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A new species of *Stenogramma* was uncovered Indian Ocean during the expedition Atimo Vatae: *Stenogramma lamyi* sp. nov.

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Abstract – The red algal genus *Stenogramma* (Gigartinales, Phyllophoraceae) was not recorded in the Indian Ocean. In this study, we used molecular assisted alpha taxonomy to assess the algal diversity of the algal flora of the southern coasts of Madagascar. Using COI-5P sequences as barcode marker and LSU and *rbc*L sequences to infer phylogenetic relationships, a novel species of *Stenogramma* has been uncovered at Manantenina, Anosy, Madagascar, which is here described. The species diversity within the genus *Stenogramma* is discussed and in light of our phylogenetic inference we hypothesized that species of northern and southern hemispheres may have diversified separately. This novel species, being found on subtidal rocks surrounded by sands, the habitat preference of the genus is discussed.

Diversity / Indian Ocean / *Stenogramma* / Phyllophoraceae / Systematics

INTRODUCTION

Within the family Phyllophoraceae (Gigartinales), notwithstanding several phylogenetic studies inferred mostly from the plastid gene coding the large subunit of RUBISCO (*rbc*L), the delineation of generic frontiers is still vague (Le Gall & Saunders, 2010; Maggs *et al.,* 2013) for many taxa. Nevertheless, the contour of the genus *Stenogramma* Harvey has never been questioned and the genus is still well characterized by the characters proposed by Harvey in its

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protolog (Harvey, 1840): "*Frons membranacea, rubra, plana, enervis. Fructificatio:1.-? 2. receptacula linearia, nerviformia, in frondem sessilia, granulis minutissimis replete*". In the current terminology, the genus *Stenogramma* is represented by erect blade-forming species, characterized by the medial lines of procarps and cystocarps on female plants resembling an interrupted midrib. At present, the red algal genus *Stenogramma* Harvey is represented worldwide by seven species that are distributed from the Pacific to the Atlantic oceans as well as the Coral Sea, the Tasmanian Sea and the Yellow sea ((Guiry & Guiry, 2015), Table 1). Nevertheless, the genus has never been reported in the Indian Ocean (Silva *et al.,* 1996; Guiry & Guiry, 2015). In the present study we will assess the phylogenetic affinities of a specimen collected during the expedition Atimo Vatae using a phylogenetic *rbc*L sequence analysis and we will provide a formal description of this novel specific entities.

Table 1. List of species of *Stenogramma* with their type localities and habitat preferences. In bold letters the type species of the genus.

Species	*Type locality*	*Hemisphere*	*Habitat*
Stenogramma bamfieldiense L. Le Gall & G.W. Saunders	Vancouver Isle, Canada	N	Subtidal at 8-11 m on cobbles, sheltered sites (sandy muddy bottom), one specimen was observed in a tidal rapid flowing on big cobble and rock (Saunders, pers. com.)
***Stenogramma californicum* Harvey**	San Francisco Harbour, USA	N	
Stenogramma guleopensis M.S. Calderón & S.M. Boo	Guleopdo Isle, Dukjeokdo Islands, Korea	N	Isolated patches on sand-covered rocks at a depth of 5-10 m (Calderon & Boo 2014)
Stenogramma interrupta (C. Agardh) Montagne	Cadiz, Spain	N	Epilithic, sublittoral to 13 m, in sheltered areas on small stones among gravel and mud (Dixon & Irvine 1977)
Stenogramma lamyi sp.nov.	Manantenina, Madagascar	S	On small pebbles spread on sand-covered substrate at a depth of 6 m
Stenogramma leptophyllum J. Agardh	Victoria, Australia	S	Depth of 10-36 m (Womersley 1994)
Stenogramma phyllophoroides (J. Agardh) A.J.K.Millar	New South Wales, Australia	S	Grows in isolated patches on sand-covered rocks at 7-20 m depths (Millar 1990)
Stenogramma rhodymenioides A.B. Joly & Alveal	Valparaiso, Chile	S	(Joly and Alveal 1970)

MATERIAL AND METHODS

Sample collection

During the Atimo Vatae Expedition, more than 2300 algal specimens were collected along the southern coast of Madagascar between April and June 2010 from Crocker Bay (western limit of the Androy Region) to Manantenina (eastern limit of the Anosy Region). Both a voucher and a tissue collection was established and housed in the herbarium (PC, Thiers, [continously updated]) of the Muséum National d'Histoire Naturelle (MNHN), Paris, France. The species here described is based on two individuals collected on June 3rd 2010 on the surface of patchy reefs located in front of the mouth of the Manantenina River which form a long stretch of a sandy shore (Fig. 1). The two individuals were logged in the field as "unidentified red blades" and mounted on a single herbarium sheet with the field number MAD2036 and the collection number PC0142763. Towards identification of this taxon both anatomical observations and molecular systematics analysis were performed.

Morphological and anatomical examination

Anatomical observations were performed on small pieces of tissue excised from pressed specimens and rehydrated in a 5% freshwater detergent solution for 1-3 min. Sections were cut by hand using a razor blade, or obtained with the aid of a freezing microtome (JUNG Frigocut 2800E; Leica Microsystems, Wetzlar, Germany obtaining sections between 10 and 15 µm thickness. Sections were stained in an acidified aniline blue solution [10 parts 1% aniline blue solution added to 90 parts 7% acetic acid solution (Kraft 1988)], then rinsed and permanently mounted on a 50% aqueous Karo® (ACH Food Companies Inc., Memphis, TN, USA) solution (4% formaldehyde to prevent microbial growth). Microphotographs were obtained using a DM750 Leica microscope equipped with a Leica ICC 50 camera (Leica Microsystems, France).

Molecular analyses

A molecular assisted taxonomic study was undertaken for a selection of unidentified specimens from the Atimo Vatae expedition. Toward this aim, DNA extraction was performed as described in (Vergés *et al.,* 2014) and subsequently the DNA barcode (the mitochondrial gene coding 670 base pairs of the 5' end of the cytochrome oxidase 1 here referred as COI-5) was amplified using the forward primers GWSFn (Le Gall & Saunders, 2010) and the reverse primers GWSRx (Saunders & McDevit, 2012). To further assess the phylogenetic relationships of this species, both *rbc*L (including the *rbc*L-*rbc*S spacer) and LSU (28S) were also amplified as described in (Vergés *et al.,* 2014). 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). Taxonomic affinities of the species here studied was tested submitting the COI-5' sequence to the identification engine within BOLD (Ratnasingham & Hebert 2007) and all the sequences to the BLAST algorithm on the NCBI portal. Both algorithms were congruent in revealing strong affinities of our unidentified taxon to the family Phyllophoraceae

Fig. 1. Map of the collected site

and more precisely to the genus *Stenogramma*. Therefore the COI-5' (GenBank accession number: KR733111), *rbc*L (GenBank accession number: KR733113) and LSU (GenBank accession number: KR733112) sequences generated here were included into the alignments constructed by (Maggs *et al.*, 2013) along with a *rbc*L sequence (KM262228) of *Stenogramma* generated since then (Calderon & Boo, 2014). Phylogenetic analyses were conducted on the *rbc*L alignment by Bayesian inference (BI) with four heated Monte Carlo Markov Chains for 2 000 000 generations using MrBayes version 3.2.5 (Ronquist *et al.*, 2012). Output trees and data were sampled every 100 generations. Appropriate burn-in for each

run was determined by plotting the overall likelihood against generations prior to estimating the posterior probability distribution. In all analyses, likelihood values were stable after the first 200 000 generations. Final results were based on the pooled samples from the stationary phase of the two independent runs. Maximum likelihood and distance bootstrap values (1000 replicates) were also calculated using SeaView with a GTR + gamma + I model (Gouy *et al.,* 2010).

RESULTS AND DISCUSSION

Phylogenetic relationships among species of *Stenogramma*

The molecular assisted identification of the specimens MAD2036 was initiated after obtaining the DNA-barcode sequences (COI-5') using both the BLAST algorithm and the identification engine in BOLD which both revealed that the taxa allied with the family Phyllophoraceae and more precisely to the genus *Stenogramma*. Phylogenetic analysis inferred from the plastid gene encoding the RUBISCO large subunit (*rbc*L) (Fig. 2) confirmed this preliminary placement as, in both bayesian and ML analysis, MAD2036 joined with full support the included species of *Stenogramma*. Interestingly, MAD2036 was resolved as sister to the lineage encompassing two Australian *Stenogramma*: *S. phyllophoroides* and a not yet described species reported by Calderon & Boo (2014); the other species of *Stenogramma* (*S. bamfieldiensis*, *S. californica*, *S. rhodymenioides*, *S. interrupta*) forming a second fully supported lineage. Distance analysis of the 124 bp of the *rbc*L for which sequences of the recently described *S. guleopensis* are available suggested that this later taxa also allied with the Australian species (Table 2). In light of these results, it seems that two ancestors of current *Stenogramma* have colonized two contrasted biogeographic regions. One of these ancestors likely occurred along the Pacific American coast and diversified into *S. bamfieldiensis*, *S. californica*, and *S. rhodymenioides.* Despite the poor intrinsic dispersal capacity of red algae, Gary Saunders has showed recently that kelps have likely played a key role in conveying other algae along the West American coast (Saunders, 2014). Likewise, the Arctic Ocean has long been seen as an impassable border between

Table 2. Distance matrix among the eight taxa of *Stenogramma* calculated based on the 124 bp of the *rbc*L available for *S. guleopensis* using uncorrected p-distances.

	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
1 *Stenogramma bamfieldiensis*		0.008	0.024	0.024	0.032	0.024	0.000	0.056
2 *Stenogramma californica*			0.032	0.032	0.040	0.032	0.008	0.048
3 *Stenogramma guleopensis*				0.032	0.040	0.016	0.024	0.040
4 *Stenogramma interrupta*					0.056	0.032	0.024	0.064
5 *Stenogramma lamyi*						0.032	0.032	0.064
6 *Stenogramma phyllophoroides*							0.024	0.032
7 *Stenogramma rhodymenioides*								0.056
8 *Stenogramma* sp.								

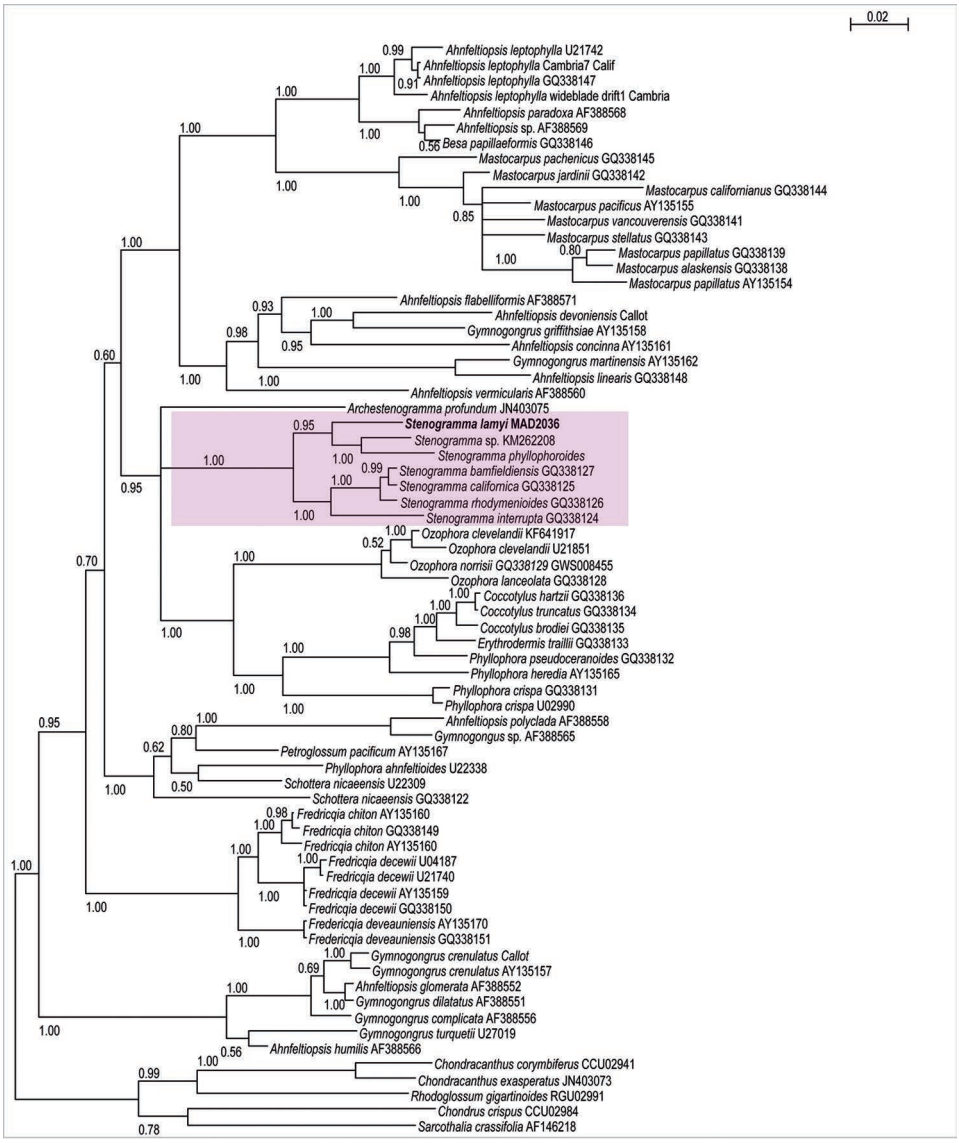


Fig. 2. Phylogram inferred from *rbc*L using Bayesian analyses. Values at nodes nodes indicate Bayesian posterior probabilities. GenBank accession numbers are indicated after the species name.

the Atlantic and Pacific oceans; nevertheless, recent insights have highlighted natural trans-Arctic interchanges (Jenkins *et al.* 2008) that have been shown successful for many algal taxa (Saunders & McDevit 2013). We can therefore hypothesize that the *S. interrupta* have a Pacific origin and colonized European waters through the Arctic route. The second ancestor likely occurred in the Asian Oceanian region and diversified to presently at least occurs in Australia (*S. phyllophoroides*), Korea (*S. guleopensis*) and Madagascar. Unfortunately there

is no molecular sequence available for the Australian species *S. leptophyllum*, to assess whether this species would also join this Asian Oceanian lineage of *Stenogramma*. In the scenario evoked here, it is worth to mention that both the west American and the Asian Oceanian ancestors have diversified and occurred presently in the northern and southern hemisphere (see Table 1).

Phylogenetic analysis inferred from *rbc*L (Fig. 2) and the very partial fragment of 124 bp (Table 1) clearly indicated that the Magalasy species was distinct to any other included species. Luckily, our analysis included sequences from the holotype of two recently described, *Stenogramma bamfieldensis* and *S. guleopensis.* In addition the two previously mentioned studies have shed light on the conundrum of the generitype and have reported sequences of all the known species of *Stenogramma* but *S. leptophyllum*. The morphology of this latter species being characterized by very narrow axes (2-3 mm) and by two or three layers of medullary cells (Womersley, 1994), it is easy to rule out the conspecificity of the magalasian taxa with this Australian species. Therefore, the magalasian species deserve to be recognised as the novel species that will be herein described as *S. lamyi*.

This novel species bring the number of formally described species of *Stenogramma* to eight and as highlighted by Kylin (1956) the distribution of those species are now seen as being restricted rather than cosmopolitan as attested by the debate of the synonymization of the generitype *S. californica* with the European species *S. interrupta*.

Ecology of *Stenogramma*

This novel species of *Stenogramma* occurred on a sandy stretch of coast where it was growing on small pebbles. This observation combined with the habitat preference of other species of *Stenogramma* recorded in the literature (see Table 1 for review) tended to show that the genus has strong affinities to sandy shores with a patchy occurrence of shells or reefs which suggest that it has good resistance to sand abrasion. In addition, the fact that *Stenogramma* is not common on substrate with a dense algal cover suggests that this species is probably not a good competitor against other algae.

Moreover, these species is always reported as subtidal and usually occur at a few meters of depth where light is attenuated in such sandy conditions.

Despite the very disjunctive distribution of species of *Stenogramma,* which occur in temperate regions of the northern and southern hemispheres, they all shared some common traits.

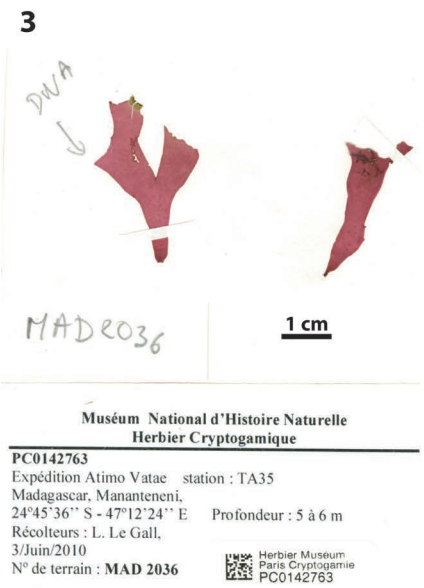
Further studies of the species diversity of the “bloody red blades” occurring in temperate waters and on subtidal environments affected by sand will most certainly unravel further species diversity within the genus *Stenogramma*.

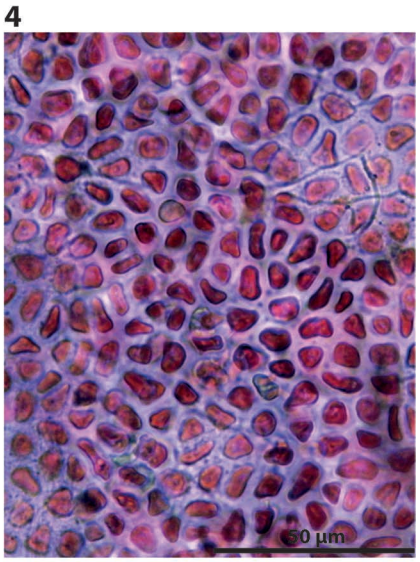
Taxonomic conclusion

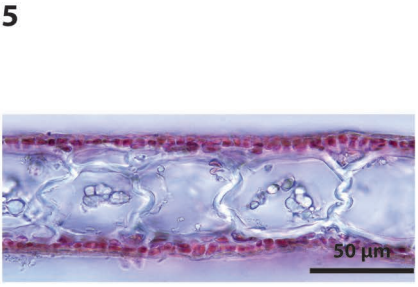
In light of molecular sequences obtained in this study, the anatomical observations which fit the genus *Stenogramma* as well as the disjunct distribution of the magalasy entity, we here proposed the thereafter described novel species.

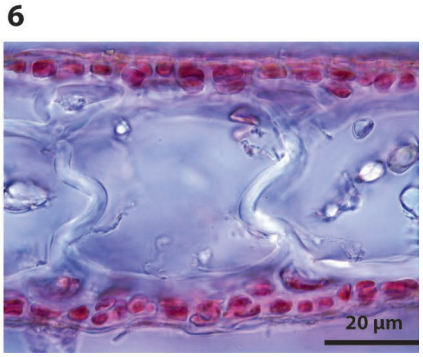
***Stenogramma lamyi* L. Le Gall sp. nov.**

Description: Small attachment disk giving rise to an erect frond three to four centimetres high and half to a centimetre wide, flattened, dichotomously divided and slightly constricted under divisions (Fig. 3). Plants rose-red to darker red in color. Surface cells (Fig. 4) angular with a maximum width from 5 to 8 µm. Structure multiaxial , thallus 80 µm thick (Figs 5-6) consisting of one layer of small cortical cells (4-8 µm) on each side of the blade and a layer of large medullary cells (50-60 µm) and one or two layers of smaller cells present between cortex and medulla. Reproduction not yet observed.









Figs 3-6. *Stenogramma lamyi:* examination of the holotype PC0142763. **3.** Gross morphology of the two specimens collected. The arrow and the hand writing DNA indicate the specimen that has been subsample for both molecular analysis and anatomical observations. **4.** Cortical cells on surface view. **5.** Transversal section showing the multiaxial structure. **6.** Transversal section showing the medullary cells and the cortex composed of small cells.

Holotype (here designated): PC0142763. Manantenina, Anosy Region, Madagascar (-24.76, 47.2067, depth -6 m), 3 June 2010, leg. L. Le Gall
Etymology: The epithet « lamyi » is in honour of our colleague Denis Lamy, who dedicated 42 years of his carrier to the study of Botany and for the main part of Cryptogamy. He showed great interest in the past, and also present, expeditions, especially the one of Madagascar during which this new species was collected.
Distribution: Plants known only from type locality (Manantenina) growing on small pebbles spread on sand-covered substrate at a depth of 6 m.
Holotype representative sequences: COI-5' (KR733111), *rbc*L (KR733113) and LSU (KR733112) available in BOLD (project STLAM).

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