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Rapid Communication

First report of the introduced isopod *Ligia exotica* Roux, 1828 in rocky intertidal habitats of Midway Atoll, Papahānaumokuākea Marine National Monument as confirmed by morphological and molecular approaches

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Abstract

The Papahānaumokuākea Marine National Monument (PMNM) is one of the world's largest marine protected areas and covers marine habitats as well as the islands and atolls of the Northwestern Hawaiian Islands. Despite its protected status, the biodiversity of the PMNM face several threats to its preservation, including the presence of introduced species. Given its history as a shipping and military outpost, Midway Atoll represents a potential entry point for introduced species to the PMNM. Thus, monitoring for new introduced species in Midway is of importance for the management and preservation of the PMNM. In this study, we use morphological and molecular approaches to confirm the presence of the coastal isopod *Ligia exotica*, a species of Asian origin that has been introduced to coastlines around the world, in Midway Atoll and thus the PMNM. The presence of *L. exotica* in Midway Atoll is of concern as islands of this marine monument harbor endemic species of *Ligia* isopods.

Key words: Northwestern Hawaiian Islands, Oniscidea, Ligiidae, Leeward Hawaiian Islands

Introduction

Midway Atoll comprises several small coral islands and sand islets in the Northern Pacific Ocean. These low-lying islands, although located over 2,000-km from the major Hawaiian Islands (i.e., Niʻihau, Kauaʻi, Oʻahu, Molokaʻi, Maui, Lānaʻi, Kahoʻolawe, Hawaiʻi), are the remnants of an older volcanic island formed ~ 28 mya by the same stationary hotspot that led to the formation of the major Hawaiian Islands. Given its location, Midway Atoll is considered part of the Northwestern Hawaiian Islands and is thus protected both as the Midway Atoll National Wildlife Refuge and Battle of Midway National Memorial and as a part of the Papahānaumokuākea Marine National Monument (hereafter PMNM), a marine monument created in 2006 to protect the biodiversity of the remote Northwestern Hawaiian Islands. Despite its protected status, the biodiversity of Midway

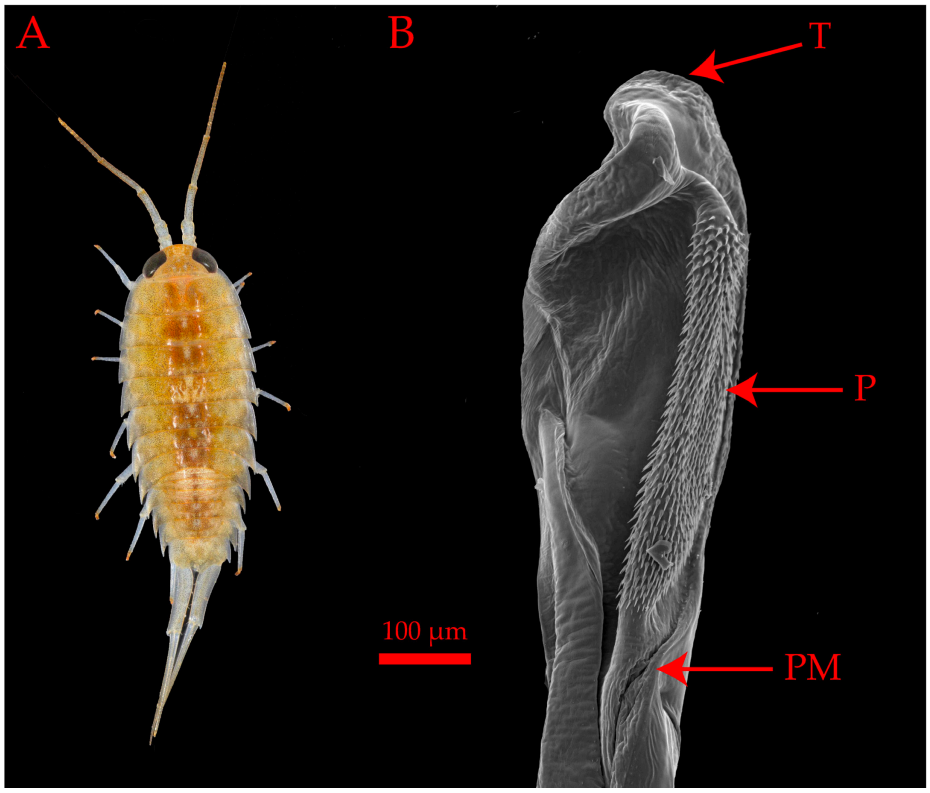

Figure 1. *Ligia exotica* and SEM photograph of the appendix masculina of individual collected in Midway Atoll. Panel A shows an edited image of *L. exotica* produced by Natalia Agudelo, Smithsonian National Museum of Natural History. Panel B shows and SEM photograph produced in this study with major characteristics shown: a rounded tip (T), a lateral pad with a large field of spines (P), and a sloping posterior margin (PM). Image of *L. exotica* in Panel A is reproduced from https://www.flickr.com/photos/serc_biodiversity/13048733914/ under a Creative Commons Attribution 2.0 license.

Atoll and the PMNM face various threats to its preservation including the presence of introduced and invasive species (Selkoe et al. 2008).

Since it was first claimed by the United States of America in 1867, Midway Atoll has served as a commercial and military shipping waypoint with this anthropogenic activity thought to have led to the introduction of several terrestrial and marine alien species (DeFelice et al. 1998). For instance, Midway Atoll's flora is comprised primarily of introduced species with these greatly outnumbering local species in richness and biomass (Starr and Martz 1999; Starr and Starr 2008). Marine introduced species are also present in the waters of Midway Atoll; however, surveys of Midway Atoll and other islands in the PMNM have recorded < 20 invasive marine fish, invertebrate, and algal species with most of these species found in artificial habitats such as harbors, rock pilings, and seawalls (See et al. 2009). Nonetheless, marine ecozones of the PMNM and Midway Atoll such as rocky intertidal and shallow coastal ecosystems appear to be at risk of the detrimental effects of introduced species (Selkoe et al. 2008). Thus, surveillance to detect the presence of previously unreported introduced species to these habitats is of value to the conservation of the PMNM.

The supralittoral isopod *Ligia exotica* Roux, 1828 (Figure 1A) is a species that has been introduced to tropical and sub-tropical coastlines around the

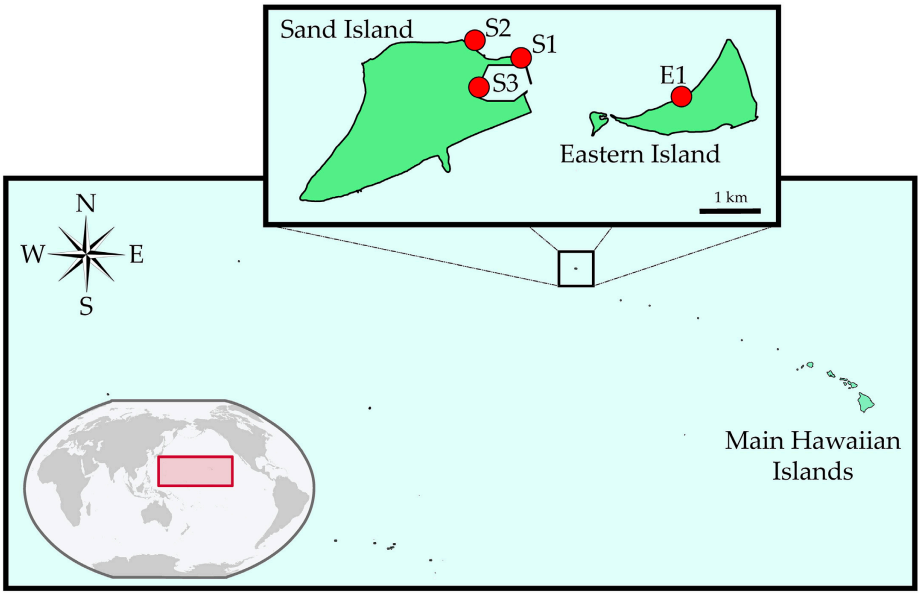

Figure 2. Localities in Midway Atoll sampled in this study. Localities and number of *Ligia* individuals collected for each location are as follows: E1 – Boat Dock, Sector 3, Eastern Is., 4 individuals; S1 – Rocky Point, Sector 31, Sand Is., 4 individuals; S2 – Cargo Pier, Sector 36, Sand Is., 6 individuals; and S3 – Harbor Boat Ramp, Sector 35, Sand Is., 14 individuals.

world and inhabits rocky intertidal habitats throughout its established range (Schmalfuss 2003; Taiti et al. 2003; Van Name 1936; Yin et al. 2013). In the Hawaiian Islands, this species is only confirmed to be present in artificial habitats such as harbors and seawalls on the islands of O'ahu and Hawai'i (DeFelice et al. 2001), with no peer-reviewed reports of its presence in Midway Atoll or the PMNM to date. The presence of *L. exotica* in the Hawaiian Islands is of concern as they may be competitors to a cryptic species complex comprised of eight species previously recognized as *L. hawaiiensis* that is endemic to the region (DeFelice et al. 2001). As these endemic *Ligia* species are known to inhabit rocky intertidal habitats in the PMNM (Taiti and Ferrara 1991; Taiti and Howarth 1996), determining whether *L. exotica* is present in Midway Atoll is of importance to the conservation and management of rocky intertidal habitats of the PMNM. In this study we use morphological and molecular approaches to identify *Ligia* specimens collected in Midway atoll to determine whether populations in the atoll correspond to *L. exotica*.

Materials and methods

Sampling

Ligia specimens were collected in four locations across Sand and Eastern islands in Midway Atoll (Figure 2). At each site, 4–14 individuals were caught by hand and field-preserved in 70% isopropanol prior to transport to The University of Tampa. Collections in Midway Atoll were carried under FWS permit PMNM-2022-001.

Morphological identification

In the laboratory, all specimens were sexed under a Leica EZ4W Stereomicroscope. Specimens were determined to be male by the presence of the appendix masculina of the second pleopod and female if absent. Male specimens were then putatively identified to species by comparing the morphology of their appendix masculina to previously published images of this structure for *L. exotica* (Yin et al. 2013, Santamaria et al. 2014) and for the *Ligia* species belonging to the cryptic species complex endemic to the Hawaiian Islands (see *L. hawaiiensis* figures in Taiti et al. 2003).

We also produced scanning electron microscope (SEM) images of the appendix masculina of the gonopod of two male specimens. To this end, two male specimens were transferred from isopropyl to 70% ethyl alcohol for 24 hours. Afterwards, we detached the left gonopod of each specimen using micro-forceps under a dissecting microscope. Once detached, gonopods were chemically fixed for at least four hours in a 3.0% glutaraldehyde and 2.0% paraformaldehyde (Karnovsky's fixative) solution with 0.2 M phosphate buffer at pH 7.4. Afterwards, specimens were dehydrated in a series of increasing concentrations of ethyl alcohol (70%, 85%, 95%, and 100%) at 15 minutes per concentration and stored in fresh 100% ethyl alcohol overnight prior. Chemical dehydration was then completed with two 20 minutes washes of hexamethyldisilane (HDMS) followed by 12 hours of air drying in a desiccation chamber. Once dried, the appendix masculina were mounted on aluminum SEM stubs with double-sided conductive tape and coated with a 3.6 nm thick layer of gold-palladium alloy in a Cressington 108 Auto Sputter Coater prior to imaging with a Jeol JSM-6010LA analytical SEM.

Molecular identifications

We used the PCRBIO Rapid Extraction PCR Kit (PCRBiosystems) following standard directions to extract total genomic DNA from pereopods for each *Ligia* individual collected in Midway. Two mitochondrial gene fragments were then amplified using previously published primers and conditions: a ~ 490-bp segment of the mitochondrial 16S rRNA gene (primers 16Sar/16Sbr; Palumbi 1996) and a ~ 495-bp segment of the 12S rDNA gene (primers crust-12Sf/crust-12Sr; Podsiadlowski and Bartolomaeus 2005). All PCR products were visualized on a 1% agarose gel stained using SYBR-Safe (Invitrogen). Positive amplicons were cleaned with Exonuclease and Shrimp Alkaline Phosphatase and cycle sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied BioSystems). Cycle sequencing products were cleaned with BigDye® XTerminator (Applied BioSystems) and run on a SeqStudio™ Genetic Analyzer (Applied BioSystems).

Sequences were assembled, checked for quality, and edited to remove primer sequences in CodonCode Aligner v10.0.2. After editing, sequences

of each gene were clustered into contigs of 100% identity prior to querying the consensus sequences against records in GenBank to establish their putative identity. GenBank searches were carried out using the BLASTn search function in May of 2022. Searches resulting in similarity scores of > 99% were considered confident species-level identifications.

Results

We collected 28 *Ligia* specimens from four locations in Midway Atoll (Figure 2). Ten of these individuals were identified as males based on the presence of the appendix masculina: one individual from Eastern Island (E1) and nine individuals from Sand Island (S1: 5 specimens; S2: 2 specimens; S3: 2 specimens). The morphology of the appendix masculina of all male specimens matched that reported for *L. exotica* (Yin et al. 2013; Santamaria et al. 2014), with SEM images of this structure showing the presence of a rounded tip (T in Figure 1B), a lateral pad (P in Figure 1B) with a large field of spines and a sloping posterior margin (PM in Figure 1B). SEM mounts and a subset of specimens have been deposited in the Invertebrate Zoology Collections of Florida Museum of Natural History in Gainesville, Florida (Catalog numbers 069961–069970).

We successfully amplified and sequenced the 16S rDNA and 12S rDNA mitochondrial genes for all 28 *Ligia* specimens collected in Midway. A single 16S rDNA haplotype was recovered from these specimens, with this haplotype being 100% identical to four previously published *L. exotica* 16S rDNA haplotypes. These haplotypes have been previously recovered from specimens collected in Toyohashi, Japan (GenBank Acc. No.: KX447741); Lutao, Taiwan (GenBank Acc. No.: KX447735); Pearl Harbor, Oʻahu (GenBank Acc. No.: KX447729); Hilo Harbor, Hawaiʻi (GenBank Acc. No.: KX447728). For the 12S rDNA, we recovered a single haplotype that was 100% identical to 12S rDNA haplotypes previously reported from Toyohashi, Japan (GenBank Acc. No.: MG676417); Pearl Harbor, Oʻahu (GenBank Acc. No.: MG676415); Hilo Harbor, Hawaiʻi (GenBank Acc. No.: MG676416). Haplotypes produced in this study were deposited in GenBank under Accession numbers ON753731 (16S rDNA) and ON753732 (12S rDNA).

Discussion

Coastal habitats in the Hawaiian Islands are home to an endemic complex of eight currently valid *Ligia* species previously recognized as a *L. hawaiiensis* (Santamaria 2019), and *L. exotica*, an introduced species of Asian origin (Hurtado et al. 2018). Endemic *Ligia* species in the Hawaiian Islands are known to occur in natural coastlines across the main Hawaiian Islands (Taiti and Howarth 1996; Taiti et al. 2003; Santamaria 2019) as well as several islands in the Papahānaumokuākea Marine National Monument (PMNM; Taiti and Howarth 1996). On the other hand, *L. exotica* is only

confirmed to be present in artificial habitats in O'ahu and Hawai'i (DeFelice et al. 2001). To date, no peer-reviewed reports confirming the presence of the introduced *L. exotica* in the PMNM exist. By applying morphological and molecular approaches, we herein confirm the presence of the introduced *L. exotica* along the coastlines of Midway Atoll, a part of the PMNM.

Inspecting the morphology of the appendix masculina of male specimens, a useful character for distinguishing amongst *Ligia* species and species complexes (Khalaji-Pirbalouty and Wägele 2010; Yin et al. 2013; Santamaria et al. 2014; Hurtado et al. 2018), we putatively identified all male *Ligia* individuals from Midway Atoll as *L. exotica*. These findings were corroborated by 16S and 12S rDNA mitochondrial gene sequences that produced identical matches to previously reported *L. exotica* sequences. Indeed, all specimens used in this study harbored a single haplotype for each gene, it being identical to a haplotype previously uncovered by Hurtado et al. (2018) from *L. exotica* specimens collected in artificial habitats in the Hawaiian Islands, and localities in Japan and Taiwan. These haplotypes were not found elsewhere in the world by Hurtado et al. (2018), despite their survey of over 40 localities in the Americas, Africa, India, and East Asia.

The sharing of the identical haplotypes for 16S and 12S rDNA between Midway Atoll, the main Hawaiian Islands, and two localities in Asia coupled with Midway's historical use suggests that *L. exotica* may have been introduced to the atoll by ship transport in the past century. Midway Atoll was claimed by the U.S. Navy in 1867 to establish a coaling station, supply depot, and emergency refuge for ships traveling between Hawai'i and Eastern Asia. In the 1900's, Midway Atoll served as Naval Air Station, submarine base, and as refueling and repair stop for military equipment travelling to and from Hawai'i to Asia. Considering its short time presence in Midway Atoll, studies of the impact of *L. exotica* on populations of endemic *Ligia* remain needed.

Ligia populations are known to harbor high levels of cryptic biodiversity, with populations often exhibiting deep genetic divergences even across small geographic scales (Taiti et al. 2003; Jung et al. 2008; Hurtado et al. 2010; Eberl et al. 2013; Santamaria et al. 2013, 2014, 2017; Greenan et al. 2018; Hurtado et al. 2018; Santamaria 2019). In the Hawaiian Islands such work led to the description of eight novel cryptic species (Santamaria 2019). Considering the remoteness of islands in the PMNM and the presence of endemic *Ligia* there, it is possible that populations of these isopods in the PMNM harbor unique lineages that are found nowhere else in the Hawaiian Islands or the world. Thus, the presence of *L. exotica* in Midway Atoll is of concern to the conservation of these isopods in the PMNM. Although the potential impact of *L. exotica* on native populations of *Ligia* in Hawaiian Islands or other regions of the world has not yet been studied, DeFelice et al. (2001) suggested competition with endemic *Ligia* in Hawaii

is likely. Given the difficulty of identification and lack of surveys in the area, the presence of *L. exotica* in the PMNM may lead to the silent replacement of endemic lineages. Thus, we recommend the survey and monitoring of localities within the PMNM to determine the distributional extent of *L. exotica* in the area as well as molecular work to determine whether these habitats harbor unique lineages of *Ligia*.

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Authors' contribution

C.A.S: conceived and designed the experiments, identified *Ligia* specimens using morphological approaches, performed molecular work, analyzed the data, contributed reagents and analytical tools, prepared figures and/or tables, authored and reviewed drafts of the paper, approved the final draft. A.J.L.: performed molecular work, analyzed molecular data, reviewed drafts of the paper, approved the final draft. L.J.A.: conceived, designed, and completed SEM imaging, contributed reagents and analytical tools, authored and reviewed drafts of the paper, approved the final draft. A.M.L.: conceived, designed, and completed SEM imaging, contributed reagents and analytical tools, authored and reviewed drafts of the paper, approved the final draft. K.W.R.: sampled *Ligia* populations in Midway, reviewed drafts of the paper, approved the final draft.

Ethics and permits

All work completed was completed in accordance with institutional and national policies and laws. Collections in Midway Atoll was undertaken under FWS permit PMNM-2022-001. Molecular and morphological work completed at the University of Tampa did not require institutional ethical permits or review. Data produced in this study has been made publicly available in GenBank.

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