DNA phylogeny of Ryukyus Leucothoidae (Crustacea: Amphipoda)

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Abstract

Commensal leucothoid amphipods collected from sponges, ascidians, and coral rubble from the Ryukyu Archipelago, Japan, were investigated using nuclear 18S ribosomal DNA sequences. Analysis of sequences from 21 species in three genera supported the current morphological species designations and the separation of the family into two clades. Additionally, a possible new generic-level clade was identified and the separation of the genera *Anamixis* and *Paranamixis* was not supported. Our results demonstrate that host specificity appears to evolve rapidly in sibling species in this family.

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Introduction

Leucothoid amphipods are commensal peracarid crustaceans, typically associated with sessile invertebrate hosts such as sponges, ascidians, or bivalve mollusks. They are also found in crevices in coral rubble, presumably associated with filter feeding organisms (*e.g.* sponges) within the rubble. Currently, there are 163 species of Leucothoidae described from coral reefs to the deep sea (White, 2011a; White and Reimer, 2012a, b, c), but it is likely many more undiscovered and undescribed species exist given the cryptic habitats and small sizes of many species in this family (Thomas and Klebba, 2006; Thomas, 2008; White, 2010; Thomas and Krapp-Schickel, 2011).

In 2012, White and Reimer described 24 new species of Leucothoidae with their host associations from

the Ryukyu Archipelago in southern Japan (2012a, b, c). Prior to this research only seven species had been reported from mainland Japan, with no reports from the Ryukyu Archipelago, and only one species had associated host information available (discussed in White and Reimer, 2012a).

Despite the progress made in species descriptions of Leucothoidae from some regions of the world, little research has been conducted on their molecular phylogeny, with only two reports in the literature. As of March 3, 2012, GenBank lists approximately 10,000 sequences for Amphipoda, with 921 of these being 18S ribosomal DNA (18S rDNA) sequences (Benson *et al.*, 2005). Molecular sequence data are scarce for Leucothoidae, comprising only 94 of the total amphipod sequences. Of these 94 sequences, 30 are 18S rDNA sequences for 10 species in two genera from Florida, Belize, and Indonesia (*Anamixis* and *Leucothoe*) (White, 2011b). The remaining 64 are partial COI sequences for three species (*Leucothoe ashleyae* Thomas and Klebba, 2006; *Leucothoe kensleyi* Thomas and Klebba, 2006, and one sequence for *Anamixis vanga* Thomas, 1997) from Florida and Belize (Richards *et al.*, 2007; White, 2011b).

White (2011b) utilized specimens from Florida, Belize, and Indonesia, and documented the utility of 18S rDNA as a species-level molecular marker for the Leucothoidae, while supporting the separation of the family into two large clades. The 'leucothoid' clade was shown to contain the genera *Leucothoe* Leach, 1814 and *Paraleucothoe* Stebbing, 1899, while the 'anamixid clade' contained *Anamixis* Stebbing, 1897, *Nepanamixis* Thomas, 1997, and *Paranamixis* Schellenberg, 1938. The genera *Anamixis* and *Paranamixis* are currently distinguished from each other based on the presence of gnathopod 1 and a cleft maxilliped inner plate in *Anamixis* species. However, in addition to the molecular results of White (2011b), recent morphological analyses

also do not support the separation of these two genera based on these characters (White, 2010). Thus, it is clear further phylogenetic analyses are needed to clarify our evolutionary understanding of this unique amphipod family.

In this study, we obtained and phylogenetically analyzed sequences from 21 species of Leucothoidae from the Ryukyu Archipelago. We examine the phylogenetic relationships within the Leucothoidae, and also discuss the potential evolutionary significance of host relationships.

Material and methods

Leucothoid amphipods for this study were collected from marine sponges, ascidians, and coral rubble from throughout the Ryukyu Archipelago, Japan, as reported in White and Reimer (2012a, b, c). These collections included detailed host association and location data (GPS coordinates, depth etc.), as such associations were previously unknown for most of the described species in the Leucothoidae. Specimens, station data, and GenBank accession numbers are listed in Table S1.

Genomic DNA was extracted from the urosomes of representative individual ethanol-preserved animals after morphological examination. In total, DNA from one to six specimens each of 24 morphologically determined Leucothoidae species was extracted. Voucher specimens are deposited in The University of the Ryukyus Museum (Fujikan), with the prefix RUMF for museum numbers. Extractions were performed using a guanidine extraction protocol (Sinniger *et al.*, 2010). Partial 18S rDNA sequences were amplified (697–851 base pairs including the V4 hypervariable region) using polymerase chain reaction (PCR) (Saiki *et al.*, 1988). PCR protocols were used following Spears *et al.* (2005). The following peracarid-specific primers were used for 18S rDNA: 329 (5'-TAATGATCCTTC-CGCAGGTT-3') and Hi- (5'-GTGCATGGCCGTTCTTAG-TTG-3') (Spears *et al.*, 2005). PCR products were purified using shrimp alkaline phosphatase (SAP) and gel purified with a Qiagen gel extraction kit if non-specific PCR products were apparent after gel electrophoresis.

Sequencing was done by Fasmac (Yokohama, Japan). Sequences were edited, combined, and initially aligned in Bioedit 7.0.5.3 (Hall, 1999) and the alignments were further edited by eye using SeaView 4.3.3 (Guoy *et al.*, 2010) and Se-Al v.2.01 (Rambaut, 2002).

Three different alignments were constructed in order to examine the influence of hypervariable regions on resulting phylogenetic trees, as some studies suggest deleting these regions (*e.g.* Kim and Abele, 1990; Spears *et al.*, 1992; Carmean *et al.*, 1992; Pashley *et al.*, 1993; Campbell *et al.*, 1994; Friedrich and Tautz, 1995; Kim *et al.*, 1996; Chalwatzis *et al.*, 1996; Giribet *et al.*, 1996; Friedrich and Tautz, 1997; Spears and Abele, 2000) while others propose retaining them (Crease and Taylor, 1998; Hwang *et al.*, 2000; White, 2011b). The first alignment contained all variable regions including the V4 hypervariable region ('long' alignment). The second alignment retained all variable regions except the V4 hypervariable region ('medium'). The final alignment retained only conserved regions ('short'). All three generated alignments contained sequences for 40 taxa (including two outgroup taxa). Representatives of the amphipod family Liljeborgiidae Stebbing, 1899 were chosen as outgroup taxa based on the loss of molar structure and rudimentary charpochelation of gnathopod 1 (more primitive than the fully carpochele gnathopod 1 in Leucothoidae) in the Liljeborgiidae (Barnard, 1974). The long alignment dataset contained 1205 sites, the medium alignment 751 sites, and the short alignment 426 sites. All three alignments are available from the corresponding author and at the homepage <http://web.me.com/miseryukyu/>.

All three alignments resulted in trees with identical topologies based on preliminary maximum likelihood (ML) and neighbor-joining (NJ) analyses, with the short and medium trees showing generally lower bootstrap support values at most nodes. For these reasons, we therefore utilized the long alignment for all subsequent analyses. Uncorrected measures of percent-sequence divergence were obtained using Mesquite V2.74 (Maddison and Maddison, 2011). The levels of divergence used were 0–0.023 between different populations of a species, 0.090–0.295 between species within a genus, 0.260–0.349 between different genera within the Leucothoidae, and greater than 0.306 between leucothoid genera and the outgroup, Liljeborgiidae (after White, 2011b).

Twenty one ingroup taxa were included in the analyses due to lack of specimens or difficulty of obtaining molecular data from the other four species collected in the Ryukyus. PhyML (Guindon and Gascuel, 2003) was used for ML analyses using an input tree generated by BIONJ (Neighbor Joining) with the Generalized Time Reversible (GTR) model, incorporating invariable sites and a discrete gamma distribution with eight

substitution rate categories. Base frequencies were estimated from the dataset. Support for the maximum likelihood tree was measured using the bootstrap method with 1,000 replicates in PhyML.

NJ analysis was accomplished in SeaView 4.3.3 using Jukes Cantor (JC) distance methods. Support was measured using the bootstrap method with 1,000 replicates in SeaView 4.3.3.

Bayesian phylogenetic inference analysis (B) was accomplished using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) under GTR + I + Γ . One cold and three heated Markov chains Monte Carlo (MCMC) with default-chain temperatures were run for 5 million generations, sampling log-likelihoods. Trees at 1000-generation intervals were saved (5000 InLs and trees saved during MCMC). The likelihood plot suggested that MCMC reached a stationary phase after the first 1,000,000 generations, and thus the remaining 4,000 trees were used to obtain clade probabilities and branch-length estimates.

Results

Maximum likelihood (ML) analysis of partial 18S rDNA sequences of all available Ryukyus species' sequences resulted in a tree with generally high bootstrap support values for most nodes. Neighbor Joining (NJ) analysis resulted in an identical distance tree with high bootstrap support values. Bayesian analysis (B) resulted in a phylogenetic tree identical to the ML tree with high posterior probabilities.

The resulting 18S rDNA tree depicted two major clades (Fig. 1), one composed of *Anamixis* and *Paranamixis* species (ML = 100%, NJ = 100%, B = 1.00) and the other containing *Leucothoe* species (ML = 92%, NJ = 72%, B = 1.00). The anamixid clade did not have prominent subclades, grouping *Anamixis sentan* White and Reimer, 2012c and *Paranamixis thomasi* White and Reimer, 2012a into one clade. The 18S rDNA sequence data also supported the connection of leucomorphs and anamorphs of *P. thomasi* (ML = 69%, NJ = 99%, B = 1.00). The connection of anamorphs and leucomorphs was not possible for *A. sentan* as only leucomorphs were sequenced. The leucothoid clade depicted two distinct subclades within the genus *Leucothoe*. A new generic-level subclade grouped morphologically similar species (*Leucothoe toribe* White and Reimer, 2012b, *Leucothoe obuchii* White and Reimer, 2012a, *Leucothoe enko* White and Reimer, 2012c, and *Leucothoe kebukai* White and Reimer,

2012c) together (ML = 100%, NJ = 100%, B = 1.00). Within the larger *Leucothoe* subclade (ML = 86%, NJ = 98%, B = 1.00), multiple specimens of each species formed subclades.

Four morphologically similar species (*Leucothoe vulgaris* White and Reimer, 2012a, *Leucothoe amamiensis* White and Reimer, 2012a, *Leucothoe akuma* White and Reimer, 2012c, and *Leucothoe akaisen* White and Reimer, 2012c) formed a subclade separate from all other *Leucothoe* species within the leucothoid subclade (ML = 92%, NJ = 100%, B = 1.00). Five specimens of *Leucothoe elegans* White and Reimer, 2012a formed a subclade with *Leucothoe hashi* White and Reimer, 2012b, *Leucothoe zanpa* White and Reimer, 2012b and *Leucothoe lecrovae* White and Reimer, 2012b, depicting minor population differences between Okinawa-jima, Amami-oshima, and Hiroshima populations of *L. elegans* (ML = 90%, NJ = 67%, B = 1.00). The final subclade in the *Leucothoe* subclade was comprised of morphologically similar species (*Leucothoe trulla* White and Reimer, 2012a, *Leucothoe bise* White and Reimer, 2012b, *Leucothoe nurunuru* White and Reimer, 2012b, *Leucothoe daisukei* White and Reimer, 2012b, and *Leucothoe akaoni* White and Reimer, 2012b) (ML = 68%, NJ = <50%, B = 1.00).

Discussion

After many unsuccessful attempts by the first author at amplifying and sequencing multiple molecular markers (cytochrome oxidase subunit 1, 12S ribosomal DNA, 16S ribosomal DNA) for Leucothoidae, this study focused on nuclear 18S rDNA. Several studies have found that the hypervariable regions of 18S are taxon specific and useful as a species-level marker (Hwang *et al.*, 2000; White, 2011b) and several amphipod molecular phylogenies have been based at least partially on 18S rDNA, including gammaridean Lake Baikal amphipods (Sherbakov *et al.*, 1999; Macdonald *et al.*, 2005), subterranean gammaridean amphipods (Englisch and Koenemann, 2001), *Gammarus* species (Hou *et al.*, 2007), and caprellid and corophioid taxa (Ito *et al.*, 2008).

While all three alignments in this study grouped the taxa into the same clades, the long alignment, containing the V4 hypervariable region, showed the strongest bootstrap support for species, suggesting that full length 18S rDNA sequences are an excellent marker at the species-level and potentially useful for

Fig. 1. Maximum likelihood (ML) phylogenetic tree of 'long' partial 18S ribosomal DNA gene sequences from replicates of 21 Leucothoid taxa collected from depths of 1-33 meters, rooted with an outgroup of two Liljeborgiidae species. Numbers at branches represent ML/Neighbor joining (NJ) bootstrap values. Thick lines represent Bayesian posterior probabilities >0.95.

population studies. Removing the variable regions in the shorter 18S rDNA alignments resulted in the loss of some resolution at the species level, but still supported the major clades with lower support and shorter distances.

Our 18S rDNA sequence data strongly support the separation of the anamixid and leucothoid clades, and grouped morphologically similar species into highly supported subclades within the genus *Leucothoe*. The unexpected new generic-level clade ('new' clade hereafter) groups *Leucothoe toribe*, *L. obuchii*, *L. kebukai*, and *L. enko* together. These species all have a one-articulate palp of maxilla 1, gnathopod 1 basis widened or inflated, gnathopod 1 propodus curved or proximally inflated (not straight), and gnathopod 1 dactylus reaching less than $0.2 \times$ propodus length. Further investigation will clarify whether these 'new' clade species belong in a new genus or if they belong in the genus *Paraleucothoe*, whose members share the diagnostic characters listed above in addition to having moderate sexual dimorphism and an elongate maxilliped inner plate. If this clade is indeed *Paraleucothoe*, the diagnostic morphological characters of *Paraleucothoe* will need to be revised. However, *Leucothoe togatta* White and Reimer, 2012b and *L. ouraensis* White and Reimer, 2012b do not share the morphological characters of this new generic level clade, suggesting that they may be placed differently in a tree containing sequences from more species; this is further supported by the relatively weak support for these species as members of the new clade (ML = 89%, NJ = 78%, B = 0.92) and their basal position in the grouping.

The three large subclades in the genus *Leucothoe* group morphologically similar species. One subclade contains *Leucothoe vulgaris*, *L. amamiensis*, *L. akuma* and *L. akaisen*, which share a rounded anterodistal head margin, gnathopod 1 dactylus reaching greater than $0.2 \times$ propodus length, and wide pereopod 5-7 bases. Additionally, these four species also all have unique color patterns. *L. vulgaris* has a red 'saddle', a red dot on coxa 4, and yellow antennae; *L. amamiensis* has a thick pink striped pattern; *L. akuma* has a thin pink striped pattern; and *L. akaisen* has a yellow 'saddle', a red dot on coxa 4, and red striped antennae.

Another subclade contains *Leucothoe elegans*, *L. hashi*, *L. zanpa*, and *L. lecrovae*, and these species also share some unique morphological characters, including a thin gnathopod 1 carpus and propodus and gnathopod 1 dactylus reaching less than $0.3 \times$ propodus length. Finally, the subclade of *L. trulla*, *L. bise*, *L. nurunuru*, *L. daisukei*, and *L. akaoni* share gnathopod

1 dactylus reaching greater than $0.2 \times$ propodus length and gnathopod 2 propodus mediofacial setal row displaced to midline.

The 18S rDNA tree depicts *P. thomasi* and *A. sentan* as sister species and; therefore, does not support the separation of the genera *Anamixis* and *Paranamixis*. Examination of both morphological and molecular data of all species in the anamixid clade is necessary to determine the status of these genera. The connection of a *P. thomasi* anamorph and leucomorphs is also confirmed by DNA sequences with levels of divergence between 0 and 0.0175. It is apparent that these two genera will need further future revision, but we do not formally merge them here due to lack of available data for more species from each genus. Once such data are available, we feel such a revision will be necessary.

As shown in White (2011b), molecular identification and analyses of leucothoid taxa supports traditional morphological species definitions. The available molecular data clusters morphologically similar species together, suggesting that the diagnostic morphological characters are not due to convergent adaptation to similar hosts, and do in fact reflect true evolutionary relationships between leucothoid species. However, higher level clades and relationships within Leucothoidae need to be further investigated using both morphological data and additional molecular markers to clarify species groupings within the different genera. Taxonomic revisions are not proposed here due to the lack of morphological and molecular data available for more of the 163 species in Leucothoidae. Further analyses including more species from more regions of the world will help us clarify our understanding of the evolutionary relationships within this family, and may lead to the formal taxonomic revisions that appear needed from this preliminary study.

Finally, from the phylogenetic analyses it is apparent that several closely related species inhabit different hosts, suggesting that host specificity can evolve and change rapidly in sister species. Sponge and ascidian habitation appears to have evolved more than once in the Leucothoidae, as species collected from sponges or ascidians occur in nearly every clade on the tree.

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On-line supplementary material

S1. Leucothoid specimens (from White and Reimer, 2012a, b, c) examined in this study and corresponding GenBank Accession Numbers.

Table S1. Leucothoid specimens (from White and Reimer, 2012a, b, c) examined in this study and corresponding GenBank Accession Numbers. Abbreviations: NA=not applicable, KNW = Kristine Nicolle White, NSW = Nathan Stuart White, DU = Daisuke Ueno; ^a Negative values represent West longitude values and South latitudes, while positive values represent East longitude values and North latitudes.

Specimen number	Taxonomic identification	Life stage	Collection locality	Host	Latitude ^a	Longitude ^a	Date collected	Depth (m)	Collected by	18S Accession number	RUMF museum number
A2	<i>Leucothoe akaoni</i>	NA	Manza, Okinawa Island	Niphatidae of van Soest, 1980 (sponge)	26°30'15"	127°50'39"	2010.9.27	12–14	KNW	JX145088	ZC-01925
A_3	<i>Leucothoe akaoni</i>	NA	Zanpa Cape, Okinawa Island	Tetillidae of Sollas, 1886 (sponge)	26°26'27"	127°43'03"	2011.4.3	30–33	KNW, NSW	JX145089	ZC-01926
A_4	<i>Leucothoe akaoni</i>	NA	Manza, Okinawa Island	Niphatidae (sponge)	26°30'15"	127°50'39"	2010.9.27	12–14	KNW	JX145090	ZC-01927
AB_2	<i>Leucothoe daisukei</i>	NA	Zanpa Cape, Okinawa Island	Tetillidae (sponge)	26°26'27"	127°43'03"	2011.2.26	30	DU	JX145091	ZC-01928
AC1	<i>Leucothoe toribe</i>	NA	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2011.1.28	3–8	KNW, NSW	JX145105	ZC-01929
AC2	<i>Leucothoe toribe</i>	NA	Manza, Okinawa Island	Coral rubble	26°30'15"	127°50'39"	2011.2.8	10–23	KNW, NSW	JX145102	ZC-01930
AD2	<i>Leucothoe akuma</i>	NA	Toguchi Beach, Okinawa Island	Coral rubble	26°21'47"	127°44'12"	2011.7.10	1–2	KNW, NSW	JX145111	ZC-01936
AE1	<i>Leucothoe elegans</i>	NA	Takehara, Hiroshima	Sponge	34°19'28"	132°54'38"	2010.12.11	15	DU	JX145095	ZC-01931
AE_2	<i>Leucothoe elegans</i>	NA	Takehara, Hiroshima	Sponge	34°19'28"	132°54'38"	2010.12.11	15	DU	JX145096	ZC-01932
B_1	<i>Leucothoe vulgaris</i>	NA	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.10.1	6–9	KNW, NSW	JX145109	ZC-01933
C_2	<i>Leucothoe vulgaris</i>	NA	Route 10 Bridge, Okinawa Island	? <i>Clathria (Thalysias) reinwardti</i> Vosmaer, 1889 (sponge)	26°19'56"	127°55'23"	2010.8.21	1	KNW	JX145110	ZC-01934
C3	<i>Leucothoe vulgaris</i>	NA	Nago Bay, Okinawa Island	Ascidians	26°34'36"	127°58'29"	2011.2.20	?	DU	JX145112	ZC-01935
D_1	<i>Paranaimixis thomasi</i>	Anamorph	Toguchi Beach, Okinawa Island	<i>Pyura</i> of Molina, 1782 (ascidian)	26°21'47"	127°44'12"	2010.10.20	1–2	KNW, NSW	JX145120	ZC-01937
D_7	<i>Paranaimixis thomasi</i>	Leucomorph	Toguchi Beach, Okinawa Island	Coral rubble	26°21'47"	127°44'12"	2011.8.3	1–3	KNW, NSW	JX145119	ZC-01938
D8	<i>Paranaimixis thomasi</i>	Leucomorph	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.12.2	3–10	KNW, NSW	JX145121	ZC-01939
F3	<i>Anaimixis sentan</i>	Leucomorph	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.11.13	3–10	KNW, NSW	JX145117	ZC-01940
F_5	<i>Anaimixis sentan</i>	Leucomorph	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.11.22	3–10	KNW, NSW	JX145118	ZC-01941
I_2	<i>Leucothoe obuchii</i>	NA	Odo, Okinawa Island	Coral rubble	26°05'08"	127°42'30"	2010.12.11	10–16	KNW, NSW	JX145103	ZC-01942
I_3	<i>Leucothoe obuchii</i>	NA	Odo, Okinawa Island	Coral rubble	26°05'08"	127°42'30"	2010.12.11	10–16	KNW, NSW	JX145104	ZC-01943
J_1	<i>Leucothoe enko</i>	NA	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.10.1	6–9	KNW, NSW	JX145106	ZC-01944
K_1	<i>Leucothoe bise</i>	NA	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.12.2	2–5	KNW, NSW	JX145082	ZC-01945
K_5	<i>Leucothoe bise</i>	NA	Toguchi Beach, Okinawa Island	Coral rubble	26°21'47"	127°44'12"	2011.7.10	1–3	KNW, NSW	JX145083	ZC-01946
M_3	<i>Leucothoe</i>	NA	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2011.2.17	3–12	KNW, NSW	JX145101	ZC-01947

Mm1	<i>Leucothoe elegans</i>	NA	Island	Shioya Bay, Okinawa Island	? <i>Pericharax</i> of Poléjaeff, 1883 (sponge)	26°40.060'	128°06.599'	2011.7.7	10–12	NSW	JX145097	ZC-01948
Mm2	<i>Leucothoe elegans</i>	NA	Shioya Bay, Okinawa Island	Shioya Bay, Okinawa Island	<i>Mycale</i> of Gray, 1867 (sponge)	26°40'03"	128°06'35"	2011.7.7	10–12	NSW	JX145098	ZC-01949
Mm3	<i>Leucothoe elegans</i>	NA	Isso, Yakushima Island	Isso, Yakushima Island	<i>Ciocalypta</i> of Bowerbank, 1862 (sponge)	30°27'29"	130°29'22"	2011.5.28	20	KNW	JX145099	ZC-01950
O2	<i>Liljeborgia</i> sp.	NA	San, Tokunoshima Island	San, Tokunoshima Island	Coral rubble	27°52'12"	128°58'12"	2010.9.21	6–12	KNW, NSW	JX145078	ZC-01951
O_3	<i>Liljeborgia</i> sp.	NA	Cook Channel back reef, Moorea	Cook Channel back reef, Moorea	Coral rubble	-17°25'55"	-149°49'29"	2010.11.9	1	KNW	JX145079	ZC-01952
Oo_1	<i>Leucothoe anamiensis</i>	NA	Nominoura Oku, Kakeroma Island	Nominoura Oku, Kakeroma Island	<i>Pyura microcosmus</i> (Savigny, 1816) (ascidian)	28°07'05"	129°17'34"	2011.3.20	15	KNW	JX145085	ZC-01953
Oo2	<i>Leucothoe anamiensis</i>	NA	Nominoura Oku, Kakeroma Island	Nominoura Oku, Kakeroma Island	<i>Pyura microcosmus</i> (Savigny, 1816) (ascidian)	28°07'05"	129°17'34"	2011.3.20	15	KNW	JX145086	ZC-01954
Oo3	<i>Leucothoe anamiensis</i>	NA	Shirahama Beach, Anami-oshima Island	Shirahama Beach, Anami-oshima Island	<i>Rhopalaea circula</i> Monniot and Monniot, 2001 (ascidian)	28°11'60"	129°16'21"	2011.3.18	10	KNW	JX145087	ZC-01955
Pp1	<i>Leucothoe imilla</i>	NA	Inoda Beach, Ishigaki-jima Island	Inoda Beach, Ishigaki-jima Island	Coral rubble	24°27'46"	124°15'13"	2011.4.20	3	KNW	JX145080	ZC-01956
Qq_1	<i>Leucothoe kebukai</i>	NA	Toguchi Beach, Okinawa Island	Toguchi Beach, Okinawa Island	Coral rubble	26°21'47"	127°44'12"	2011.7.10	1–3	KNW, NSW	JX145107	ZC-01957
Ss_2	<i>Leucothoe lecrovae</i>	NA	Kuse, Kakeroma Island	Kuse, Kakeroma Island	<i>Rhabdastrella</i> of Thiele, 1903 (sponge)	28°06'05"	129°21'12"	2011.3.19	10	KNW	JX145081	ZC-01958
Tt1	<i>Leucothoe bise</i>	NA	Sunabe Seawall, Okinawa Island	Sunabe Seawall, Okinawa Island	Coral rubble	26°19'25"	127°44'43"	2010.7.4	5–8	KNW, NSW	JX145084	ZC-01959
Uu2_2	<i>Leucothoe zanpa</i>	NA	Zanpa Cape, Okinawa Island	Zanpa Cape, Okinawa Island	Tetillidae of Sollas, 1886 (sponge)	26°26'27"	127°43'03"	2011.4.3	30–33	KNW, NSW	JX145100	ZC-01966
Vv2	<i>Leucothoe akaisen</i>	NA	Isso, Yakushima Island	Isso, Yakushima Island	Coral rubble	30°27'29"	130°29'22"	2011.5.28	20	KNW, NSW	JX145114	ZC-01967
Vv3	<i>Leucothoe akaisen</i>	NA	Toguchi Beach, Okinawa Island	Toguchi Beach, Okinawa Island	Coral rubble	26°21'47"	127°44'12"	2011.9.27	1–3	NSW	JX145113	ZC-01960
Ww_1	<i>Leucothoe nurunuru</i>	NA	channel between Iriomote-jima and Hatoma-jima Islands	channel between Iriomote-jima and Hatoma-jima Islands	Ietrochotidae of Dendy, 1922 (sponge)	24°26'34"	123°49'18"	2011.4.22	10	KNW, NSW	JX145092	ZC-01961
Ww2	<i>Leucothoe nurunuru</i>	NA	channel between Iriomote-jima and Hatoma-jima Islands	channel between Iriomote-jima and Hatoma-jima Islands	Ietrochotidae (sponge)	24°26'34"	123°49'18"	2011.4.22	10	KNW, NSW	JX145093	ZC-01962
Ww3	<i>Leucothoe nurunuru</i>	NA	channel between Iriomote-jima and Hatoma-jima Islands	channel between Iriomote-jima and Hatoma-jima Islands	Ietrochotidae (sponge)	24°26'34"	123°49'18"	2011.4.22	10	KNW, NSW	JX145094	ZC-01963
Xx1	<i>Leucothoe ouraensis</i>	NA	Kita-nakase, Oura Bay, Okinawa Island	Kita-nakase, Oura Bay, Okinawa Island	Coral rubble	26°32'40"	128°03'36"	2011.3.3	10	KNW, NSW	JX145108	ZC-01968
Zz_1	<i>Leucothoe togatta</i>	NA	Kurio, Yakushima Island	Kurio, Yakushima Island	Coral rubble	30°16'00"	130°24'55"	2011.5.26	7–10	KNW, NSW	JX145115	ZC-01964
Zz_2	<i>Leucothoe togatta</i>	NA	Isso, Yakushima Island	Isso, Yakushima Island	Coral rubble	30°27'29"	130°29'22"	2011.5.28	20	KNW, NSW	JX145116	ZC-01965