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Two novel species of *Yonagunia* (Halymeniales, Rhodophyta) were uncovered in the South of Madagascar during the Atimo-Vatae Expedition

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Abstract – *Yonagunia*, a genus of the red algal order Halymeniales, has never been recorded in the Western Indian Ocean. In this study, we used molecular assisted alpha taxonomy to assess the diversity of the marine algal flora of southern Madagascar. A combination of COI-5P and *rbc*L sequences, used as a DNA-barcode and phylogenetic marker respectively, revealed two species of *Yonagunia* from the Toliara region in the South of Madagascar. One species, is described a species new to science, *Yonagunia atimo-vatae sp. nov.* Sequences of the other species, matched of *Polyopes ligulata*, but it was resolved within the genus *Yonagunia* rather than *Polyopes*. Hence, a new combination *Yonagunia ligulata comb. nov.* is established.

***Yonagunia* / Madagascar / Atimo Vatae / Rhodophyta / Halymeniales / COI / *rbc*L / phylogeny / morphology / integrative systematics / reproduction**

INTRODUCTION

The genus *Yonagunia* Kawaguchi & Masuda (in Kawaguchi *et al.*, 2004) was described a decade ago to accommodate the generitype, *Y. tenuifolia* Kawaguchi *et* Masuda, and *Y. formosana* (Okamura) Kawaguchi *et* Masuda. The latter was initially described as a species of the genus *Carpopeltis*. Diagnostic

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characters of *Yonagunia* included the unique auxiliary cell ampulla morphology as well as the results of phylogenetic analyses of halymeniacean species inferred from the chloroplast *rbc*L gene. More recently a third species was added to *Yonagunia* with the transfer of *Gelidium zollingeri* by G.H. Boo and co-authors (2013). Boo and colleagues based their decision on both the examination of the type specimen of *G. zollingeri* and the study of freshly collected specimens, in combination with COI and *rbc*L molecular data.

In the course of the Atimo Vatae expedition, which means "Deep South" in the regional Antandroy language, a comprehensive sampling was made of the marine benthic algal flora in southern Madagascar. The expedition, named "Our Planet Reviewed", was a joint initiative of an academic institution – Muséum National d'Histoire Naturelle (MNHN) – and an NGO – Pro-Natura International (PNI) – aimed at documenting, sampling and describing the novel components of biodiversity in key ecosystems of the world. In the spring 2010, the Muséum National d'Histoire Naturelle (MNHN, Paris), the Institut d'Halieutique et des Sciences Marines, University of Toliara (IH.SM), and the Wildlife Conservation Society (WCS) Madagascar Programme united their efforts to explore the coastal marine fauna and flora from Androka to Fort Dauphin, a cold water region where the algal flora is luxuriant and where endemism is supposedly high. The aim of the present paper is to describe and assess the phylogenetic relationships of the specimens of *Yonagunia* collected in the framework of the Atimo Vatae expedition.

MATERIAL AND METHODS

During the Atimo Vatae Expedition, specimens of *Yonagunia* were collected in 15 localities of southern Madagascar between April and June 2010 (Table 1). A total of 18 herbarium sheets including one to seven individuals (35 individuals in total) were mounted as voucher specimen. For subsequent molecular analysis, a piece of tissue was subsampled from a selected individual marked by an arrow and the mention "DNA" on the herbarium sheet. Anatomical observations were performed on hand sections from plants at different degrees of development and at different habit levels (basal, middle, and subapical) and stained with a 1% aniline blue solution. Microphotographs were obtained using a Diaplan Leica microscope equipped with a Leica DFC 500 camera (Leica Microsystems, Italy). Voucher specimens are housed in the herbarium (PC, Thiers, [continously updated]) of the Muséum National d'Histoire Naturelle (MNHN), Paris, France.

DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions except for the extraction buffer, which was prepared in the lab as follows: 1M Tris-base, 1M Tris-HCl, 0.05M Na_2EDTA, 0.2M NaCl, 2.5M potassium acetate, 10% Tween 20 and 0.2 $\text{mg}\cdot\text{ml}^1$ Proteinase K (Saunders, 1993). A total of 18 specimens were used for genomic DNA extraction. From each specimen, the COI-5P region was amplified using the forward primers GazF1 (Saunders, 2005) and GWSFn (Le Gall & Saunders, 2010) variously combined with the reverse primers GazR1 (Saunders, 2005) and GWSRx (Saunders, 2009). *rbc*L was amplified for both species uncovered in this study in one-to-three fragments, covering a total of 1365-1528 bp, using the following combinations of primers: FrbcLstart/R753, F577/R1381, and F993/RrbcS

Table 1. List of studied material. N/A data non available

Species	*Field Number*	*Herbarium Number*	*BOLD*	*Place*	*Coordinates*	*Depth*	*Collectors*	*Date*	*Reproductive structure*	*COI*	*rbcL*
Y. atimo-vatae	MAD0096	PC0166063	YOMAD001-15	Pointe d'Itaperina	–25.0417, 46.9900	6-8,5 m	F. Rousseau, R. Anderson, J. Tsarahevitra, P. Laboute, A. Barrere	28/04/2010	Tetrasporangia	X	X
Y. atimo-vatae	MAD0756	PC0166723	YOMAD002-15	Cap Andavaka	–25.3500, 46.6333	0-1 m	F. Rousseau, R. Anderson, J. Tsarahevitra	14/05/2010	–	X	–
Y. atimo-vatae	MAD1167	PC0171407	YOMAD003-15	Cap Malaimpioka, Est du cap	–25.3583, 44.8333	0 m	P. Mahatante	30/05/2010	Tetrasporangia	X	–
Y. atimo-vatae	MAD1372	PC0171623	YOMAD004-15	Cap Malaimpioka, Est du cap	–25.3583, 44.8333	0 m	B. de Reviers	09/06/2010	–	X	X
Y. atimo-vatae	MAD2233	PC0142960	YOMAD005-15	Roche Choumare	–24.8367, 47.1783	12-24 m	L. Le Gall	07/06/2010	Tetrasporangia	X	–
Y. ligulata	O155SRI	GENT HEC11712	YOMAD006-15	Hikkaduwa, Sri Lanka	6.13211, 80.09999	N/A	E. Coppejans	14/01/1997	–	–	X
Y. ligulata	O60Mad	GENT HEC15198	YOMAD007-15	Libanona, cap d'Antsirabe	–25.0397, 46.9952	N/A	E. Coppejans, D. Douterlungne, I. Razanakoto,	30/08/2002	–	–	X
Y. ligulata	MAD0009	PC0165976	YOMAD008-15	Fort Dauphin, plage Monseigneur	–25.0358, 46.9983	0 m	F. Rousseau, R. Anderson, J. Tsarahevitra	27/04/2010	Tetrasporangia	X	–
Y. ligulata	MAD0193	PC0166160	YOMAD009-15	Fort Dauphin, plage Monseigneur	–25.0358, 46.9983	0-1 m	F. Rousseau, R. Anderson, J. Tsarahevitra	30/04/2010	Tetrasporangia	X	–
Y. ligulata	MAD0196	PC0166163	YOMAD010-15	Mahano, proche du Rocher de la Baleine	–25.0033, 47.1033	22-28 m	F. Rousseau, R. Anderson, J. Tsarahevitra, A. Barrere, P. Laboute	30/04/2010	–	X	–
Y. ligulata	MAD0211	PC0166178	YOMAD011-15	Cap Antsirabe	–25.0467, 46.9983	22 m	F. Rousseau, R. Anderson, J. Tsarahevitra, A. Barrere, P. Laboute	01/05/2010	Tetrasporangia	X	–
Y. ligulata	MAD0214	PC0166181	YOMAD012-15	Phare Flacourt	–25.0217, 47.0033	12-16 m	F Rousseau, R. Anderson, J. Tsarahevitra, A. Barrere, P. Laboute	01/05/2010	–	X	–

Table 1. List of studied material. N/A data non available

Species	*Field Number*	*Herbarium Number*	*BOLD*	*Place*	*Coordinates*	*Depth*	*Collectors*	*Date*	*Reproductive structure*	*COI*	*rbcL*
Y. ligulata	MAD0361	PC0166328	YOMAD013-15	En face de la plage Monseigneur	– 25.0350, 47.0017	11-12 m	F. Rousseau, R. Anderson, J. Tsarahevitra	07/05/2010	–	X	–
Y. ligulata	MAD0427	PC0166394	YOMAD014-15	Cap Ranavalona, pointe Ehoala, plage Bevava	– 25.0750, 46.9617	2-4 m	F. Rousseau, R. Anderson, J. Tsarahevitra	08/05/2010	Tetrasporangia	X	–
Y. ligulata	MAD0483	PC0166450	YOMAD015-15	Libanona, cap d'Antsirabe	– 25.0433, 46.9967	0-3 m	F. Rousseau, R. Anderson, J. Tsarahevitra	11/05/2010	Tetrasporangia	X	–
Y. ligulata	MAD0484	PC0166451	YOMAD016-15	Libanona, cap d'Antsirabe	– 25.0433, 46.9967	0-3 m	F. Rousseau, R. Anderson, J. Tsarahevitra	11/05/2010	Tetrasporangia	X	–
Y. ligulata	MAD0670	PC0166637	YOMAD017-15	Flacourt	– 25.0283, 47.0017	0-1 m	F. Rousseau, R. Anderson, J. Tsarahevitra	13/05/2010	Tetrasporangia	X	–
Y. ligulata	MAD1388	PC0171639	YOMAD018-15	Cap Sainte Marie	– 25.6050, 45.1617	0 m	B. de Reviers	10/06/2010	Tetrasporangia	X	X
Y. ligulata	MAD2328	PC0143055	YOMAD019-15	Baie d'Itaperina, Crique	– 24.9917, 47.1000	10 m	L. Le Gall	10/06/2010	–	X	–
Y. ligulata	MAD2334	PC0143061	YOMAD020-15	Reef de la fausse baie des Galions	– 25.0400, 46.9933	8-14 m	L. Le Gall	11/06/2010	Tetrasporangia	X	–

start (Freshwater *et al.*, 1995). Purification and sequencing reactions were performed by Genoscope (www.genoscope.fr, Evry, France). Forward and reverse electropherograms were edited and assembled with Codoncode (Dedham, Massachusetts, USA) and multiple sequence alignments were constructed for each marker using SeaView (Gouy *et al.*, 2010). For the COI dataset, species were determined by distance analyses using the neighbour-joining algorithm in SeaView. Similarly, distance analyses were performed for the *rbc*L dataset. Phylogenetic analyses were conducted on a larger *rbc*L alignment, which included selected sequences from the Halymeniaceae that were downloaded from Genbank (Benson *et al.*, 2013). Phylogenetic analyses were conducted by Bayesian inference using MrBayes version 3.2.1 (MPI versions for Unix clusters; Ronquist & Huelsenbeck, 2003; Altekar *et al.*, 2004), RAxML (on line version, http://embnet.vital-it.ch/raxml-bb/; Stamatakis *et al.*, 2008), and PhyML 3.0 (on line version, http://atgc.lirmm.fr/phyml; Guindon & Gascuel, 2003). For Bayesian inference, the dataset was partitioned by codon position. The covarion-like model (Huelsenbeck, 2002) was combined with the GTR+G model of sequence evolution unlinking parameters among partitions (shape, statefreq, revmat, switchrates) and setting the prior for the site specific rates as "variable". Each analyses consisted of two parallel runs, each run using four Monte Carlo Markov Chains, one cold and three incrementally heated (temp = 0.10). A single run consisted of 5 million generations that were sampled every 1000 th tree. After completion of the two runs, likelihood values were plotted against the number of generations to evaluate when MCMC chains reached stability, in order to set an appropriate burn-in value for each analysis. Only trees saved during the stationary phase were used to reconstruct a majority rule consensus tree and to calculate the distribution of posterior probability. Maximum Likelihood (ML) analyses were performed with bootstrap resampling to estimate robustness of the internal nodes (Felsenstein, 1985), based on 1000 replicates in PhyML, with a GTR+G+I substitution model (with all parameters estimated during the search), starting from ten random BIONJ trees (Gascuel, 1997) with subtree pruning and regrafting (SPR) as the branch-swapping algorithm. ML analyses were also performed with bootstrap resampling based on 100 replicates in RAxML, with a GTR+G+I substitution model partitioned by codon position.

In all phylogenetic analyses, unrooted trees were constructed, and the root was subsequently designated based on previous knowledge of the Halymeniales.

RESULTS

Molecular systematics

Following a DNA-barcoding approach, COI-5P was sequenced for each of the 18 subsamples of *Yonagunia* taken for molecular analyses. The COI cluster analysis (Fig. 1) of these sequences revealed the presence of two distinct groups which are both distinct to *Yonagunia zollingeri,* the only species of *Yonagunia* for which a COI-5P sequence is available. The two clusters displayed a divergence of 6.5%.

The *rbc*L cluster analysis (Fig. 2) included all known species of *Yonagunia* and confirmed that the two clusters from Madagascar were significantly divergent (2.3-5.1%) from the three currently recognised species and

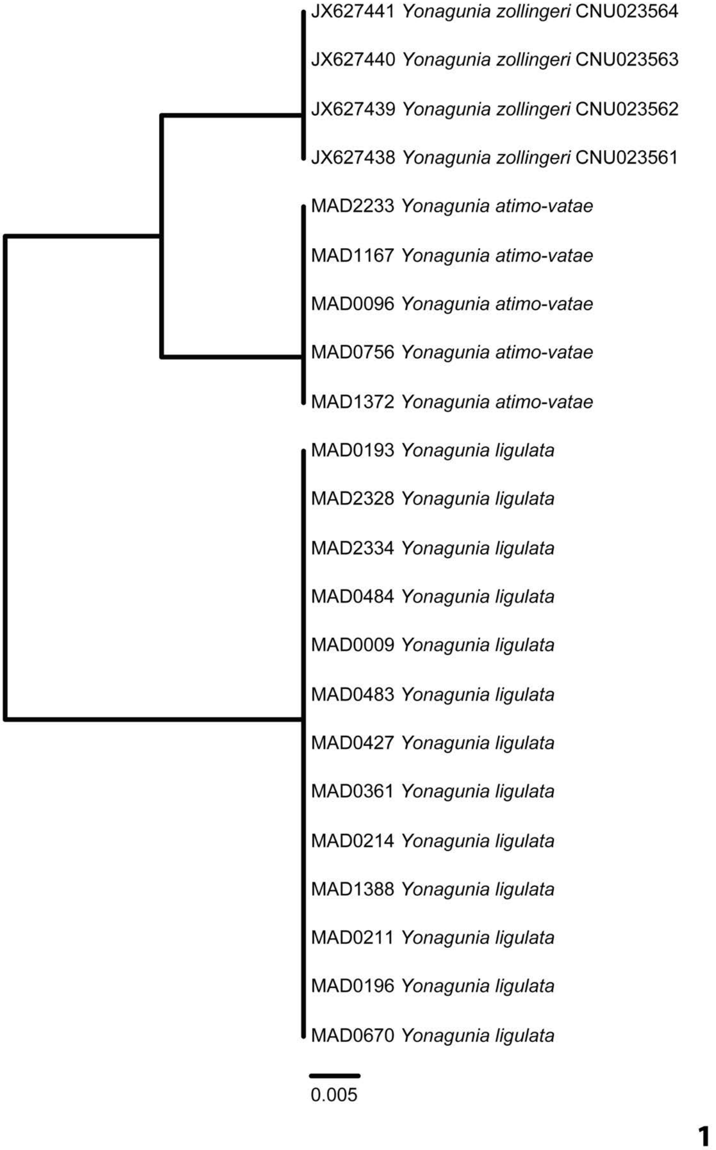

Fig. 1. Neighbor-joining tree inferred from the COI-5P dataset.

between themselves (3.8-4.1%). The cluster including specimens MAD0096 and MAD1372 diverged of 2.3-2.4% from *Yonagunia zollingeri*, 3.68-3.76% from *Yonagunia formosana*, 3.6-3.9% from *Yonagunia tenuifolia*. The specimen MAD1388 clustered together with specimens of *Polyopes ligulatus,* HEC11712 (from Sri Lanka, type locality) and HEC15198 (from Madagascar), diverging only 0.0-0.1% each other. This cluster diverged of 2.4-2.5% from *Y. formosana*, 3.7-3.9% from *Y. tenuifolia*, 4.9-5.1% from *Y. zollingeri*.

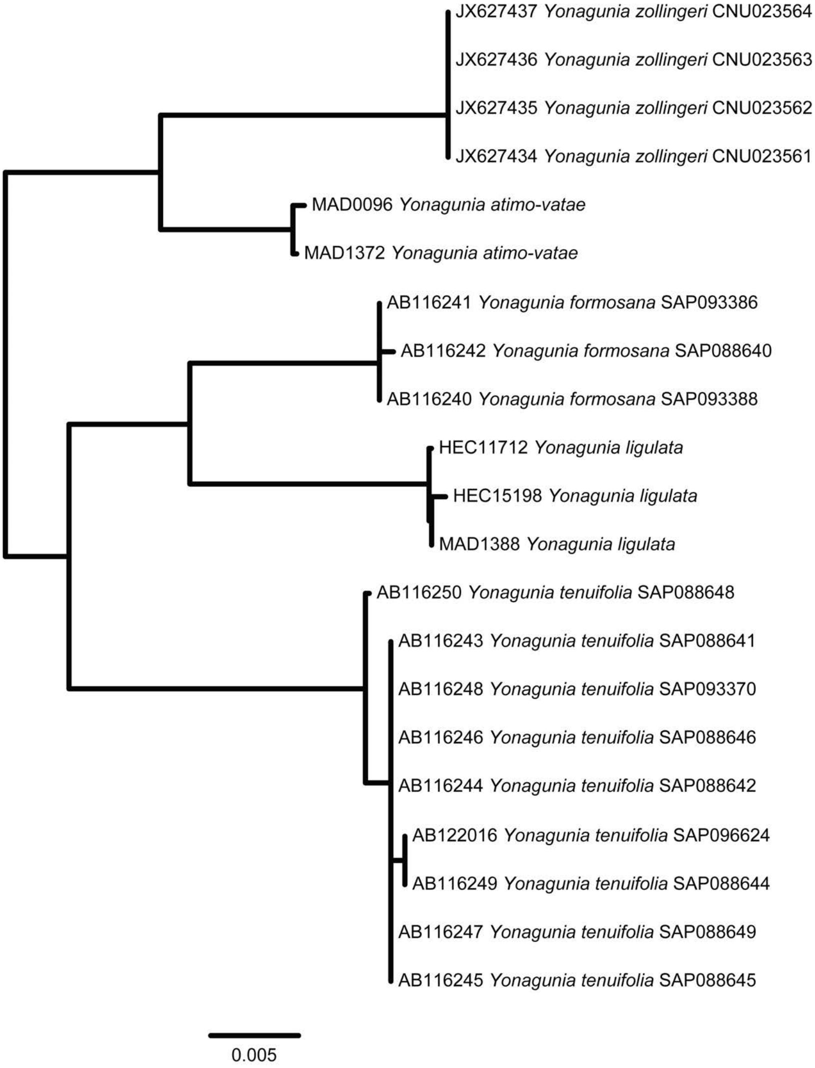

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Fig. 2. Neighbor-joining tree inferred from the rbcL dataset.

Phylogenetic analyses, inferred from the *rbc*L gene, were performed to assess the relationships of the Malagasy taxa with the three previously described species of *Yonagunia* (Fig. 3). One of the species strongly allied with *Y. formosana (*1/98%/99% Bayesian posterior probability/PhyML/RAxML bootstrap proportion values), whereas the second species joined *Y. zollingeri* with a slightly lower support (0.84/86% Bayesian posterior probability/PhyML bootstrap proportion values).

The genus *Yonagunia* consisted of a fully supported lineage among halymeniacean taxa in all analyses.

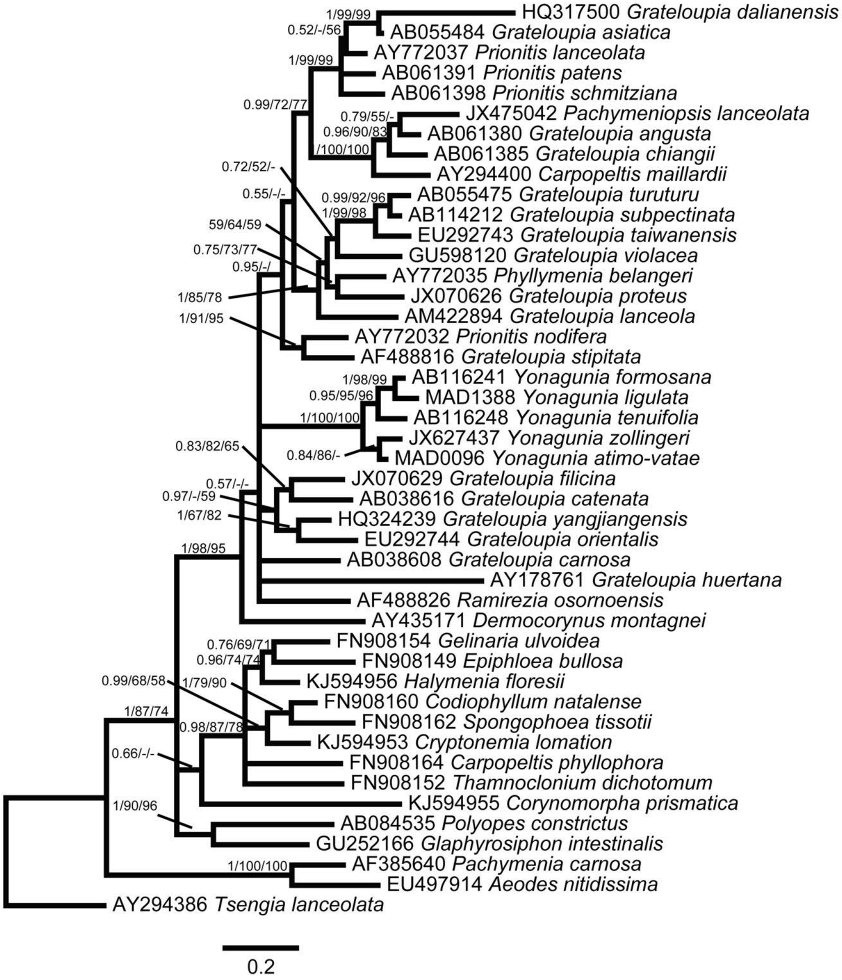

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Fig. 3. Bayesian phylogram inferred from the *rbc*L dataset. Supports at nodes indicate Bayesian posterior probabilities and bootstrap values inferred from ML analyses by PhyML and RAxML respectively.

Both DNA barcode COI-5P data and *rbc*L phylogenies suggested that the two Malagasy *Yonagunia* deserved to be recognised as novel species.

Anato morphological observations and taxonomic conclusions

Yonagunia atimo-vatae Manghisi, M. Morabito, G.H. Boo, S.M. Boo & Le Gall *sp. nov.*

Diagnosis: Thallus epilithic, with usually a single blade arising from a discoid holdfast; blades cartilaginous, purplish red to dark red, 3-5 cm tall, subcostate, complanate, 1-2 mm wide, 160-270 µm thick in the center, the basal (from 5 to 15 mm) region compressed; blades alternate to pseudo-dichotomously branched 3-4 times, rarely with a few proliferations on the surfaces; medulla occupying about three fifth of the blade thickness; cortex up to 10 cells thick, the inner layer consisting of 5-6, large, polygonal or stellate cells and the outer layer consisting of 3-4, small, rounded to ellipsoidal cells. Sexual reproduction not observed. Tetrasporangia cruciately or decussately divided, narrowly ellipsoidal, 10-15 µm in diameter and 35-55 µm long.

Holotype: specimen B on herbarium sheet PC0166063 (MAD0096), tetrasporic (Fig. 5), collected at Pointe d'Itaperina (Madagascar) on 28 April 2010 by F. Rousseau, R. Anderson, J. Tsarahevitra, P. Laboute and A. Barrere.

Isotype: specimen A on herbarium sheet PC0166063 (MAD0096), tetrasporic.

Paratypes: PC0142960 (MAD2233, tetrasporic), PC0166723 (MAD0756), PC0171407 (MAD1167, tetrasporic), PC0171623 (MAD1372).

Etymology: from the name of the expedition Atimo Vatae, that means 'Deep South' in the regional Antandroy language.

Habit: Each plant (Figs 4, 5) consists of a small discoid holdfast (1-2 mm in diameter) that usually supports a single erect blades 3-5 cm in length. The erect blades are cartilaginous, purplish red to dark red, subcostate at the base (Fig. 6) and gradually become flattened. Blades have a compressed basal portion from 5 to 15 mm in length and gradually become complanate, 1-2 mm wide and 160-270 µm thick in the centre. They are alternately branched 3-4 times, ending in blunt, bifurcate (pseudo-dichotomous) apices. Rare proliferations (to 3 mm in length) are present on the surfaces and margins of blades (Fig. 7).

Vegetative morphology: The erect blades have multiaxial structure (Fig. 8) and are internally composed of a compactly constructed anticlinal cortex and a dense filamentous medulla (Fig. 9). In Superficial view cortical cells appear singles or in pairs (Fig. 10). The cortex (Figs 9, 11, 12) is 50-65 µm thick and consists of three to four outer layers of small rounded to ellipsoidal cells (3-5 µm wide by 4-6 µm long) and five to six inner layers of larger polygonal or stellate cells (6-8 µm wide by 6-12 µm long) that are interconnected by secondary pit-connections (Fig. 12). The medulla occupies about three fifth of the blade thickness (Fig. 9). The medullary filaments are 3-10 µm in diameter and mainly periclinally directed (Fig. 13).

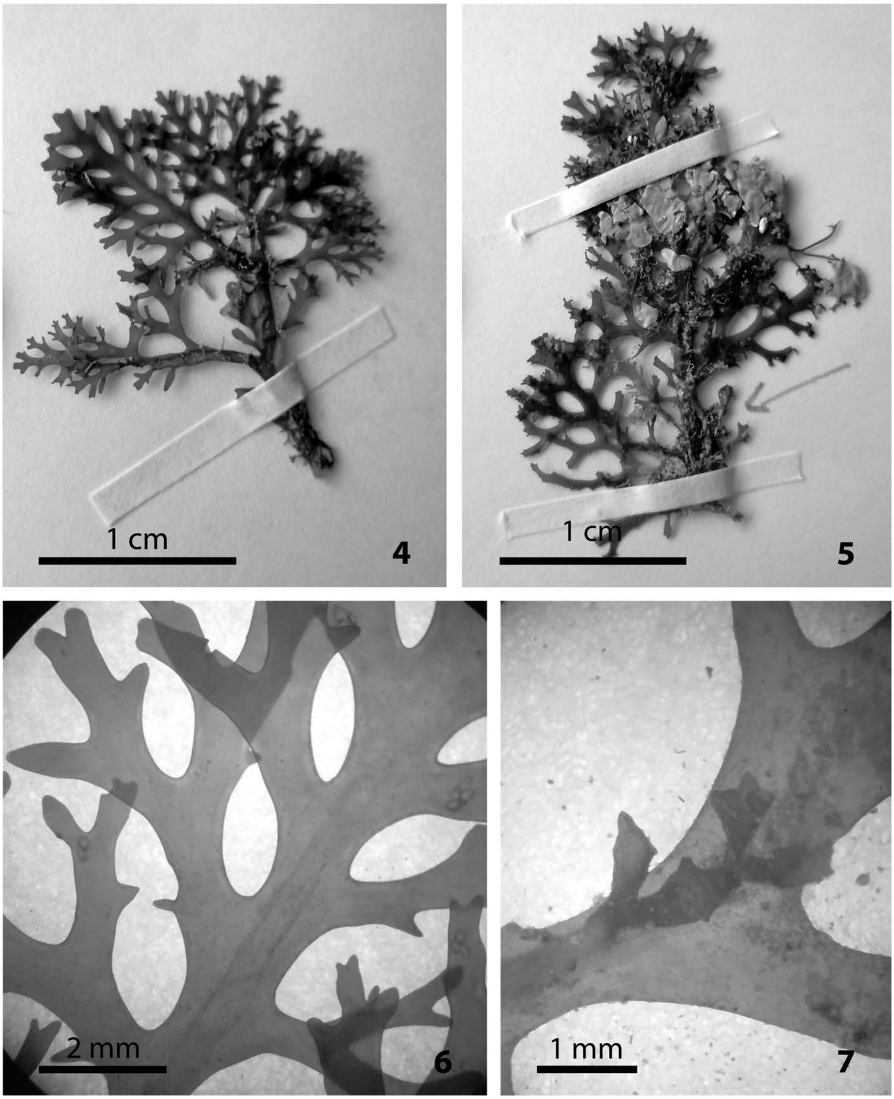
Reproductive morphology: Tetrasporangia are irregularly aggregated in the highest/ultimate order segments (Fig. 14). The segments bearing tetrasporangia are usually swollen (Fig. 15). Tetrasporangial initials are cut off from the cortical cells four or five layers proximal to the thallus surface (Fig. 16) and are cruciately or decussately divided (Figs 16-18), mature tetrasporangia being narrowly ellipsoidal and measuring 10-15 µm in diameter by 35-55 µm long. Female and male plants were not found in our collection.

Yonagunia ligulata **(Harvey *ex* Kützing) Manghisi, M. Morabito, De Clerck & Le Gall *comb. nov.***

Gymnogongrus ligulatus Harvey *ex* Kützing 1869: 23-24, pl. 63: figs. a,b. Type: in Sonder Herbarium (Kützing, 1869: 23). Isosyntypes: in W.H. Harvey's Algae of Ceylon (1857), exsiccate n. 50 (De Toni 1905: 1596). Type locality: Sri Lanka (Kützing, 1869: 23; De Toni, 1905: 1596; Silva *et al.*, 1996: 203).

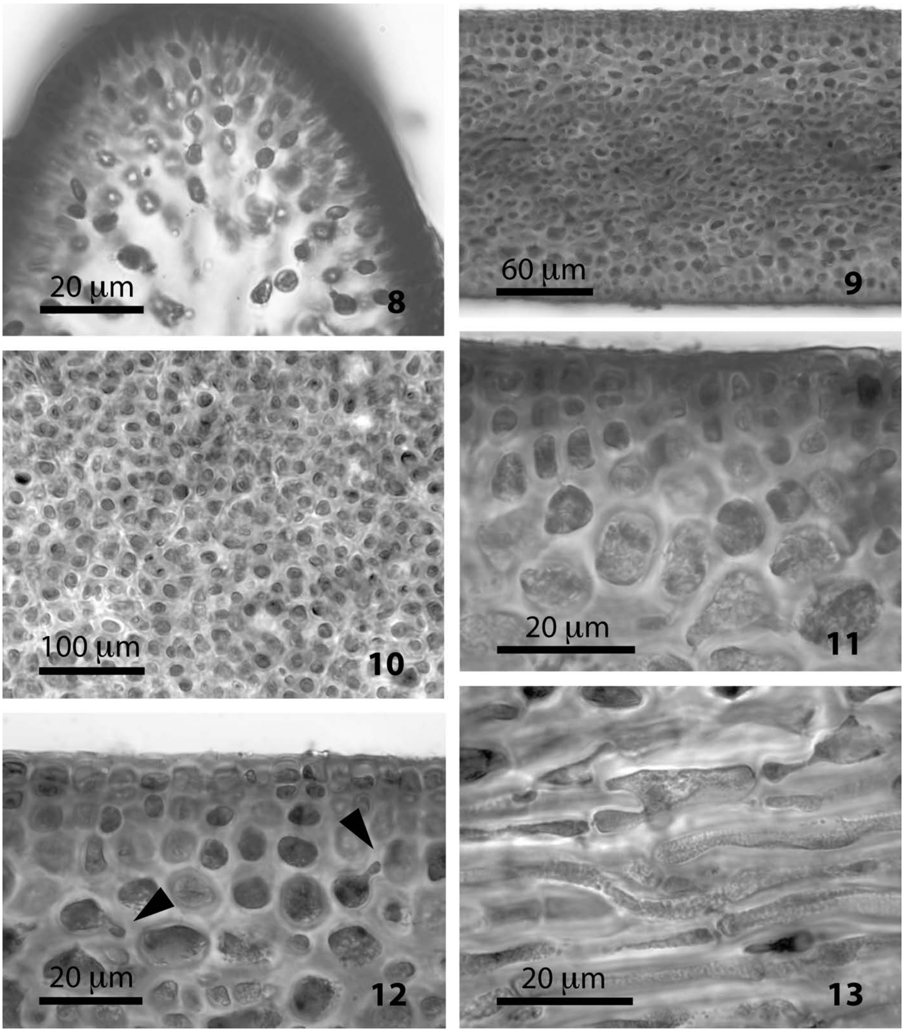
Cryptonemia ligulata (Harvey *ex* Kützing) J. Agardh 1876: 162

Polyopes ligulatus (Harvey *ex* Kützing) De Toni 1905: 1596



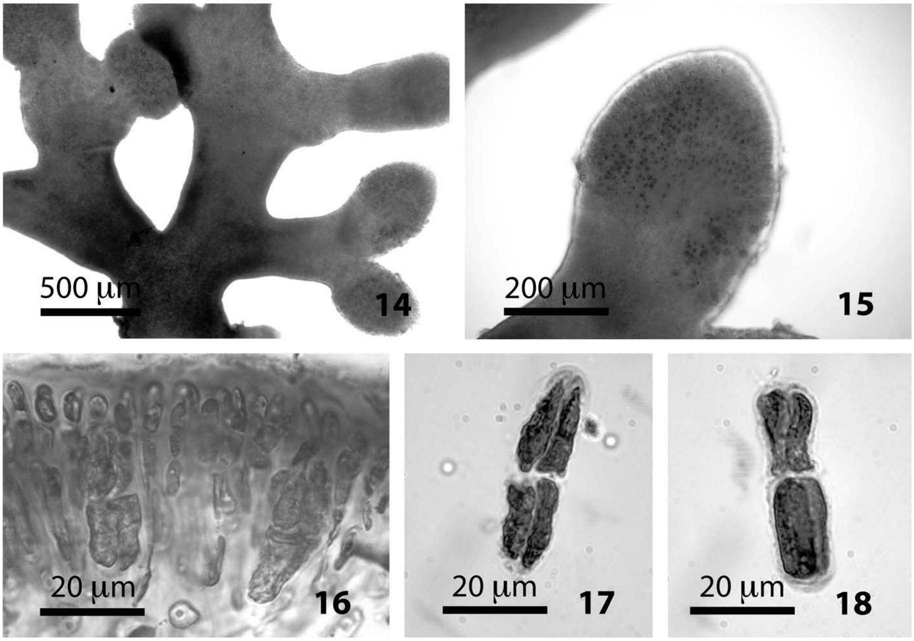
Figs. 4-7. Habit of *Yonagunia atimo-vatae* Manghisi, M. Morabito, G.H. Boo, S.M. Boo & Le Gall *sp. nov.* **4.** Morphology of a sterile specimen from voucher MAD0756/PC0166723. **5.** Morphology of the holotype, tetrasporic, specimen B from voucher MAD0096/PC0166063. **6.** Detail of the subcostate blade (voucher MAD0756/PC0166723). **7.** Detail of a small proliferation on the blade surface (voucher MAD2233/PC0142960).

Studied material: PC0143055 (MAD2328), PC0143061 (MAD2334, tetrasporic), PC0165976 (MAD0009, tetrasporic), PC0166160 (MAD0193, tetrasporic), PC0166163 (MAD0196), PC0166178 (MAD0211, tetrasporic), PC0166181 (MAD0214), PC0166328 (MAD0361), PC0166394 (MAD0427, tetrasporic), PC0166450 (MAD0483, tetrasporic), PC0166451 (MAD0484, tetrasporic), PC0166637 (MAD0670, tetrasporic), PC0171639 (MAD1388, tetrasporic).



Figs. 8-13. Morphology of *Yonagunia atimo-vatae* Manghisi, M. Morabito, G.H. Boo, S.M. Boo & Le Gall *sp. nov.* **8.** Longitudinal section of an apex, showing multiaxial structure (voucher MAD1167/PC0171407). **9.** Cross section showing cortex and medulla (voucher MAD1167/PC0171407). **10.** Superficial view of the cortical cells, singles or in pairs (voucher MAD2233/PC0142960). **11.** Longitudinal section showing a detail of the composition of cortical layers of cells (voucher MAD1167/PC0171407). **12.** Cross section showing a detail of the cortex, with secondary pit connections between adjacent cortical cells (arrowheads) (voucher MAD1167/PC0171407). **13.** Longitudinal section showing a detail of the filamentous medulla (voucher MAD1167/PC0171407).

Habit: Thalli (Figs 19-22) are attached to the substratum with a discoid holdfast (1-2 mm in diameter) from which up to 8-10 erect blades issue, the blades being terete and 0.5 mm in diameter for 2-3 mm basally and then gradually becoming compressed to uniformly flattened and 2-3mm wide and 430-570 µm thick. They reach 4-20 cm in length at maturity and are cartilaginous and dark red (rarely

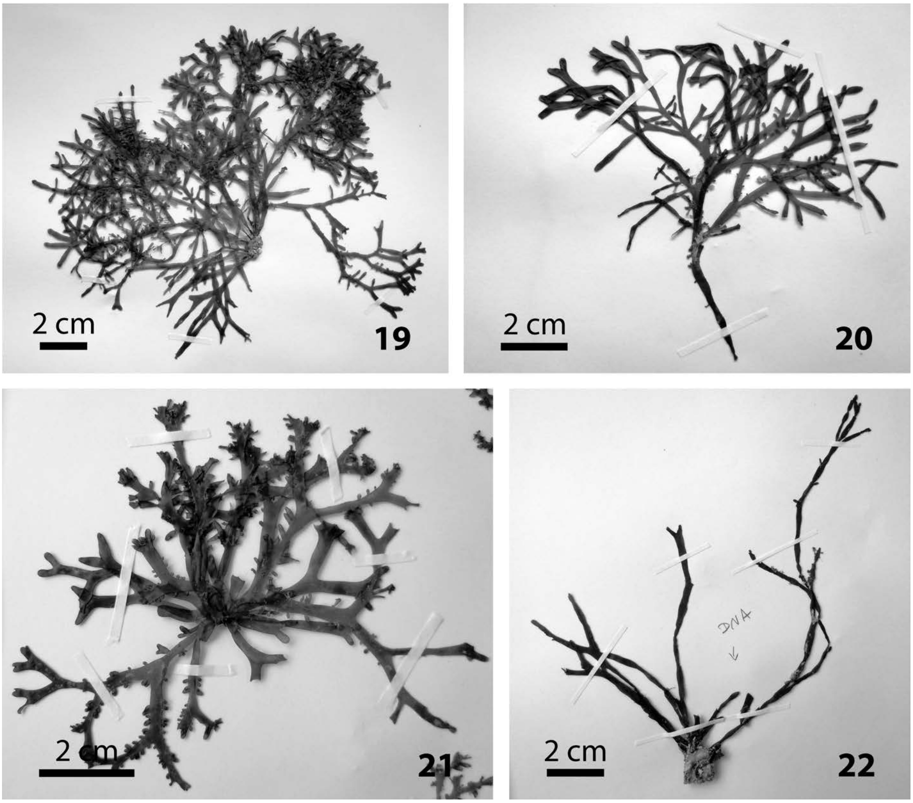


Figs. 14-18. Tetrasporangia of *Yonagunia atimo-vatae* Manghisi, M. Morabito, G.H. Boo, S.M. Boo & Le Gall *sp. nov.* **14.** Detail of a blade with various swollen ultimate order segments with tetrasporangia (voucher MAD0096/PC0166063). **15.** Detail of a swollen ultimate order segment with tetrasporangia (voucher MAD0096/PC0166063). **16.** Detail of two terasporangia immersed in the cortex (voucher MAD0096/PC0166063). **17.** A cruciately divided tetrasporangium (voucher MAD0096/PC0166063). **18.** A decussately divided tetrasporangium (voucher MAD0096/PC0166063).

brownish red) in colour. Branching is 5-7 times dichotomous or subdichotomous with wide axils (from acute to almost square angle), the linear segments slightly constricted at irregular intervals and ultimately ending in blunt or bifurcate apices. In most plants, numerous proliferations from 1 to 15 mm in length are formed at the margins (rarely on the surfaces) (Figs 19-21).

Vegetative morphology: The blades internally have multiaxial structure (Fig. 23) and consist of a compact anticlinal cortex and a dense filamentous medulla (Fig. 24). In superficial view cortical cells appear singles or in pairs (Fig. 25). The cortex is 120-160 µm thick, consisting of four to five outer layers of small ellipsoidal to rounded cells (2-4 µm wide by 5-7 µm long) and five or six inner layers of larger polygonal cells (5-16 µm wide by 7-20 µm long) (Figs 24, 26, 27) that are connected with one another by secondary pit-connections (Fig. 27). The medulla occupies three fifths to seven tenths of the blade thickness, is 220-400 µm thick, and consists of intermeshed filaments 4-13 µm in diameter that run mainly in a longitudinal direction (Figs 24, 28).

Reproductive morphology: Tetrasporangia are irregularly aggregated in the proliferations or the highest/ultimate order segments (Fig. 29). The segments are usually slightly swollen (Fig. 30). Tetrasporangial initials are produced from the fifth or sixth cortical cells proximal to the thallus surface (Figs 31-32); mature

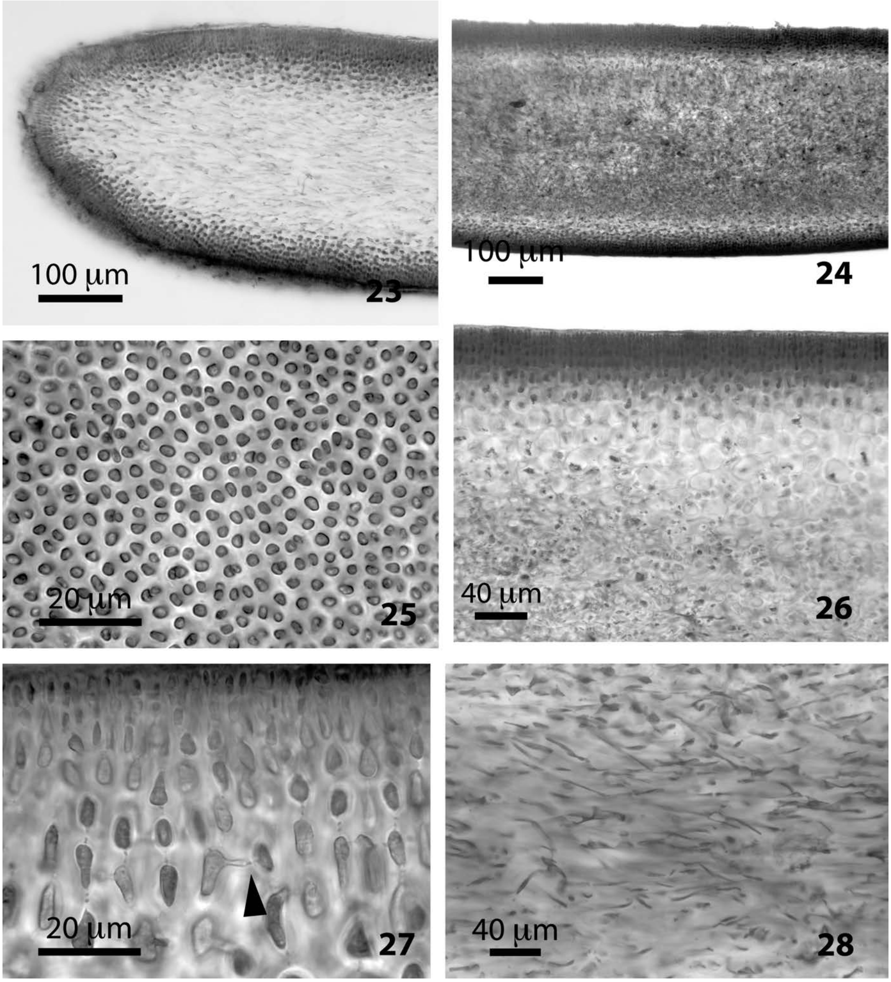


Figs. 19-22. Habit of *Yonagunia ligulata* (Harvey *ex* Kützing) Manghisi, M. Morabito, De Clerck & Le Gall *comb. nov.* **19-21.** Morphology of three tetrasporic specimens (vouchers MAD0009/PC0165976, MAD0193/PC0166160, MAD0484/PC0166451, respectively). **22.** Morphology of a sterile specimen (voucher MAD0196/PC0166163).

tetrasporangia are narrowly ellipsoidal, cruciate or decussate, 9-12 in diameter and 38-56 μm long (Figs 31-32). Female and male plants were not found in our material.

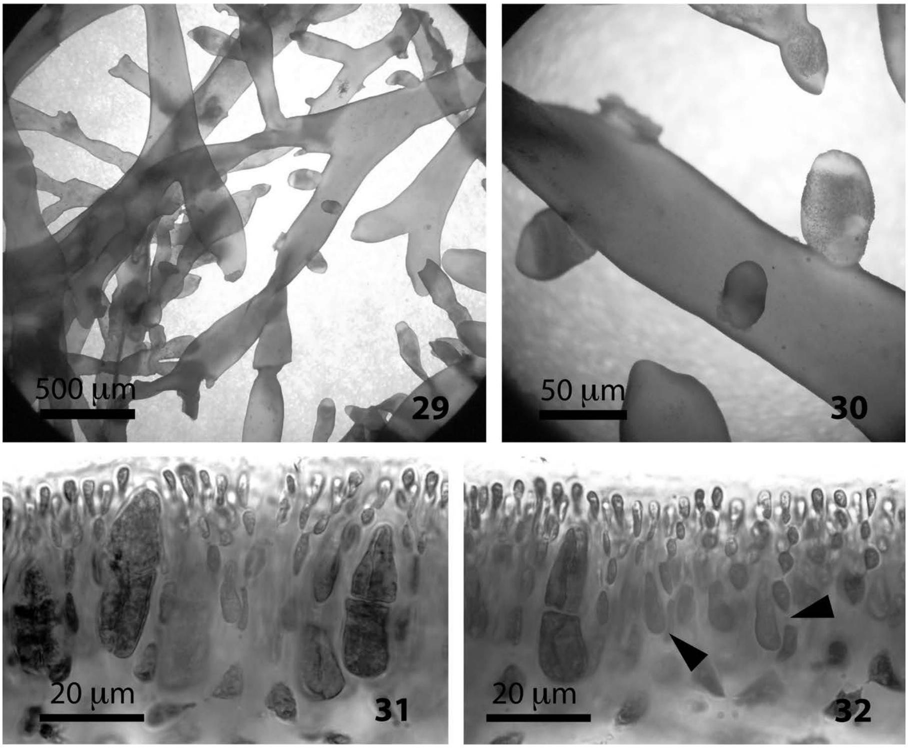
DISCUSSION

The genus *Yonagunia* Kawaguchi & Masuda was first established to accommodate *Y. tenuifolia* and *Y. formosana* (Kawaguchi *et al.*, 2004), and now also contains the recently described *Y. zollingeri* (Sonder) G.H. Boo, J.K. Park, G.S. Gerung & S.M. Boo (2013) and finally the two species herein described *Y. atimo-vatae* Manghisi, M. Morabito, G.H. Boo, S.M. Boo & Le Gall *sp. nov.* and *Y. ligulata* (Harvey *ex* Kützing) Manghisi, M. Morabito, De Clerck & Le Gall *comb. nov.*. The genus is characterized by flattened, dichotomously or irregularly branched thalli, internally multiaxial blades with compact cortical filaments, a medulla of densely intermeshed filaments, and reproductive structures formed on



Figs. 23-28. Morphology of *Yonagunia ligulata* (Harvey *ex* Kützing) Manghisi, M. Morabito, De Clerck & Le Gall *comb. nov.* **23.** Longitudinal section of an apex, showing multiaxial structure (voucher MAD0196/ PC0166163). **24.** Cross section showing relative abundance cortex and medulla (voucher MAD0196/ PC0166163). **25.** Superficial view of the cortical cells, singles or in pairs (voucher MAD0009/ PC0165976). **26.** A detail of a cross section showing the cortex and part of the medulla (voucher MAD0009/ PC0165976). **27.** Cross section showing a detail of the cortex, with secondary pit connections between adjacent cortical cell (arrowheads) (voucher MAD0484/ PC0166451). **28.** Longitudinal section showing a detail of the filamentous medulla (voucher MAD0196/ PC0166163).

ultimate and penultimate segments or in proliferations. Female reproductive structures consist of, depending on the interpretation, 2 or 5-celled carpogonial branch (Kawaguchi *et al.*, 2004; Gargiulo *et al.*, 2013) and an auxiliary cell situated at the base of relatively simple auxiliary cell ampulla. No fusion is observed of the diploidized auxiliary cell with other cells. Grape-like clusters of small globular cells which elongate subsequently to early gonimoblast formation and produce



Figs. 29-32. Tetrasporangia of *Yonagunia ligulata* (Harvey *ex* Kützing) Manghisi, M. Morabito, De Clerck & Le Gall *comb. nov.* **29.** Detail of a blade with various marginal proliferations bearing tetrasporangia (voucher MAD0096/PC0166063). **30.** Detail of a swollen marginal proliferation bearing tetrasporangia (voucher MAD0096/PC0166063). **31.** Detail of decussately divided tetrasporangia embedded in the cortex (voucher MAD0193/ PC0166160). **32.** Detail of a cruciately divided tetrasporangia and two immature tetrasporangia (arrowheads) embedded in the cortex.

involucral filaments laxly surrounding the carposporophyte form a diagnostic character for the genus (Kawaguchi & Nguyen, 1998; Kawaguchi *et al.*, 2004; Boo *et al.*, 2013; Gargiulo *et al.*, 2013). Our morphological and molecular study strongly supports the distinct position of *Yonagunia* (Kawaguchi *et al.*, 2004; Boo *et al.*, 2013; Gargiulo *et al.*, 2013) in the Halymeniaceae.

Yonagunia tenuifolia, the generitype, was characterized by strongly twisted – rather than planar – axes, blades with thickened margins, and thinner and more or less proliferous blades. It occurs only in Yonaguni Island, Okinawa, Japan (Kawaguchi *et al.*, 2004).

Yonagunia formosana, previously known as *Carpopeltis formosana* Okamura (Okamura, 1931; Pham, 1969; Silva *et al.*, 1996; Huang, 2000), is distinguished by terete to flattened blades and numerous proliferations. Recent papers (Kawaguchi *et al.*, 2004; Boo *et al.*, 2013) reported *Y. formosana* in Vietnam, Malaysia, and Taiwan. The reports at the first two localities have been verified by molecular data (Kawaguchi *et al.*, 2004), while there are no DNA sequences of specimens from Taiwan, which is the type locality of the basionym *Carpopeltis* *formosana* (Okamura, 1931). However, the type material was not located (fide Kawaguchi & Nguyen, 1998). Other reports (Guiry & Guiry, 2015), also unverified by DNA sequences, extend its distribution to Japan, China, Philippines, central Polynesia. Furthermore Farghaly (1980) as well as Combres and colleagues (1986) reported *Y. formosana* (as *Carpopeltis formosana*) in Madagascar, however this species was not found during the Atimo Vatae expedition.

Yonagunia zollingeri, previously known as *Gelidium zollingeri* Sonder (Zollinger, 1854), was characterized by a cartilaginous, flattened blade that is subcostate in the lower parts, with cystocarpic leaflets arising on terminal branchlets, and with round to heart-shaped tetrasporangial leaflets. It has been reported in Indonesia (Boo *et al.*, 2013).

Yonagunia atimo-vatae, similarly to *Y. zollingeri*, was characterized by a thallus subcostate in lower parts and alternate branching pattern. It is so far only known from southern Madagascar.

A morphological comparison of *Y. atimo-vatae* and *Y. ligulata* with the previously described species of the genus is given in Table 2. From the comparison, two gross morphologies are evident among *Yonagunia* species, which characterize two groups in *rbc*L phylogenies: species with a thallus subcostate in lower parts and alternate branching pattern, shown in *Y. atimo-vatae* and *Y. zollingeri* (0.84/86/- Bayesian posterior probability/PhyML/RAxML bootstrap proportion values), and species with flattened thalli with slight constrictions at irregular intervals, dichotomous branching and numerous proliferations, in *Y. tenuifolia*, *Y. ligulata* and *Y. formosana* (0.95/95/96).The second Malagasy species of *Yonagunia* proved to be conspecific with *Polyopes ligulatus* (Harvey ex Kützing) De Toni. *Gymnogongrus ligulatus* was a name used by Harvey (1857) to designate the exsiccatum n. 50 of his Ceylon Algae, but the specimen has neither a description nor a reference to a validly published name, so it is not valid by art. 38.1 of the International Code of Nomenclature for algae, fungi, and plants (McNeill *et al.*, 2012). The name *Gymnogongrus ligulatus* was later validly published by Kützing (1869) who added a description. Later, J. Agardh (1876) reputed that it had not vegetative nor reproductive structures of a *Gymnogongrus*, so he moved the species to the Cryptonemiaceae (now Halymeniaceae), making the combination *Cryptonemia ligulata*, even if he wondered whether it was a *Prionitis*. Finally, De Toni (1905) moved the species to another halymeniacean genus, *Polyopes*. *Yonagunia ligulata*, similarly to *Y. formosana*, was characterized by a flattened thallus with slight constrictions at irregular intervals, dichotomous branching and numerous proliferations. It is reported in this paper, with the support of DNA sequences, from southern Madagascar and Sri Lanka. Previous report of this taxa, as *Polyopes ligulatus*, are from Kenya (Coppejans *et al.*, 2000; Bolton *et al.*, 2007), Tanzania (Coppejans *et al.*, 2000), Sri Lanka (Børgesen, 1936; Silva *et al.*, 1996; Coppejans *et al.*, 2009; Atmadja, 2010), and Vietnam (Pham, 1969; Van Nguyen *et al.*, 2013). All the above mentioned records are unverified by molecular data. Note also that *Y. ligulata* has never been reported in Madagascar before the present work, while, as above mentioned, *Y. formosana* (as *Carpopeltis formosana*) was recorded. It can be conjectured that the previous reports of *Y. formosana* in Madagascar might instead refer to *Y. ligulata*, because of the morphological similarity of the two species.

These novel records of *Yonagunia* from southern Madagascar suggest that this genus has an affinity for warm temperate water in both hemispheres. Since Madagascar is a region where endemism is extremely high (Goodman & Benstead, 2005), it is likely that additional novel species of *Yonagunia* will continue to be found in the western Indian Ocean.

Table 2. Morphological comparison of *Yonagunia* species from Madagascar and previously described species of the genus. (*) for comprehensive distribution see Discussion.

	Y. atimo-vatae	*Y. ligulata*	*Y. tenuifolia*	*Y. formosana*	*Y. zollingeri*
Height	3-5 cm	4-20 cm	4-10 cm	3-8 cm	up to 7 cm
Blades	usually a single blade, cartilaginous, subcostata, few proliferations (to 3 mm)	up to 8-10 blades, cartilaginous, slight constrictions at irregular intervals, numerous proliferations (to 1,5 cm)	up to 10 erect blades, cartilaginous, slight constrictions at irregular intervals, strongly twisted in irregular manner, few proliferations (to 2 mm)	3-5 blades, cartilaginous, slight constrictions at irregular intervals, numerous proliferations (to 5 mm)	usually a single blade, cartilaginous, subcostata, few proliferations (to 3 mm)
Basal portion	compressed (0.5-1 mm wide) from 5 mm to 1.5 cm long	terete (0.5 mm in diam.) up to 2-3 mm	terete up to 2 mm	terete (1-1.5 mm in diam.) up to 2-3 mm	terete (1-3 mm in diam.)
Blade at center	1-2 mm wide, 160-270 µm thick	2-3 mm wide, 430-570 µm thick	2-4 mm wide, 230-280 µm thick	2-3 mm wide, 350-500 µm thick	2-3 mm wide, 130-320 µm thick
Branching	Pseudo-dichotomous to alternate, 3-4 times	dichotomous, subdichotomous, 4-7times	dichotomous, subdichotomous, 2-12 times	dichotomous, subdichotomous, 5-11 times	Pseudo-dichotomous to alternate, 3-7 times
Cortex	compact anticlinal cortex, 50-65 µm thick, consisting of 3-4 outer layers of small spherical to ellipsoidal cells (3-5 × 4-6 µm in size), and 5-6 inner layers of larger polygonal or stellate cells (6-8 × 6-12 µm in size), which were interconnected by secondary pit-connections.	compact anticlinal cortex, 120-160 µm thick, consisting of 4-5 outer layers of small spherical to ellipsoidal cells (2-4 × 5-7 µm in size), and 5-6 inner layers of larger polygonal or stellate cells (5-16 × 7-20 µm in size), which were interconnected by secondary pit-connections.	compact anticlinal cortex, 40-50 µm thick, consisting of 3-5 outer layers of small, rounded to ellipsoidal cells (3-4 × 4-5 µm) and 3-5 inner layers of, large, polygonal or stellate cells (5-10 × 5-12 µm) that are interconnected by secondary pit-connections.	compact anticlinal cortex, 60-75 µm thick, consisting of 3-7 outer layers of small ellipsoidal to rounded cells (2-3 × 3-4 µm) and 3-4 inner layers of larger polygonal cells (5-12 µm in diam.) that are inter connected by secondary pit-connections.	compact anticlinal cortex, ± 100 µm thick, consisting of 3-4 outer layers of small spherical to ellipsoidal cells (3-4 × 3-5 µm), and 3-4 inner layers of larger polygonal or stellate cells (5-8 × 5-13 µm), which were interconnected by secondary pit-connections.
Medulla	filamentous, 3/5 thickness of blade, 3-10 µm in diam., periclinally directed	filamentous, 3/5 to7/10 thickness of blade, 4-13 µm in diam., periclinally directed	filamentous, 2/3 thickness of blade, 8-10 µm in diam., periclinally directed	filamentous, 5/7 to 4/5 thickness of blade, 5-10 µm diam., periclinally directed	filamentous, 1/2 thickness of blade, 5-13 µm in diam., periclinally directed
Cystocarps	not found	not found	spherical, 150 µm in diam.	spherical to subspherical, up to 200 µm in diam.	spherical to subspherical, up to 130 µm in diam.
Tetrasporangia	ellipsoidal, cruciate or decussate, 10-15 × 35-55 µm, initial from 4-5th cortical cells	ellipsoidal, cruciate or decussate, 9-12 × 38-56 µm, initial from 5-6th cortical cells	ellipsoidal, cruciate or decussate, 13-15 × 40-43 µm, initial from 4-5th cortical cells	fully mature not observed, initial from 5-6th cortical cells	ellipsoidal, cruciate or zonate, 10-15 × 40-46 µm, initial from 4-5th cortical cells
Type Locality	Pointe d'Itaperina, Madagascar	Fort Dauphin, plage Monseigneur, Madagascar	Yonaguni Island, Okinawa, Japan	Orchid Island, Taiwan	Sempu, Malang, Java, Indonesia
Distribution	Madagascar (W Indian Ocean)	Sri Lanka, Madagascar (W Indian Ocean)*	Japan (Pacific Ocean)	Malaysia, Taiwan, Vietnam (Pacific Ocean)*	Indonesia (E Indian Ocean)
Source	Present study	Present study	Kawaguchi *et al.*, 2004	Kawaguchi *et al.* 2004	Boo *et al.*, 2013

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