沖縄島から得られた日本初記録のナンヨウテナガエビ (新称) (甲殻亜門: 十脚目: テナガエビ科)

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First Japanese record of *Macrobrachium ustulatum* (Crustacea: Decapoda: Palaemonidae) from Okinawa-jima Island, Japan

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Abstract. This paper reports the finding of one of the Palaemonidae freshwater prawns, *Macrobrachium ustulatum*, on Okinawa-jima Island, Ryukyu Archipelago, Japan. This represents the first record of this species in this country and also the northernmost distributional record of the species. *Macrobrachium ustulatum* redescribed by Castelin et al. (2017) is a cryptic species of *M. australe*. However, there was uncertainty in the assignation of two nomina to Castelin's et al. (2017) molecularly identified two species. Moreover, among females, it is difficult to recognize clear morphological differences between *M. ustulatum* and *M. australe*. Therefore, we examine their validity of the taxonomic identification based on DNA sequences using the mitochondrial 16S rRNA gene and the nuclear 28S rDNA gene as well as morphological analyses. Consequently, these results supported the identification of the previous study.

Introduction

Freshwater prawns of the genus *Macrobrachium* Bate, 1864 (Crustacea: Decapoda: Palaemonidae) are mainly distributed throughout temperate to tropical freshwater and brackish water environments around the world. The genus includes about 250 species and is one of the most diverse genera in the family Palaemonidae (De Grave & Fransen 2011). This genus still includes many undescribed species (Chace & Bruce 1993; Short 2004). For example, Liu et al. (2007) suggested that there are two unknown species from the Ryukyu Archipelago, Japan, via research using mitochondrial DNA. Furthermore, another three unknown *Macrobrachium* species have been collected from the Ryukyu Archipelago (Fuke, unpublished data). The actual state of the biodiversity of this genus has not been revealed yet in the Ryukyu Archipelago.

Macrobrachium australe (Guérin-Méneville, 1838) is widely distributed throughout the Indo-Pacific region (Holthuis 1950). In Japan, it has been recorded from the south of Kyushu (Suzuki et al. 1993) and was also recently recorded in the south of Kanagawa Prefecture (Imai et al. 2008; 2015; Imai & Oonuki 2013a; 2013b; Kitano & Terada 2015; Maruyama 2017; 2018). Due to its broad distribution, *M. australe* has been considered to include seven junior synonyms in various regions (De Grave & Fransen 2011). Recently, Castelin et al. (2017) conducted molecular and morphological studies on seven junior synonyms of *M. australe* and resurrected one of the junior synonyms, "*ustulatus*" described by Nobili (1899). Six morphological characters, the proportions of the pereiopods lengths and joints of the male second pereiopod, the range of the velvety setae of the male second pereiopod, the shape of the epistome lobe and the armature of the fourth thoracic sternite, distinguished *M. ustulatum* from *M. australe* (Castelin et al. 2017).

In the course of our study on 18 individuals of *Macrobrachium* species from the Ryukyu Archipelago, some specimens collected from Okinawa Island were molecularly identified as what Castelin et al. (2017) referred to as *M. ustulatum* as well as *M. australe*. Here we examine the validity of their taxonomic identification.

Materials and Methods

A total of 18 individuals of *Macrobrachium* species were collected from July 2015 to September 2017 in the rivers in Wakayama Prefecture, Kuchinoerabu-jima Island in Kagoshima Prefecture, and Okinawa-jima Island and Ishigaki-jima Island in Okinawa Prefecture. The samples were brought back to the laboratory in a fresh or frozen state. About 0.05 g of muscle tissue was excised for DNA analyses from

Table. 1. Species of *Macrobrachium* and outgroup used for phylogenetic analyses in this study. * Specimens from Two locations are mixed.
表 1. 本研究で用いたテナガエビ類と外群の標本データ . * 2 つの産地からの標本が混在している .

Species 種名	Specimen 標本番号	Sex 性別	CL (mm) 頭胸甲長	GenBank accession number アクセッション番号 16S rDNA	28S rDNA	Locality 産地	Data 採集日 (yyyy/mm/dd)
M. ustulatum ナンヨウテナガエビ	RUMF-ZC-4626	F	15.26	LC316199	LC316214	Nishiyabu River, Nago, Okinawa-jima Is. 沖縄島名護市西屋部川	2015/7/18
M. australe ザラテテナガエビ	RUMF-ZC-4625	F	15.76	LC316198	LC316213	Nishiyabu River, Nago, Okinawa-jima Is. 沖縄島名護市西屋部川	2015/7/18
M. australe ザラテテナガエビ	RUMF-ZC-4639	M	26.29	LC316200	LC316215	Nishiyabu River, Nago, Okinawa-jima Is. 沖縄島名護市西屋部川	2015/10/2
M. australe ザラテテナガエビ	RUMF-ZC-4643			LC316201	LC316216	Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2015/10/27
M. australe ザラテテナガエビ	RUMF-ZC-4645	M	15.43	LC316192	LC316211	Arakawa, Ishigaki, Ishigaki-jima Is. 石垣島石垣市新川	2015/11/3
M. australe ザラテテナガエビ	RUMF-ZC-4645	F	11.17	LC316193		Arakawa, Ishigaki, Ishigaki-jima Is. 石垣島石垣市新川	2015/11/3
M. australe ザラテテナガエビ	RUMF-ZC-4654	M	15.87	LC316194	LC316212	Fukai, Ishigaki, Ishigaki-jima Is. 石垣島石垣市桴海	2015/12/3
M. australe ザラテテナガエビ	RUMF-ZC-4654	F	10.64	LC316195		Fukai, Ishigaki, Ishigaki-jima Is. 石垣島石垣市桴海	2015/12/3
M. australe ザラテテナガエビ	RUMF-ZC-4622	M	13.93	LC316191	LC316120	Kabira, Ishigaki-jima Is. 石垣島石垣市川平	2013/9/9
M. australe ザラテテナガエビ	RUMF-ZC-4661 (1)	M	18.18	LC316202		Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2016/5/19
M. australe ザラテテナガエビ	RUMF-ZC-4661 (2)	M	15.39	LC316203		Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2016/5/19
M. australe ザラテテナガエビ	RUMF-ZC-4661 (3)	M	16.86	LC316204		Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2016/5/19
M. australe ザラテテナガエビ	RUMF-ZC-4661 (4)	F	12.14	LC316205		Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2016/5/19
M. australe ザラテテナガエビ	RUMF-ZC-4661 (5)	F	12.46	LC316206		Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2016/5/19
M. australe ザラテテナガエビ	RUMF-ZC-4661 (6)		10.12	LC316207		Ohyama, Ginowan, Okinawa-jima Is. 沖縄島宜野湾市大山	2016/5/19
M. australe ザラテテナガエビ	RUMF-ZC-4676	F		LC316196		Kuchinoerabu-jima Is. 口永良部島	2016/8/23
M. australe ザラテテナガエビ	RUMF-ZC-4676	F		LC316197		Kuchinoerabu-jima Is. 口永良部島	2016/8/23
M. australe ザラテテナガエビ	RUMF-ZC-5019		5.04	LC366024		Kushimoto or Susami*, Wakayama Pref. 和歌山県串本町 , すさみ町 *	2017/9/24
M. lar コンジンテナガエビ	RUMF-ZC-4624	F	29.64	LC316208	LC316217	Nishiyabu River, Nago, Okinawa-jima Is. 沖縄島名護市西屋部川	2015/7/18
Palaemon debilis スネナガエビ	RUMF-ZC-4658			LC316209	LC316218	Urauchi River, Taketomi, Iriomote-jima Is. 西表島竹富町浦内川	2016/1/27

the abdomen and preserved in 500 µl of TNES-8M urea buffer (Asahida et al. 1996) until DNA extraction. Total DNA was extracted from each sample by proteinase K digestion at 38°C overnight, followed by standard phenol–chloroform and diethyl–ether methods (Imai et al. 2004). Specimens were preserved in 70% ethanol and registered and deposited at Ryukyu University Museum, Fujyukan (RUMF) (Table 1). Measurements of carapace length and each segment of the second pereiopod were performed with digital calipers (CD67-S20PM; Mitutoyo) after preservation. 10 randomly selected eggs were measured using ImageJ 1.50i (US National Institutes of Health, Bethesda, Maryland, USA; https://imagej.nih.gov/ij/).

A fragment of the mitochondrial 16S rRNA gene and of the nuclear 28S rDNA gene were amplified using PCR using the following primers: 16Sar-L (5′-CGCCTGTTTATCAAAAACAT-3′) and 16S-R (5′-GGTTTGAACTCAGATCATGT-3′) (Palumbi et al. 2002) for the 16S rRNA; C1 (5′-ACCCGCTGAATTTAAGCAT-3′) and D2

Fig. 1. *Macrobrachium ustulatum* from Okinawa-jima Island, Japan (RUMF-ZC-4626).
図 1. 沖縄島から得られたナンヨウテナガエビ (新称) (RUMF-ZC-4626).

(5′-TGGTCCGTGTTTCAAGAC-3′) (Hassouna et al. 1984) for the 28S rDNA. The 16S rRNA region was amplified in a 25 µl reaction mixture volume containing 12.5 µl of EmeraldAmp® PCR Master Mix (Takara Bio), 0.25 µl of each 25 µM primer, 1 µl of template DNA, and 11 µl of dH_2O. PCR amplification proceeded on a thermal cycler GeneAmp® PCR System 9700 (Applied Biosystems) or SimpliAmp® Thermal Cycler (Applied Biosystems). The amplification for the 16S rRNA region involved initial denaturation (94°C, 3 min); 30 cycles of denaturation (98°C, 15 s), annealing (52°C, 45 s), and extension (72°C, 60 s); and a single final extension (72°C, 7 min). The 28S rDNA region was amplified in a 25 µl reaction mixture volume containing 12.5 µl of EmeraldAmp® PCR Master Mix (Takara Bio), 0.5 µl of each 25 µM primer, 1 µl of template DNA, and 10.5 µl of dH_2O. The amplification for the 28S rDNA region involved initial denaturation (94°C, 5 min); 30 cycles of denaturation (94°C, 60 s), annealing (52°C, 60 s), and extension (72°C, 60 s); and a single final extension (72°C, 5 min). We outsourced the sequencing work of the amplified PCR product using 3730xl DNA Analyzer (Applied Biosystems) to Macrogen Japan. Sequences were deposited in the DNA Data Bank of Japan (DDBJ) (Table 1). In the phylogenetic analyses, we included the sequences of "*M. australe*" and "*M. ustulatum*" used in Castelin et al. (2017) with a set of 16S rRNA and 28S rDNA that registered in GenBank to be compared with our samples. We also used two samples, *M. lar* and *Palaemon debilis,* as outgroups. Multiple alignments were performed using Clustal W (Thompson et al. 1994) in MEGA 7.0.20 (Kumar et al. 2016), and sequences of both 16S rRNA and 28S rDNA genes were combined. A phylogenetic tree was reconstructed by the maximum likelihood (ML) method (Felsenstein 1981) with the GTR+G+I model (Yang 1994), and bootstrap analyses (Felsenstein 1985) were performed with 500 replicates. Similarly, the phylogenetic tree of 16S rRNA only was reconstructed with the HKY+G model (Hasegawa et al. 1985).

Abbreviations are as follows: CAL, carpus length; CHL, chela length; CL, carapace length; FL, finger length; PL, palm length.

Results

Phylogenetic analyses. We determined a total of 1,219 bp (475 bp for 16S rRNA and 744 bp for 28S rDNA). As a result of comparison with the individuals used in the study by Castelin et al. (2017), one ovigerous female (RUMF-ZC-4626) collected from Okinawa-jima Island was identified as "*M. ustulatum*" (= group 1, sensu Castelin et al. 2017) (Figs. 1, 2), whereas all other individuals were identified as "*M. australe*" (= group 2, sensu Castelin et al. 2017) (Figs. 2, 3, 4).

Morphological analyses. The present study compared the Castelin's et al. (2017) data of two male specimens of "group 1" (MNHN-IU-2013-13201, 13203) that were examined for both phylogenetic and morphological analyses and those of the types of *M. australe* and *M. ustulatum* (Table

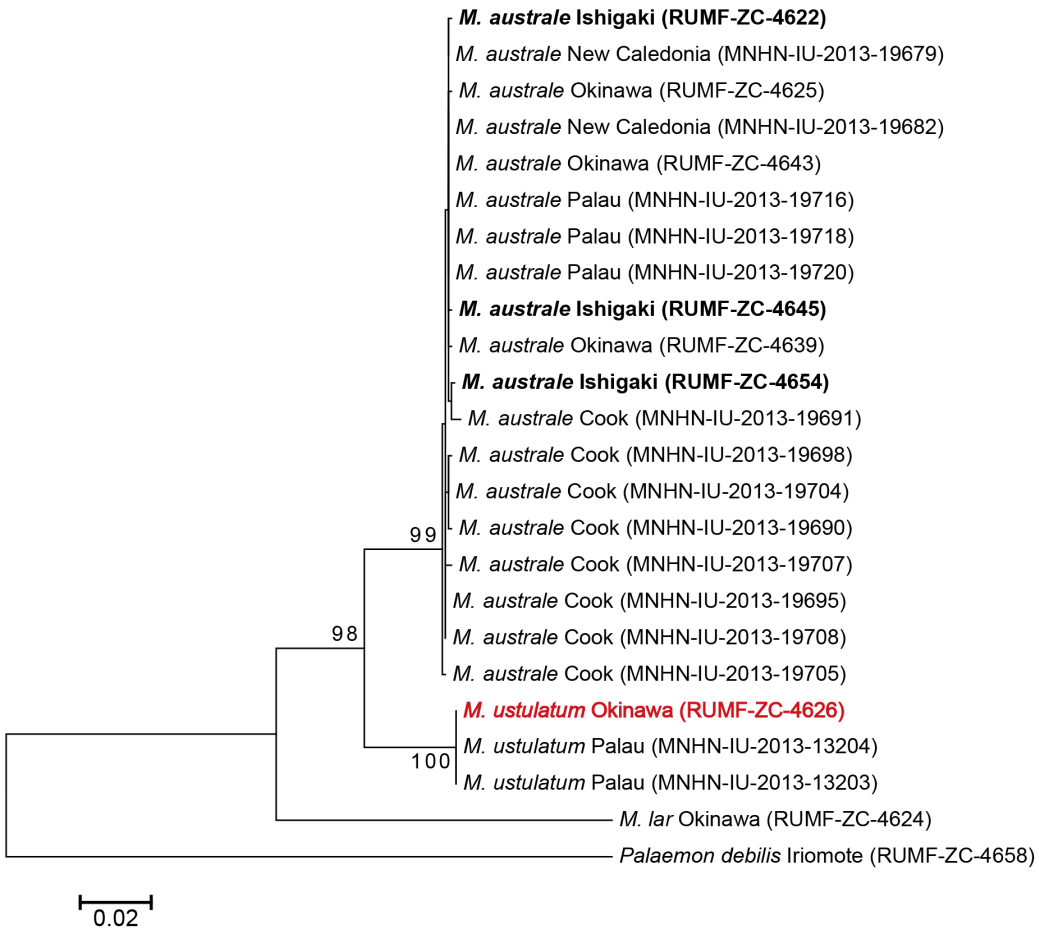

Fig. 2. Phylogenetic tree of *Macrobrachium australe* and *M. ustulatum* by the maximum likelihood (ML) method based on 16S rRNA and 28S rDNA sequence data. Numbers at each node are bootstrap values from 500 replicates. Bootstrap values > 70% are shown.
図 2. 16S rRNA と 28S rDNA 領域 (1,219 bp) を用いた ML 法によるザラテテナガエビとナンヨウテナガエビの分子系統樹 . 500 回の解析によるブートストラップ値は 70% 以上のものを表示している .

2). Since the holotype of *M. ustulatum* lacks the major second pereiopod, only 3 out of 6 diagnostic ratio characters (proportion of the joint of the minor P2: CAL/CHL, CAL/PL, FL/PL; for diagnostic characters, see Castelin et al. 2017: table 3) are available. There are, however, no clear tendencies to support that “group 1” is *M. ustulatum* (Fig. 5).
Morphology of 6 adult males of molecularly identified "group 2" from Okinawa-jima Island (RUMF-ZC-4622, 4645, 4654, 4661) was compared with the data of the type specimens of the above two nominal species. The ratios of both major and minor second pereiopods showed that these Okinawan specimens have very close values as the neotype of *M. australe* (Table 2; Fig. 5).

Discussion

Castelin et al. (2017) conducted phylogenetic analyses of *Macrobrachium* species and recognized two distinct clades that can be referable to *M. australe* and *M. ustulatum*. The type specimens of neither species were examined in the phylogenetic analyses. Morphological comparison between the types and molecularly identified specimens could help to assign correct names to the two clades, but Castelin et al. (2017) did not include phylogenetically analyzed specimens for morphological comparison, the effect of which on the identification is uncertain. Therefore, we verified the validity of the taxonomic identification based on phylogenetic and morphological analyses. These results indicate that “group 2” is likely *M. australe*, and therefore “group 1” is *M. ustulatum*.

Macrobrachium ustulatum **(Nobili, 1899)**
[New Japanese name: Nanyou-tenagaebi]
(Fig. 1)

Material examined. RUMF-ZC-4626, 1 ovigerous female, 15.26 mm (CL), Nishiyabu River, Nago, Okinawa-jima Island, coll. Yusuke Fuke, July 18,

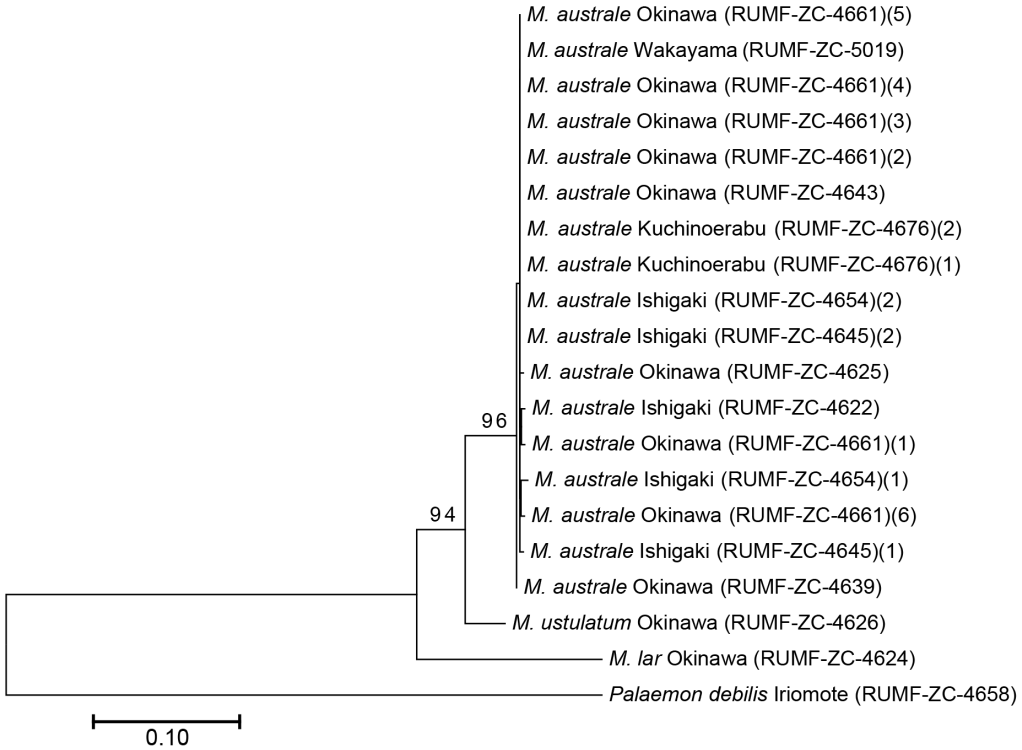

Fig. 3. Phylogenetic tree of *Macrobrachium australe* and *M. ustulatum* by the maximum likelihood (ML) method based on 16S rRNA sequence data. Numbers at each node are bootstrap values from 500 replicates. Bootstrap values >70% are shown.

図 3. 16S rRNA 領域 (475 bp) を用いた ML 法によるザラテテナガエビとナンヨウテナガエビの分子系統樹 . 500 回の解析によるブートストラップ値は 70% 以上のものを表示している .

2015.

Diagnosis of examined specimen. Rostrum reaching distal end of scaphocerite, upcurved distally; dorsal margin with 10 teeth, including 3 teeth on carapace, distances between distal first and second teeth, and proximal second last and third last teeth being larger than those between the others; ventral margin with 3 teeth. Epistome divided into two lobes, lobes produced antero-ventrally. Fourth thoracic sternite with median process. Second pereiopods unequal in length, right larger than left, similar in form. Major second pereiopod (right) length (from ischium to dactylus) 1.32 of that of minor second pereiopod (left). Major second pereiopod (right) slender, ischium 1.05 of merus length, merus 0.63 of carpus length, carpus 0.81 of propodus length, palm 0.56 of propodus length, dactylus 0.79 of palm length and 0.44 of propodus length, respectively. Minor second pereiopod (left) slender, ischium 1.02 of merus length, merus 0.73 of carpus length, carpus 0.85 of propodus length, palm 0.57 of propodus length, dactylus 0.77 of palm length and 0.43 of propodus length, respectively. Dactylus and part of the propodus of right fourth pereiopod and dactylus of left fifth pereiopod missing. Eggs numerous, average egg size 0.65 mm × 0.56 mm.

Coloration. Original coloration unknown. Preserved specimen with body milk-white. Tip of fingers of both second pereiopods with dark spots. Ovary dark olive green. Eggs yellow (Fig. 1).

Habitat. This specimen was collected in a pool of the Nishiyabu River, about 2.5 km upstream from the sea. *Macrobrachium australe* also inhabited the same place.

Distribution. Japan (Okinawa-jima Is.); Taiwan; Philippines; Papua New Guinea [type locality; Rigo]; Indonesia; Palau; Vanuatu (Nobili 1899; Castelin et al. 2017; present study).

Remarks. Castelin et al (2017: table 3) listed six morphological characters to distinguish *M. australe* and *M. ustulatum*, four of them were only applicable to adult males. We examined the other two diagnostic characters to identify the female specimen collected from Okinawa-jima Island (RUMF-ZC-4626). The shape of the epistome lobes and the armature of the fourth thoracic sternite appear to be common between males and females (Short 2004). Although the conditions of those characters in the Okinawan specimen were relatively similar to those of *M. ustulatum* shown in Castelin et al (2017: table 3), the differences are subtle and difficult to distinguish the

Fig. 4. *Macrobrachium australe* from Ishigaki-jima Island, Japan (RUMF-ZC-4645).
図 4. 石垣島から得られたザラテテナガエビ (RUMF-ZC-4645).

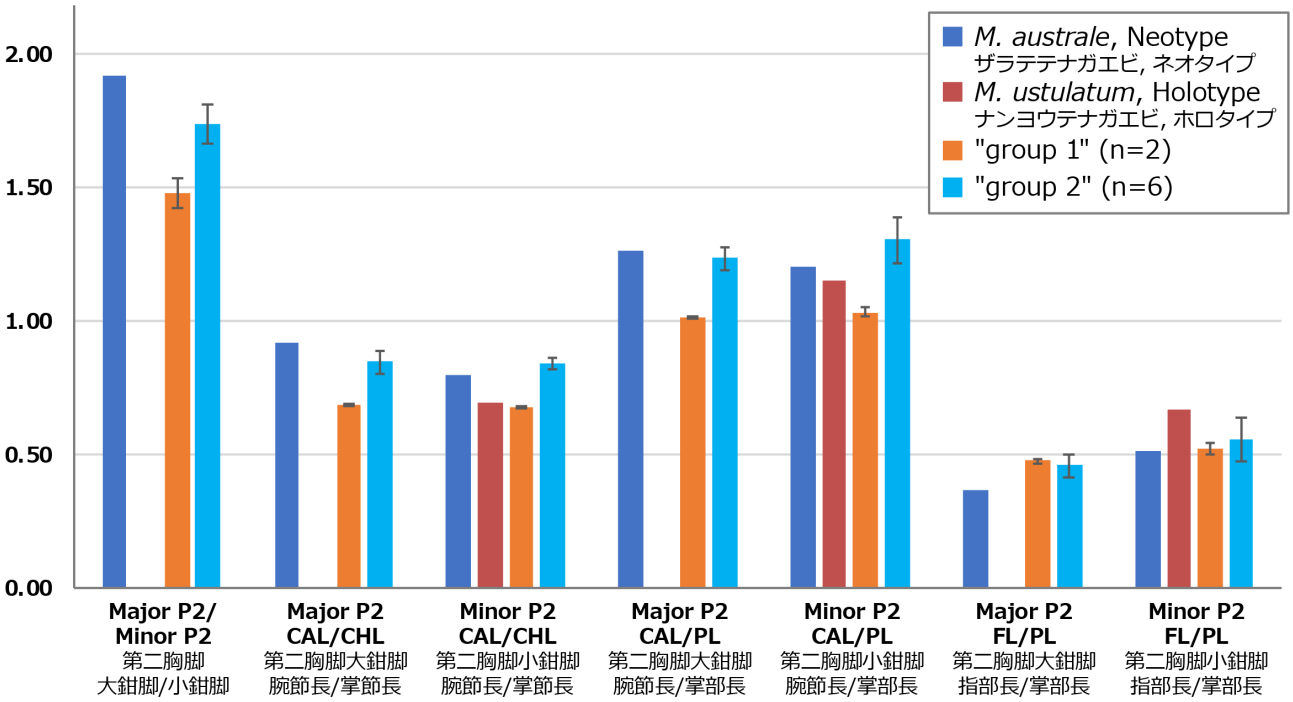

Fig. 5. Seven diagnostic ratio characters of the types of *Macrobrachium australe* and *M. ustulatum*, and two specimens of "*M. ustulatum*" (= group 1) that were examined for both phylogenetic and morphological analyses in Castelin et al. (2017) and six specimens of "*M. australe*" (= group 2; examined phylogenetically and morphologically). Bars show standard deviations. The data source of these ratios for the types and "group 1" is Castelin et al. (2017).
図 5. *Macrobrachium australe* と *M. ustulatum* のタイプ標本 , Castelin et al. (2017) で形態解析と系統解析の両方に用いられた "*M. ustulatum*" (= group 1) 2 個体の標本 , および本研究で形態解析と系統解析の両方に用いた成体オスの "*M. australe*" (= group 2) 6 個体の標本の判別形質の値 . タイプ標本および "group 1" の計測値は Castelin et al. (2017) から引用した .

two species by these qualitative traits alone.

This paper represents the first record of *M. ustulatum* from Japan and also extends the distributional area to the north. The ovigerous female

Table 2. Measurements of diagnostic characters of *Macrobrachium australe* and *M. ustulatum*. Dataset excluding RUMF specimens is cited from Castelin et al. (2017). Red color represents type specimen. Since Castelin et al. (2017) did not show the measurement value of the basis and the coxa, the ratio of Major P2 length / Minor P2 length was calculated using the length from the ischium to the tip of chela. Therefore, the ratio values are different from those in Castelin et al. (2017).
表 2. ザラテテナガエビおよびナンヨウテナガエビの判別形質の測定値 . RUMF 標本を除くデータセットは Castelin et al. (2017) から引用した . 赤字はタイプ標本を示す . 大鉗脚と小鉗脚の比率は Castelin et al. (2017) に底節と基節の測定値情報がなかったため , 座節から掌節の先端部までの長さで計算した . そのため , 元論文と数値が異なっている .

Species 種名	Specimen 標本番号	CL (mm) 頭胸甲長	Sex 性別	Major P2 length/ Minor P2 length 第二胸脚 大鉗脚 / 小鉗脚	CAL/CHL 第二胸脚 腕節長 / 掌節長		CAL/PL 第二胸脚 腕節長 / 掌部長		FL/PL 第二胸脚 指部長 / 掌部長	
					Major 大鉗脚	Minor 小鉗脚	Major 大鉗脚	Minor 小鉗脚	Major 大鉗脚	Minor 小鉗脚
M. australe ザラテテナガエビ	Neotype, MNHN-IU-2013-13198	15.3	M	1.92	0.92	0.79	1.26	1.20	0.37	0.51
M. ustulatum ナンヨウテナガエビ	Holotype, GENOVA Grande vaso VII-106	23.5	M	-	-	0.69	-	1.15	-	0.67
M. ustulatum ナンヨウテナガエビ	MNHN-IU-2013-13201	20.7	M	1.42	0.68	0.68	1.01	1.02	0.48	0.50
M. ustulatum ナンヨウテナガエビ	MNHN-IU-2013-13203	20.7	M	1.53	0.69	0.68	1.00	1.05	0.46	0.54
M. australe ザラテテナガエビ	RUMF-ZC-4622	14.1	M	1.76	0.79	0.85	1.18	1.45	0.49	0.71
M. australe ザラテテナガエビ	RUMF-ZC-4645	15.43	M	1.80	0.92	0.86	1.26	1.29	0.37	0.51
M. australe ザラテテナガエビ	RUMF-ZC-4654	16.26	M	1.73	0.88	0.83	1.28	1.24	0.46	0.49
M. australe ザラテテナガエビ	RUMF-ZC-4661(1)	18.18	M	-	0.85	-	1.24	-	0.46	-
M. australe ザラテテナガエビ	RUMF-ZC-4661(2)	15.39	M	1.78	0.81	0.80	1.17	1.20	0.45	0.51
M. australe ザラテテナガエビ	RUMF-ZC-4661(3)	16.86	M	1.60	0.83	0.84	1.25	1.32	0.51	0.57
M. ustulatum ナンヨウテナガエビ	RUMF-ZC-4626	15.26	F	1.32	0.81	0.85	1.45	1.5	0.79	0.77

of *M. ustulatum* examined in this study bears a large number of small eggs, which infers that the species has amphidromous life history (Wowor et al. 2009). It is unknown whether the specimen arrived from the south via the Kuroshio Current by chance or being reproduced on Okinawa-jima Island. In the future, it is possible that this species will be found not only south of Okinawa-jima Island but also north of it. *Macrobrachium ustulatum* is morphologically so close to *M. australe* (see above) that it might have already been collected from Japan but misidentified as *M. australe*. Detailed investigations of previously collected specimens may find further knowledge on this species.

The standard Japanese name “Nanyou-tenagaebi” is here proposed for *M. ustulatum*. The name is derived from the main distribution of this species. “Nanyou” means the islands on the Pacific Ocean along the equator. RUMF-ZC-4626 (Fig. 1) is designated as a standard specimen for the Japanese name “Nanyou-tenagaebi” of *M. ustulatum*. Morphologically similar *M. australe* has the standard Japanese name “Zarate-tenagaebi”, but no standard specimen for the Japanese name has been proposed. RUMF-ZC-4645 (Fig. 4) is designated as a standard specimen for “Zarate-tenagaebi” of *M. australe* to avoid any confusion.

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沖縄島から得られた日本初記録のナンヨウテナガエビ（新称）（甲殻亜門：十脚目：テナガエビ科）

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要旨. 琉球列島沖縄島北部の河川から得られたテナガエビ科の *Macrobrachium ustulatum* の標本を記録した . これは本種の日本初記録であると同時に , 分布の北限記録となる . 本種は長らくザラテテナガエビ *M. australe* の新参異名とされてきたが , Castelin et al. (2017) により独立種として再記載された . しかし , 分子系統解析によって認識された 2 種への名義の割り当ての妥当性には不明確な部分があった . そこで本報告では , 両種について形態形質とミトコンドリア DNA の 16S rRNA と核 DNA の 28S rDNA 領域を用いた相同性検索を併用することで , 先行研究で行われた分類の妥当性を検証した . その結果 , 先行研究の結果を支持する証拠が得られ , それに従って両種の同定を行った . 併せて , *Macrobrachium ustulatum* の標準和名としてナンヨウテナガエビを提唱し , その基準標本に RUMF-ZC-4626 を指定した . *Macrobrachium australe* の標準和名は従来通りザラテテナガエビとし , 和名の基準標本に RUMF-ZC-4645 を指定した .

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