A New Quadrannulate Species of Orobdella (Hirudinida: Arhynchobdellida: Orobdellidae) from Pingtung, Taiwan

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(Received 17 August 2017; Accepted 12 October 2017)

http://zoobank.org/90220F49-CD28-47AB-A627-9C4C8682F50E

A quadrannulate leech species, Orobdella meisai sp. nov., from southern Taiwan is described. This new species is characterized by its camouflage coloration, which is unique among the known Orobdella species. Orobdella meisai is the second species of the genus to be described from Taiwan. Phylogenetic analyses using nuclear 18S rRNA, histone H3, mitochondrial cytochrome c oxidase subunit I, tRNA Cys, tRNAMet, 12S rRNA, tRNA¹⁰⁰, 16S rRNA, tRNA¹⁰⁰ and NADH dehydrogenase subunit 1 markers showed that O. meisai and another Taiwanese species, O. ketagalan Nakano and Lai, 2012, formed a supported clade. According to the results of the morphological examination and the phylogenetic position of O. meisai, morphological-phylogenetic relationships of this species and close congeners are briefly discussed.

Key Words: Erpobdelliformes, terrestrial, macrophagous, molecular phylogeny.

Introduction

The genus Orobdella Oka, 1895 comprises 18 known species inhabiting terrestrial habitats in the Japanese Archipelago, Korean Peninsula, Taiwan, and adjacent islets (Nakano 2017a, b); an Orobdella leech, whose taxonomic status remains uncertain, has been recorded from the Russian Far East (Gilyarov et al. 1969; Nakano 2012a). Orobdella species can be identified by the following three characteristics (Nakano 2017a): 1) the possession of a gastroporal duct, which is an accessory copulatory organ that receives a spermatoaphore during copulation (Nakano 2017b); 2) mid-body somite annulation that can be subdivided into quadrannulate (four annuli per one somite; ten species), sexannulate (six annuli; six species), and octannulate (eight annuli; two species); and 3) the body length of mature individuals, grouped into small (the body length is ~5 cm; five species), middle (reaching ~10 cm; 11 species), and large types (reaching ~20 cm; two species). Whereas only quadrannulate species are assigned to the small type, the two octannulate species are designated large types (Nakano 2017a, b).

Orobdella leeches are particularly diversified in the Japanese Archipelago. All sexannulate, octannulate and small-type quadrannulate species are indigenous to the archipelago (Nakano 2017a). By contrast, only one middle-type quadrannulate species O. ketagalan Nakano and Lai, 2012 is known from Taiwan (Nakano and Lai 2012); and only O. tsushimensis Nakano, 2011 (middle-type quadrannulate), is indigenous to the Korean Peninsula (Nakano and Seo 2012, 2014). However, several undescribed species of Orobdella have been recognized in Taiwan (Nakano and Lai 2012).

Additional Orobdella leeches, which were designated as middle-type quadrannulate, were collected from Pingtung County, Taiwan, and they are described herein as a new species. Additionally, the phylogenetic position of the new species within Orobdella was estimated using nuclear and mitochondrial genetic markers.

Materials and Methods

Sampling and morphological examination. Leeches were collected from a locality in Pingtung County, Taiwan (Fig. 1). When possible, elevation and geographical coordinates for the collection site were obtained using a Garmin eTrex® GPS unit.

Specimens were relaxed by the gradual addition to absolute ethanol (EtOH) to freshwater, or directly fixed in absolute EtOH. For DNA extraction, botryoidal tissue was removed from the posterior part around the caudal sucker of every specimen, and then preserved in absolute EtOH. The remainder of the body was fixed in 10% formalin and preserved in 70% EtOH. Four measurements were taken: body length (BL) from the anterior margin of the oral sucker to the posterior margin of the caudal sucker, maximum body width (BW), caudal sucker length (CL) from the anterior to the posterior margin of the caudal sucker, and caudal sucker...
Molecular phylogenetic analyses. The phylogenetic position of the newly identified *Orobdella* species within the genus was estimated based on the 18S, H3, COI, tRNA^{Cys–16S}, and tRNA^{Leu–ND1} sequences. The four erpobdelliform total, ten sequences from the unidentified *Orobdella* species within the genus were identical to those in Nakano (2016a). In the Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.6 (Ronquist et al. 2012). The best-fit partition scheme and models for each partition were selected with the Bayesian information criterion using PartitionFinder with the “greedy” algorithm: for 18S and H3 1st position/H3 2nd position/COI 1st position/COI 2nd position/H3 3rd position/tRNA^{Cys–16S}, tRNA^{16S} and tRNA^{Leu–ND1} in 12S/16S/ND1 1st position/ND1 2nd position/ND1 3rd position. BI and Bayesian posterior probabilities (PPs) were estimated using MrBayes v. 3.2.6 (Ronquist et al. 2012). The best-fit partition scheme and models for each partition were selected with the Bayesian information criterion using PartitionFinder with the “greedy” algorithm: for 18S and H3 1st position, K80+I; for H3 2nd position, JC+I; for H3 3rd position, HKY+G; for COI 1st position, GTR+G; for 2nd position of COI and ND1, HKY+I+G; for 3rd positions of COI and ND1, 16S, HKY+G; and for tRNA^{16S}, tRNA^{Cys}, 12S, tRNA^{Leu}, tRNA^{Met}, and ND1 1st position, GTR+I+G. Two independent runs of four Markov chains were conducted for 20 million generations, and the tree was sampled every 100 generations. The parameter estimates and convergence were checked using Tracer v. 1.6.0 (Rambaut and Drummond 2013), and the first 50001 trees were discarded based on the results.

**Orobdella meisai** sp. nov. (Figs 2–4)

**Diagnosis.** Body length of mature individual exceeding 6cm. Somite IV uniannulate, somites VIII–XXV quadran-nulate. Clitellum in somite XI b5 to somite XIII a2. Male gonopore in somite XI b5/b6, female gonopore slightly posterior to anterior margin of somite XIII a1, behind gastropore, gonopores separated by 5+1/4 annuli. Pharynx reaching to somite XV b5–b6. Gastropore conspicuous, slightly posterior to anterior margin of somite XIII a1. Gastroporal duct rudimentary tubular. Paired vasa deferentia thick. Epipodiumis lacking. Pre-atrial cornu present. Atrial cornu lacking.

**Material examined.** Holotype: KUZ Z1917, dissected, collected from under a stone along the Jinshui Ying Old Trail, Fanliao, Pingtung County, Taiwan (22.40730°N, 120.758568°E; elev. 1470 m) by Takafumi Nakano (TN) on 9 March 2017. Paratypes (12 specimens collected from around the type locality): KUZ Z1908 (22.407°N, 120.756°E; elev. 1490 m) by Wei-Ren Liang on 26 August 2014; KUZ Z1909–Z1910 (22.407°N, 120.756°E; elev. 1490 m) by Fu-Sheng Huang on 23 February 2014; KUZ Z1911–Z1912 (22.40824°N, 120.75264°E; elev. 1650 m) by Yi-Te Lai (YL) on 9 March 2017; KUZ Z1913 (22.40809°N, 120.75288°E; elev. 1620 m), KUZ Z1914–Z1915 (22.40790°N, 120.75304°E; elev. 1620 m) by Yi-Te Lai (YL) on 9 March 2017; KUZ Z1916 (22.40772°N, 120.75662°E; elev. 1450 m) by TN on 9 March 2017; KUZ Z1918–Z1919 (22.40793°N, 120.756530°E) by YL on 22 January 2017; and KUZ Z1920 (22.409192°N, 120.754820°E) by Kao-Shir Shiang on 9 December 2016.
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(KUZ Z1910, Z1914, and Z1915 were dissected).

**Description.** Body firm and muscular, elongate, with constant width in caudal direction, dorsoventrally compressed, BL 62.5 mm, BW 4.7 mm (Fig. 2A, B). Caudal sucker ventral, slightly elliptic, CL 2.7 mm, CW 2.9 mm (Figs 2B, 3D). Somite I completely merged with prostomium (Fig. 3A). Somites II (=peristomium), III and IV uniannulate (Fig. 3A); somite II not separated from somite I. Somite V biannulate, \(a_1 + a_2 = a_3\); \(a_3\) forming posterior margin of oral sucker (Fig. 3A, B). Somites VI and VII triannulate, a1 = a2 = a3 (Fig. 3A, B). Somites VIII–XXV quadrannulate, a1 = a2 = b5 = b6 (Fig. 3A–E). Somite XXVI triannulate, a1 > a2 < a3; a3 being ventrally last complete annulus (Fig. 3C, D). Somite XXVII uniannulate with slight dorsolateral furrow on respective sides (Fig. 3C). Anus behind somite XXVII; post-anal annulus absent (Fig. 3C).

Somite X b5 and somite XIII a2, respectively, being first and last annuli of clitellum (Fig. 3E). Male gonopore in somite XI b5/b6 (Fig. 3E). Female gonopore slightly posterior to anterior margin of somite XIII a1, inconspicuous, located posterior to gastropore (Fig. 3E, F). Gonopores separated by 5+1/4 annuli (Fig. 3E).

Anterior ganglionic mass in somite VI a2 and a3. Ganglion VII in a1 and a2. Ganglia VIII–XIX, of each somite, in a2 (Fig. 4B). Ganglia XX–XXII, of each somite, in a1 and a2. Ganglion XXIII in a2. Ganglia XXIV–XXV, of each somite, in a1 and a2. Ganglion XXVI in somite XXV b6. Posterior ganglionic mass in somite XXVI a2 and a3.

Eyes in 3 pairs, 1st pair dorsally on anterior margin of somite III, 2nd and 3rd pairs dorsolaterally on posterior mar-

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### Table 1. Samples used for the phylogenetic analyses. The information on the vouchers is accompanied by the INSDC accession numbers.

| Species Voucher | INSDC accession # |
|-----------------|--------------------|
| **Orobdella**   |                    |
| *O. meisai* sp. nov. | KUZ Z1915 Paratype LC314418* LC314420* LC314419* LC314417* LC314421* |
| *O. meisai* sp. nov. | KUZ Z1917 Holotype LC314423* LC314425* LC314424* LC314422* LC314426* |
| *O. brachyepididymis* Nakano, 2016 | KUZ Z1673 Holotype LC106319 LC106321 LC106320 LC106318 LC106322 |
| *O. dolichopharynx* Nakano, 2011 | KUZ Z120 Holotype AB663665 AB698876 AB679680 AB679681 AB828558 |
| *O. esulessa* Nakano, 2010 | KUZ Z29 Holotype AB663665 AB698873 AB679664 AB679665 AB828555 |
| *O. iijima* Oka, 1895 | KUZ Z110 Topotype AB663659 AB698877 AB679672 AB679673 AB828559 |
| *O. kanaeokae* Nakano, 2017 | KUZ Z1747 Holotype LC184551 LC184553 LC184552 LC184550 LC184554 |
| *O. kawakatsusorum* Richardson, 1975 | KUZ Z167 Topotype AB663661 AB698878 AB679704 AB679705 AB828561 |
| *O. ketagalan* Nakano and Lai, 2012 | KUZ Z228 Holotype AB704785 AB704786 AB704787 AB828582 AB828563 |
| *O. kokai* Nakano, 2012 | KUZ Z156 Holotype AB698883 AB698882 AB679688 AB679689 AB828560 |
| *O. maoakokurowai* Nakano, 2014 | KUZ Z694 Holotype AB938003 AB938013 AB938006 AB937997 AB938016 |
| *O. mononoke* Nakano, 2012 | KUZ Z224 Holotype AB698868 AB698866 AB698867 AB828564 |
| *O. nakahama* Nakano, 2016 | KUZ Z1672 Holotype LC106330 LC106332 LC106331 LC106329 LC106333 |
| *O. naraiharaetmagarum* Nakano, 2016 | KUZ Z1652 Holotype LC087143 LC087145 LC087144 LC087142 LC087146 |
| *O. octonaria* Oka, 1895 | KUZ Z181 Topotype AB698870 AB698871 AB679708 AB679709 AB828562 |
| *O. okanori* Nakano, 2016 | KUZ Z1671 Holotype LC106341 LC106343 LC106342 LC106340 LC106344 |
| *O. shimadai* Nakano, 2011 | KUZ Z128 Holotype AB663663 AB698875 AB679676 AB679677 AB828557 |
| *O. tsushimini* Nakano, 2011 | KUZ Z134 Holotype AB663653 AB698872 AB679662 AB679663 AB828554 |
| *O. whitmani* Oka, 1895 | KUZ Z45 Topotype AB663657 AB698874 AB679668 AB679669 AB828556 |
| *O. yamaneae* Nakano, 2016 | KUZ Z1678 Holotype LC106349 LC106351 LC106350 LC106348 LC106352 |

| Outgroup | | |
|----------|-----------------------------|
| *Erpobdella japonica* Pawłowski, 1962 | KUZ Z178 AB663648 AB698879 AB679654 AB679655 AB828542 |
| *Gastrostomobdella monticola* Moore, 1929 | UNIMAS/A3/BH01/10 AB663649 AB698880 AB679656 AB679657 AB828543 |
| *Mimobdella japonica* Blanchard, 1897 | KUZ Z179 AB663650 AB698881 AB679658 AB679659 AB828544 |
| *Odontobdella blandrachi* (Oka, 1910) | KUZ Z180 AB663651 AB938012 AB938004 AB937995 AB938014 |

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![Fig. 2. Orobdella meisai sp. nov., holotype, KUZ Z1917. A, dorsal view; B, ventral view; C, dorsal view of live animal. Scale bars: 5 mm.](image-url)
gin of somite V (a1 + a2) (Fig. 3A). Papillae numerous, minute, hardly visible, on row on every annulus.

Nephridiopores in 17 pairs, each situated ventrally at posterior margin of a1 of each somite in somites VIII–XXIV (Fig. 3B, D, E).

Pharynx agnathous, euthylaematous, reaching to somite XV b5 (Fig. 4A). Crop tubular, acecate, reaching to somite XXI a2. Gastropore conspicuous, ventral, slightly posterior to anterior margin of somite XIII a1 (Fig. 3E, F). Gastrointestinal duct rudimentary tubular, reaching to somite XV a1/a2 (Fig. 4A); its pore opening to crop hardly detectable. Intestine tubular, acecate, reaching to somite XXIV a1/a2. Rectum tubular, thin-walled, straight.

Testisacs multiple (Fig. 4B); on right side, in somite XVIII

Fig. 3. Orobdella meisai sp. nov., holotype, KUZ Z1917. A, dorsal view of somites I–VIII; B, ventral view of somites I–VIII; C, dorsal view of somites XXIV–XXVII and caudal sucker; D, ventral view of somites XXIV–XXVI and caudal sucker; E, ventral view of somites X–XIII; F ventral view of gastropore and female gonopore. Scale bars: 1 mm (A, B); 2 mm (C–E); 0.25 mm (F). Abbreviations: af, annular furrow; an, anus; cl, clitellum; fg, female gonopore; gp, gastropore; mg, male gonopore; np, nephridiopore.
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...a2 to somite XXV a2, in total ~40 testisacs, 4 in XVIII, 5 in XIX, 6 in XX, 6 in XXI, 5 in XXII, 5 in XXIII, 6 in XXIV, 3 in XXV; on left side, in somite XVIII a2 to somite XXIV b6, in total ~35 testisacs, 3 in XVIII, 4 in XIX, 7 in XX, 5 in XXI, 5 in XXII, 5 in XXIII, 6 in XXIV. Paired vasa deferentia thick (Fig. 4B). Epididymis absent. Paired ejaculatory duct ...
ducts in somite XI a2 to somite XVIII a1 (Fig. 4B); coiled in position posterior to ovisacs; each duct crossing ventrally beneath each ovisac, then coiled in position anterior to ovisacs; each winding from respective junction with vas deferens, narrowing toward atrium with pre-atrial loop extending to middle of somite XI a2. Atrial cornu absent. Atrium small, globular, in somite XI b5 and b6 (Fig. 4B, C). Penis sheath and penis absent.

Paired ovisacs globular, in somite XIII a2 and b5 (Fig. 4B, D). Oviducts thin-walled (Fig. 4D); both oviducts converging in to common oviduct in somite XIII a1; dorsal surface of junction of both oviducts covered with ventral nerve cord. Common oviduct thin-walled, short, directly descending to female gonopore (Fig. 4D).

**Variation.** Measurements (mean ± 1SD, followed by ranges in parentheses; n = 13, including holotype): BL 48.9 ± 16.3 mm (27.4–73.1 mm), BW 4.2 ± 1.5 mm (2.1–7.2 mm), CL 2.1 ± 0.68 mm (1.2–3.0 mm), CW 2.5 ± 0.79 mm (1.4–4.2 mm). Somite XXVI a3 sometimes with slight dorsal furrow, or dorsolateral furrow on respective sides. Somite XXVII generally uniannulate without secondary furrow, or rarely biannulate. First pair of eyes rarely dorsally on posterior margin of somite II, or on somites II/III; small unpaired eye dorsally on left posterior margin of somite III (KUZ Z1912). Pharynx reaching to somite XV b5/b6–b6. Crop reaching to somite XXI a1–a2. Gastroptoral duct reaching to female gonopore (Fig. 4D).

**Remarks.** 

Orobdella meisai clearly belongs to Orobdella, because it possesses the generic diagnostic characteristics defined by Nakano (2016a): post-anal annulus absent; male gonopore in posterior part of somite XI; female gonopore in anterior part of somite XIII; gonopores separated by more than one full somite; pharynx agnathous euthylaematous; gastropore in anterior part of somite XIII; gastroptoral duct
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The authors are grateful to Mr Fu-Sheng Huang, Mr Wei-Ren Liang and Mr Kao-Shr Shiang for providing specimens of the new species. We are also grateful to Dr Ko Tomikawa (Hiroshima University) for his generous assistance with our

Table 2. Comparisons of morphological characters between Orobdella meisai sp. nov. and ten quadrannulate congeneric species.

| Species                  | Body length | Somite IV | Somite XXV | Annulli between gonopores | Pharynx length | Gastroporal duct | Epididymides | Atrial cornua |
|--------------------------|-------------|-----------|------------|---------------------------|----------------|------------------|--------------|--------------|
| Orobdella meisai         | middle      | 1         | 4          | 5 + 1/4                   | to posterior XV | rudimentary tubular | absent       | absent       |
| Orobdella brachyepididymis| small       | 1         | 4          | 1/2 + 4[+ (<1/2)]         | to anterior XIV | tubular           | XX to X     | small, ovate |
| Orobdella esulcata       | middle      | 1         | 4          | 2/3 + 4 + 1/3             | to anterior to posterior XIV | tubular, but bulbous at junction with gastropore | XVI to X | developed, ovate |
| Orobdella kanaakoikoei   | small       | 1         | 4          | 1/2 + 4 + 1/2             | to posterior XIII to anterior XIV | bulbous | XIV to XVIII | developed, ovate |
| Orobdella wakatassumurum | middle      | 2         | 4          | 6                         | to middle to posterior XIV | simple tubular | XVI to XVII | undeveloped |
| Orobdella ketagalan      | middle      | 1         | 4          | 1/2 + 4 + 1/2             | to posterior XIV | simple tubular | absent       | undeveloped |
| Orobdella koikeyi        | small       | 1         | 3          | 1/2 + 4 + 1/2             | to posterior XIII to anterior XIV | bulbous | XX to XV     | developed, ovate |
| Orobdella masaakiikureiwi| small       | 1         | 4          | 1/2 + 4 + 1/2             | to anterior to middle XIV | bulbous | XVI to XVIII | developed, ovate |
| Orobdella naraharaetmagarum| small    | 1         | 4          | 1/2 + 4 + 1/2             | to posterior XIII | bulbous | XV to XX     | developed, ovate |
| Orobdella tsushimensis   | middle      | 1         | 4          | 1/2 + 5                   | to posterior XIII to posterior XIV | bulbous | XVII to XIX | developed, ovate |
| Orobdella whitmani       | middle      | 1 or 2    | 4          | 1/2 + 4 + 1/2             | to anterior to posterior of XIV | bulbous | XVI to XVIII | developed, ovate |

lying on female organ; testisacs multiple; male atrium part of somite XI, without penis sheath or penis; ovisacs globular in somite XIII; female median reproductive system essentially lacking.

According to taxonomic studies (Nakano 2010, 2011b, 2012a; Nakano and Lai 2012; Nakano and Seo 2014; Nakano 2016a, b, 2017b), the new species is distinguishable from the ten quadrannulate species (O. brachyepididymis, O. esulcata, O. kanaakoikoei, O. wakatassumurum Richardson, 1975, O. ketagalan, O. koikeyi Nakano 2012, O. masaakiikureiwi Nakano 2014, O. naraharaetmagarum, O. tsushimensis, and O. whitmani Oka, 1895) by the following combination of characters (Table 2): body length, annulation of somites IV and XXV, number of annuli between gonopores, pharynx length, gastroporal duct, epididymidal lengths and atrial cornua. Orobdella meisai can clearly differ from these known quadrannulate species in having 5 + 1/4 annuli between the gonopores, the pharynx reaching to the posterior part of somite XV, the rudimentary tubular gastroporal duct, and in lacking atrial cornua in the male median reproductive system.

The new species is obviously distinguished from the six quadrannulate species in having 5 + 1/4 annuli between the gonopores, the pharynx reaching to the posterior part of somite XV, and the undeveloped gastroporal duct and atrial cornua. These characteristics are congruent with the recent finding that the Orobdella gastroporal duct is a secondary copulatory organ that receives a spermatophore (Nakano 2017b); a leech spermatophore is secreted from the male atrium (Brumpt 1900). It remains uncertain whether the gastroporal duct of O. meisai plays a role as a spermatophore receptor. However, it is highly possible that the rudimentary gastroporal duct of O. meisai has lost its function, and that this species transfers spermatozoa directly to the female organ.

Undeveloped gastroporal duct and male atrium are shared characteristics within a clade comprising four Orobdella species distributed in the Ryukyu Islands and Taiwan. Orobdella shimadae and O. dolichopharynx, which are respectively indigenous to Okinawajima and Amamioshima islands, possess a rudimentary gastroporal duct, and they completely lack an atrial cornu (Nakano 2011a). Although the Taiwanese O. ketagalan bears atrial cornua and a functional gastroporal duct, these organs are generally undeveloped (Nakano and Lai 2012). Since O. mononoke and the other phylogenetically related species, e.g., O. esulcata, O. kanaakoikoei and O. naraharaetmagarum, possess a developed gastroporal duct and atrial cornua (Nakano 2010, 2012b, 2016b, 2017b), the undeveloped gastroporal duct and atrium may be synapomorphic characters within the clade containing O. shimadae, O. dolichopharynx, O. ketagalan and O. meisai. Moreover, the pharynx of O. shimadae, O. dolichopharynx, and O. meisai, of which the gastroporal duct is vestigial, is relatively longer than those of the close congeners with a developed gastroporal duct (Nakano 2011a, 2012b; Nakano and Lai 2012). The pharyngeal elongation and degeneration of the gastroporal duct within Orobdella species may correlate with each other.

The obtained phylogenies showed that O. meisai is genetically highly diverged from O. ketagalan compared with the phyletogenetic relationships of O. shimadae and O. dolichopharynx. These relationships suggest that the diversification of the Taiwanese Orobdella species had occurred prior to the speciation between O. shimadae and O. dolichopharynx. A biogeographical study with divergence time estimates of Orobdella species will help to improve our understanding of the evolutionary history of this East-Asian specific terrestrial leech group.

Discussion

Orobdella meisai possesses a rudimentary gastroporal duct and an undeveloped male median reproductive system without atrial cornua. These characteristics are congruent with the recent finding that the Orobdella gastroporal duct is a secondary copulatory organ that receives a spermatophore (Nakano 2017b); a leech spermatophore is secreted from the male atrium (Brumpt 1900). It remains uncertain whether the gastroporal duct of O. meisai plays a role as a spermatophore receptor. However, it is highly possible that the rudimentary gastroporal duct of O. meisai has lost its function, and that this species transfers spermatozoa directly to the female organ.

Acknowledgments

The authors are grateful to Mr Fu-Sheng Huang, Mr Wei-Ren Liang and Mr Kao-Shr Shiang for providing specimens of the new species. We are also grateful to Dr Ko Tomikawa (Hiroshima University) for his generous assistance with our
filed survey in 2017, and to two anonymous reviewers and Dr Nozomu Muto (Tokai University) for their constructive comments on this manuscript. This study was financially supported by JSPS KAKENHI Grant numbers JP26840127 and JP15J00720.

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