUN622 | JQ12493 | Resolute | 75.08 | −94.86 | |
| Rhachotropis inflata | CCNUN334-07 | NUN-0334 | JQ12487 | Igloolik | 69.37 | −81.79 | |
| Rhachotropis inflata | CCNUN154-07 | NUN-0154 | JQ12489 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN155-07 | NUN-0155 | JQ12498 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN156-07 | NUN-0156 | JQ12488 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN157-07 | NUN-0157 | JQ12497 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN158-07 | NUN-0158 | JQ12499 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN159-07 | NUN-0159 | JQ12495 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN160-07 | NUN-0160 | JQ12494 | Resolute | 74.68 | −94.86 | |
| Rhachotropis inflata | CCNUN161-07 | NUN-0161 | JQ12490 | Resolute | 74.68 | −94.86 | |
bears six plumose setae. Maxilla 1 inner plate bearing 1 subterminal seta, outer plate with 9 denticulate spines. Maxilliped outer plate 2.5 times as long as inner plate, reaching half of article 2 of maxillipedal palp; inner margins of palp, outer plate and terminal end of inner plate setose. Labrum entire, smooth and broadly rounded. Hypopharynx setose, outer lobes with broad gap.

Gnathopod 1 coxa 1 produced, reaching to end of head, coxa 2, 3 and 4 subquadrate. Gnathopods similar in shape, subchelate. Gnathopod 1 slightly smaller than gnathopod 2, basis bearing several small spines at anterior side; merus with long setae at posteroventral corner; carpus lobe extending width of propodus, spines at terminal end of lobe; propodus widened, oval; dactylus slender, reaching end of palm.

Pereopod 5 basis small, narrow; merus longer than carpus. Pereopod 6 basis larger than of pereopod 5, posteroventral angle produced. Pereopod 7 basis widened, posterior margin serrate, posteroventral angle strongly produced.

Uropod 1 rami same length.

Remarks. *Rhachotropis novazealandica* spec. nov. differs from the other four *Rhachotropis* species from New Zealand (*R. chathamensis* Lorz 2010; *R. delicata* Lorz 2010; *R. levantis* Barnard 1961 and *R. spec* Dahl, 1959) by the combination of following characters: rounded coxa 1 (vs *R. chathamensis*), coxa 2 smaller than coxa 3 (vs *R. chathamensis*), coxa 3 and 4 ventral margin slightly bilobed (vs straight *R. chathamensis*), second segment of maxillipedal palp not broadend (vs *R. spec* Dahl, 1959), gnathopod 2 propodus extension exceeding article (as *R. delicata* vs *R. levantis*), uropod 1 rami same length (as *R. delicata*, vs *R. levantis*), gnathopod 1 and 2 dactylus as long as palm, basis pereopod 6 and 7 strong dorsolateral projection (vs *R. delicata*).

Distribution. New Zealand, Chatham Rise, 520 m.

Discussion

This is the first molecular study of *Rhachotropis* and has revealed a high level of diversity among specimens from the northern and southern hemispheres. The historic *Rhachotropis* collections, including the type material for most the species, were preserved in formalin or other DNA degrading media and are therefore not suitable for routine molecular investigations. Some fragile *Rhachotropis* specimens collected on recent expeditions were damaged and unsuitable for detailed morphological descriptions, but were fixed in ethanol to enable molecular studies. This study

| Species                        | BOLD          | Sample ID | GenBank Acc# | Area       | Lat   | Lon   | Depth |
|-------------------------------|---------------|-----------|--------------|------------|-------|-------|-------|
| *Rhachotropis inflata*        | CCNZ1162-07   | N10-0162  | JQ412496     | Resolute   | 74.68 | -94.86|       |
| *Rhachotropis inflata*        | RBGC0388-03   | MaEus002  | JQ412486     | Resolute   |        |       |       |
| *Rhachotropis inflata*        | GBCMA0081-06  | AY271854  | AY271854     | Resolute   |        |       |       |
| *Rhachotropis novazealandica* | AMPNZ128-09   | 42664     | GU04309      | New Zealand| -44.13| 174.85| 520   |
| *Rhachotropis rossi*          | ANZR470-08    | 45813     | JF498593     | Ross Sea   | -76.59| 176.83| 369   |
| *Rhachotropis sp.* 28         | GBCMA1154-08  | EF989704  | EF989704     | California | 36.33 | 122.90| 300–700|
| *Rhachotropis sp.* A           | AMPNZ184-10   | 60487     | JF498594     | New Zealand| -36.52| 179.20| 5173  |
| *Rhachotropis sp.* B           | AMPNZ102-09   | 42768.e   | HM372956     | New Zealand| -43.80| 175.32| 418   |
| *Eusirus sp.* (outgroup)       | ANZR028-08    | 35955     | JQ412464     | Ross Sea   | -72.08| 175.55| 1620  |

N = number of specimens.

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| N within R. aculeata R. inflata R. helleri R. abyssalis chathamensis R. sp. 28 R. rossi R. novazealandica R. sp. A R. sp. B Outgroup |
|---------------------------------------------------------------|-----------------|----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| R. aculeata                                                   | 24              | 0.00887        |                 |                 |                 |                 |                 |                 |                 |                 |                 |
| R. inflata                                                    | 13              | 0.03672        | 0.27756         |                 |                 |                 |                 |                 |                 |                 |                 |
| R. helleri                                                   | 9               | 0.00353        | 0.26149         | 0.27599         |                 |                 |                 |                 |                 |                 |                 |
| R. abyssalis                                                 | 2               | 0.25087        | 0.26891         | 0.29778         |                 |                 |                 |                 |                 |                 |                 |
| R. chathamensis                                              | 3               | 0.22547        | 0.25206         | 0.19702         | 0.28893         |                 |                 |                 |                 |                 |                 |
| R. sp. 28                                                    | 1               | 0.24149        | 0.30575         | 0.27101         | 0.31554         | 0.2622          |                 |                 |                 |                 |                 |
| R. rossi                                                     | 1               | 0.2502         | 0.28218         | 0.28517         | 0.28076         | 0.2164          | 0.26698         |                 |                 |                 |                 |
| R. novazealandica                                            | 1               | 0.23667        | 0.26022         | 0.24123         | 0.27004         | 0.2435          | 0.14342         | 0.24142         |                 |                 |                 |
| R. sp. A                                                     | 1               | 0.30815        | 0.32293         | 0.31698         | 0.30095         | 0.3544          | 0.31781         | 0.31284         | 0.3328          |                 |                 |
| R. sp. B                                                     | 1               | 0.26806        | 0.30257         | 0.25556         | 0.29027         | 0.3214          | 0.30855         | 0.30804         | 0.315           | 0.3699          |                 |
| Outgroup                                                     | 1               | 0.30998        | 0.30515         | 0.29003         | 0.30271         | 0.3229          | 0.28589         | 0.3078          | 0.3151          | 0.2916          | 0.365          |

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continues the integrative approach of DNA barcoding and classic taxonomy.

Most barcode projects aim to develop open-access libraries derived from referenced (vouchered) specimens that will improve understanding of biodiversity, highlight cryptic species, and provide rapid tools for identification of a wide range of species [5,6,7]. While barcoding has its limitations, especially the discrimination of recently diverged species that underwent introgressive hybridisation, the COI barcode region has been shown to be appropriate for discrimination between closely related species across diverse animal phyla [7–10]. Barcoding can highlight potentially cryptic species that appear in discrete clades with high sequence divergences as in the Rhachotropis case here. High intra-specific divergences indicate that additional data are required to distinguish potential new species from known species. The barcode databases, once established can be applied to the DNA identification of specimens where traditional morphological methods are inappropriate such as stomach contents in fishes [11,12], fish fillets [13,14] and environmental barcoding for biomonitoring [15].

Although there are ongoing discussions about the level of intra- and inter-specific divergences in amphipods and the concept of species to be used [16,17] molecular species recognition is mostly based on the barcode “gap” between intra- and interspecific variations, with high inter- and low intra-clade sequence divergences indicative of cryptic species. Based on the barcode gap and consistent morphological differences, Lötz et al. [18] described and redescribed species of Antarctic Amphipoda and suggested that benthic species of Amphipoda do not occur circum Antarctic.

The inter- and intra-specific divergences of the Rhachotropis clades are in the same order of magnitude as for other deep sea Amphipoda (e.g. [10,18,19]). Interspecific uncorrected COI sequence distances in the Antarctic Iphimiellidae varied from 7.9% (Echiniphimella scotti to E. hodgsoni) to 29.5% (Iphimiella cyclogena to I. gorgon) [18]. The deepwater Antarctic Rhachotrois species from the Admiralty seamount and Scott Island, to the north of the Ross Sea were in the same range, 28%.

Within the Epimeriidae sequence divergence varied from 8.5% (E. schiaparelli to E. macrodonta) to 26.15% (E. horsti to E. annabellae) [18]. Sequences of species from New Zealand’s seamounts, Epimera horsti and E. bruuni were more similar to each other than to any of the remaining Antarctic Epimeria species, but the distance between them was high with nearly 20%. The Antarctic Epimera species formed a monophyletic clade [18] while this study found the New Zealand Rhachotrois not to be monophyletic with the largest genetic distance of 37% between species.

Rhachotropis specimens are found in all major oceans of the world: Arctic, Atlantic Ocean, Mediterranean Sea, Caribbean Sea, Indian Ocean, Pacific Ocean and the Southern Ocean (see Fig. 1 map). Rhachotropis specimens have been collected in all water depths (see Fig. 8a,b), from the shelf (e.g. [20]) to abyssal and hadal sampling sites (R. rossi, R. abyssalis, R. annabellae, R. flemmingi, R. spec A) [18]. Within the Rhachotropis clades are in the same order of magnitude as for other deep sea Amphipoda (e.g. [10,18,19]). Interspecific uncorrected COI sequence distances in the Antarctic Iphimiellidae varied from 7.9% (Echiniphimella scotti to E. hodgsoni) to 29.5% (Iphimiella cyclogena to I. gorgon) [18]. The deepwater Antarctic Rhachotrois species from the Admiralty seamount and Scott Island, to the north of the Ross Sea were in the same range, 28%.

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(e.g. [21]). Specimens used in this study are from three oceans, the Arctic, Southern and Pacific Oceans. Generally more species are currently known from the shelf and upper slope area, however, the observed depth pattern is heavily sample/collection biased and areas with more stations show more species. For example, detailed sampling at one deepwater location (2700 m Iceland Basin) shows four species. Similar results are found for Southern Ocean species in general [22] and in specific groups, such as isopods and gastropods [23].

The worldwide and broad depth distribution makes Rhachotropis an ideal model group to test the relationship between shelf and trench faunas or biogeographic “processes” such as sub- or emergence events. Submergence describes the downwards movement/shift of taxa from the shelf/shallow water depth to deeper waters (continental slopes and abyss) while emergence represents the upward movement of taxa from the shelf/shallow water depth to deeper depths (Emergence) in oceans around the world.

Materials and Methods

All necessary permits were obtained for the described field studies. Studies in the Ross Sea were undertaken under permit number AMLR07/005/Tangaroa/ZMFRI, issued by the New Zealand Government by the Minister of Fisheries Jim Anderton on 19 December 2007 under New Zealand Antarctic Marine Living Resources Act 1981, for the CCAMLR statistical subareas 88.1 and 88.2. Collection of bio samples from the Kermadec Trench expedition (KAH0910) and for the Oceans Survey 2020 Chatham Challenger project (TAN0705) was undertaken under Special Permits (421 and 318) issued by the Ministry of Fisheries pursuant to section 97 (1)(i) and (ii) of the New Zealand Fisheries Act 1996.

Taxon sampling

Rhachotropis amphipods were collected during the Ocean Survey 2020 voyages of RV Tangaroa to the Chatham Rise 2007 (TAN0705) east of New Zealand and to the western Ross Sea 2008 (IPY-CAML, TAN0802), and during the RV Kaharoa voyage HADEEP #6 to the Kermadec Trench north of New Zealand 2009 (KAH0910). Specimens were immediately sorted on deck, often photographed alive on board to record live coloration, fixed in 98% ethanol and later transferred to 70% ethanol.

The amphipod specimens were identified to species level by the first author using identification keys based on morphological characters.

The amphipod specimens including the type material have been registered and curated at the National Institute for Water & Atmospheric Research (NIWA) Invertebrate Collection (NIC) in Wellington, New Zealand.
DNA extraction and analyses

DNA was extracted from a sub-sample of muscle tissue from nine specimens using an automated Glass Fiber protocol [27]. The 650 bp barcode region of COI was amplified under the following thermal conditions: 1 min at 94 °C; 5 cycles of 94 °C for 40 s, 45 °C for 40 s and 72 °C for 1 min, followed by 35 cycles at 94 °C for 40 s, 45 s at 51 °C, and 1 min at 72 °C, and a final step of 72 °C for 1 min. The 12.5 μl PCR reaction mixes included 6.25 μl of 10% trehalose, 2.00 μl of ultrapure water, 1.25 μl 10× PCR buffer [200 mM Tris-HCl (pH 8.4), 500 mM KCl], 0.625 μl MgCl2 (50 mM), 0.125 μl of each primer [0.01 mM, using LCO1490/HCO2198 [28] with M13 tails], 0.625 μl of each dNTP (10 mM), 0.060 μl of Platinum® Taq Polymerase (Invitrogen), and 2.0 μl of DNA template. PCR amplicons were visualized on a 1.2% agarose gel E-Gel (Invitrogen) and bidirectionally sequenced using sequencing primers M13F or M13R and the BigDye terminator criteria, and the TIM analyses were performed using a nucleotide substitution model MEGA v 5.0 [30]. Net sequence divergences among taxa were evaluated by 1000 bootstrap replications [33]. Bayesian analyses was done using PAUP v. 4b10 [32], with support for each clade determined using 10000 generations, saving the current tree every 1000 generations.

Morphological description

The specimen of the new species was dissected under a Leica MZ12 stereomicroscope and drawn using a camera lucida. All illustrations were digitally ‘inked’ following Coleman [35,36]. Inking was done with the software Adobe Illustrator 14.0 and an A3 drawing table (Wacom Intuos 9×12).

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