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Morphological and Molecular Delineation of *Riccia (Ricciaceae, Marchantiophyta)* present In Ethiopia and Madagascar (East Africa)

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Abstract **Original Research Article**

Riccia constitutes a group of species suffering from a liverworts complaint in thalles very diversified and with which a big part of these species are still described in the especially African tropical zones more particulary in Ethiopia and in Madagascar. The identification of certain species of bryophytes according to their morphological characteristics remains even today problematic notably because of a strong phenotypic plasticity or of phenomena of anvergence. The objective is to characterize and to study the genetic diversity of several species of bryophytes of these genera *Riccia*, on all sides the sides the channel of Mozambique, by an approach of integrative taxonomy. In a first phase, we developed with the morphological data of the primary hypotheses of delimitation of species and the second part, we tested theses hypotheses by molecular analyses based on the adapted genes. The results obtained show, on a set 13 morphological hypotheses, 10 groups were confirmed by the analysis of ABGD. We shall discuss the congruence of these results of molecular delimitation with our morphological hypotheses.

Key words: *Marchantiophyta*, *Riccia*, Morphological and molecular delineation, Ethiopia, Madagascar.

INTRODUCTION

Species delineation is an old problem in biology that continues to attract considerable attention [37, 44]. In the current global biodiversity crisis, it is of paramount importance to delineate and identify taxa at the specific level as objectively as possible [33]. The existence of cryptic species [28] poses problems in ecological, physiological and genetic studies [3]. Molecular approaches to species delineation have developed rapidly over the past twenty years, generating a wide variety of methods and data. Currently, the use and comparison of several molecular approaches is advocated [10].

Bryophytes include three phyla (Anthocerotes, liverworts, mosses) that diverged early in terrestrial plants [35]. Liverworts or *Marchantiophytes* form the earliest diverging lineage in terrestrial plants probably dating from the Silurian era [18, 31, 41, 13] with monophyly [20, 23, 5, 11] supported by most molecular analyzes using, for example, the mitochondrial gene *nda5* [2], mitochondrial *19S rDNA* [9], chloroplast genes [24] or cpITS sequences [32]. Liverworts include about 391 genera and 5000 species [7] including 80 percent of leaf liverworts, with a cosmopolitan distribution taking into account the results of recent monographic works [12, 34, 36, 7].

Recent analyzes support the recognition of two groups within liverworts: *Jungermanniopsida* (*Haplomitriales*, *Metzgeriales*, *Treubiales* and *Jungermanniales*) and *Marchantiopsida* (*Sphaerocarpales*, *Marchantiales* and *Monocleales*) [1] 4].

The *Marchantiopsida* group together several orders including that of the *Marchantiales* which include the *Ricciaceae* family. It is made up of cosmopolitan species with two genera (*Riccia*, *Ricciocarpos*). These genera *Riccia* is represented by around 200 species worldwide [42], including 88 for sub-Sahelian Africa, including the Indian Ocean [43]. Only *Riccia* from North

Mediterranean Africa [15] and South Africa have been explored and described quite precisely [25]. The *Riccia* are very diverse in South Africa (around 80 species) with a high rate of endemism. For the other African countries, only five species are cited for Madagascar [22] and seven for Ethiopia [29]. One can reasonably hypothesize a sub-sampling and an analysis deficit for gender in these countries.

The objective of this work is, thanks to morphological and molecular approaches to propose hypotheses of species for the Ethiopian and Malagasy taxa, then to highlight the possible relations between these two little-known floras and with the African flora already described. A molecular approach using independent barcode markers (chloroplast *trn*L-F and nuclear *ITS*2) will complete the morphological study.

MATERIALS AND METHODS

Morpho-anatomical characterization of the study group: *Riccia* (*Ricciaceae*, *Marchantiales*, *Marchantiophyta*).

Characters common to all Riccia

The very constant morphology of the thallus develops into more or less complete lobes or rosettes, one to four times dichotomously branched. The thallus contains bottle-shaped, acropetal-growing antheridia and archegonia, followed by a reduced sporogonium (without pedicel, buried in the thallus) devoid of protective parts. The spore mass surrounds the belly of the archegonia with an absence of oil bodies and elaters [17]. Among the *Riccia*, their absence is probably due to a secondary loss, these characters have tended to simplify during the evolution of the *Marchantiidae*. It is noted that the absence of elaters associated with the burial of the sporophyte and the large size of the spores, lead to a decrease in dispersive power in *Riccia*.

Physiological characters characterize the *Riccia*, in particular the revival of the thallus and the ability to remain dehydrated, in particular over a long period (several months or even years) [21].

Characteristics that differentiate species and / or groups of species:

Dorsal tissue and pores: formed of cells arranged in files leaving between them filiform channels open to the outside by often triangular perforations appearing between the epidermal cells (*Riccia congoana*, *Riccia atropurpurea*) [17]. Aquatic *Riccia* or permanently wet muds contain in their dorsal tissue either very irregular cavities open widely to the outside (Spongodes section) or air chambers (elongated gaps) opening by simple perforations (pores) or by ostioles surrounded by small well-organized cells resembling stomata (*Riccia fluitans*).

Ornamentation of the thallus: In some *Riccia*, the dorsal surface of the thallus may be lined with short or long cilia (*Riccia microcilliata*). It can have papillae on the margin, at the top of the lateral surfaces (*Riccia papillosa*, *Riccia atromarginata*) or be covered with them (*Riccia section Pilifer*).

The walls of epidermal and subepidermal cells may thicken (*Riccia sorocarpa* and *Riccia sommieri*) [17].

Ventral scales: are sometimes well developed to cover the dorsal surfaces when the thallus is dry (*Riccia lamellosa*). The scales are sometimes pigmented pink, light purple, black purple (*Riccia nigrella*) or orange (*R. macrocarpa*).

Position of the capsule: In terrestrial species, the capsule protrudes from the dorsal surface of the thallus. It tears when ripe, abandoning the spores that spread on the ground. On the other hand, in aquatic or hydrophilic species, the capsule protrudes on the ventral surface of the thallus, tears, releases the spores which accumulate under the thallus and which are carried away by the water current [14].

Spore characters

Welding of spores: Spores form within a tetrad, the result of meiosis. In general, they are released individually except in a small number of species, *Riccia personii*, *Riccia curtisii* [16]. In the latter, they remain locked in an exine common to all 4 spores which remain in the form of a tetrad. Free spores are considered to be newer and more derived than tetrad-fused spores [26].

Sporoderm ornamentation: The micromorphology of the sporoderm surface is very variable between species or group of species but very constant within the same species. Its description provides strong characters for the identification of taxa. Seven main types of ornamentation have been described, including the honeycomb type, the most frequent. The distal face and, very often, the two faces are adorned with small or large alveoli which bear (or not) tubercles at the angles of the walls, and whose walls are smooth or granular (*Riccia trichocarpa*, *Riccia atromarginata* and *Riccia sorocarpa*).

DATA ACQUISITION

Sampling

Collection sites: In our present study, samples were collected between 2011 and 2013 in two countries in East Africa and the Indian Ocean: in the highlands of Ethiopia (Mount Guna, Nile Valley, Mount Ambafarit and Abuna Youssef in the North and in the Sanetti plateaus in the South) and in the South-West and North-East of Madagascar (Makay and Marojejy National Park).

Selection of specimens: The specimens were selected so as to retain one to five individuals of each spotted morphotype (depending on the number available). 73 specimens were selected. In order to

complete the sampling and to test the DNA quality for older specimens kept in herbarium, 17 samples from the the Muséum National d'Histoire Naturelle collections were added. A total of 90 samples were analyzed, extracted and amplified.

Identification and preparation of samples

Identification of the specimens: For those which could not be assigned to species described in the flora, primary hypotheses of morpho-species grouping together individuals which seem to us to be morphologically similar have been proposed. Based on the morphological description of the external appearance of the thallus, a table of the different morphological hypotheses encountered was established. The characters studied are as follows: 1) the form of insertion of the thallus (see if it is rosette or not); 2) the shapes of the branches (or ramifications) of the thallus: in ovals, in strips or in short and wide. 3) The color of the young and old parts and of the thallus margin. 4) The covering or not of the dorsal face of the thallus by the dry blanks. 5) The external aspect of the thallus surface (spongy or smooth appearance). 6) The ventral or dorsal aspect of the scale covering the margin of the thallus if present and the color of the scales. 7) Presence and appearance of the gutter. 8) Presence or absence of cilia or papillae on the external surfaces of the thallus; 9) the ornamentation of the spores (diameter, external structure, patterns of the proximal and distal face and the triradial mark either strong or weaker in some cases). To complete the whole set of series of images were taken for each species from different angles (Table 1).

Preparation of samples: The sample should be cleaned in order to get rid of the species of all debris (soil, roots and other species stuck on it, especially many cyanobacteria) and to avoid contamination of the DNA during of the following steps. At the end, the clean samples are placed in 2ml Eppendorf tubes and dried at 65 ° C in an oven in order to prevent the development of fungi, especially on the wet samples.

DNA extraction: Three extraction sessions were necessary. The protocol is based on a method commonly used in plants [8].

Grinding: Two tungsten beads are added to each 2ml tube, as well as a pinch of Fontainebleau sand using Lyser II Qiagen tissue at 30,000 rpm repeated 1 to 3 times.

Extraction: The DNeasy Plant Mini Kit, suitable for very small quantities of tissues, was used after a preliminary treatment with 400 µl AP1 and under the hood 30 µl of CTAB and 30 µl of proteinase K under the hood then they are placed in the thermal cycler for overnight (gentle agitation, about 300 rpm). The next day, they were purified with one volume of CIA (490 µl), mixed and centrifuged. Then the protocol is followed according to the instructions of the manufacturer of the DNeasy Plant Mini Kit QIAGEN for all the samples.

Amplification

Choice of markers: The most important characteristic of an appropriate DNA marker is its variability at the taxonomic level considered [10]. Other properties must be retained, in particular the ease of amplification, the independence or not and the propensity of a marker to convergent evolution. For the choice of markers, preliminary tests were carried out on samples from 5 markers usually used in the molecular delineation of bryophytes (*rps*4, *mat*K, rbcL, *trn*L-F, *ITS*2) [38]. The *trn*L-F (chloroplast marker) and *ITS*2 (nuclear marker) appeared to be the most regularly amplified.

Choice of primers: An important property of a primer is its universality [10] for the group of interest. This is the case with the primers used which are variable and universal within bryophytes. Too specific primers might not amplify some species of these genera (Table 2). For the trnL-F gene, a pair of external primers cf was used first, then two internal pairs (cd and ef) when the long amplification was negative (Table 2).

Amplification protocol: The amplifications were carried out in Eppendorf thermal cyclers in 20 µl of reaction containing a mixture of Taq buffer, 2 µl dNTP, 1 µl DMSO, 1 µl of BSA, the primers for the marker considered and 0.12 µl or 0.2 µl of Taq Qbiotaq or Taq polymerase and 2 to 10 µl of DNA. The extracted DNA was first used at dilution 1. PCR conditions included an initial denaturation step of 7 minutes at 94 ° C, followed by 40 or 45 cycles (one-minute denaturation at 94 ° C, one-minute annealing at an optimum temperature depending on the primer used, one-minute elongation at 72 ° C) and a final elongation step 10 min at 72 ° C.

Electrophoresis: In order to check if the amplifications are potentially to be sequenced, visualize them with the solidified agarose gel consisting of 40 ml of Tris, Acetate, Ethylene Diamine Tetra-Acetic (EDTA) commonly called TAE, 0.8 g agarose and 0.8 µl of Ethidium Bromide (BET).

Data processing

Sequencing: three plates are prepared, the first P1 containing the PCRs retained and the plates P2 and P3 respectively the Forward and Reverse primers.

Sequence cleaning and alignment: After cleaning the sequences on CodonCode Aligner and aligned via MEGA version 6 [44], the primer sequences are subtracted. Alignment was done by favoring transitions to transversions and transversions to gaps, sometimes shifting certain portions of the bases with respect to each other in order to align the bases that we consider to be homologous. In addition, *Riccia*

sequences from previous studies can be obtained from GenBank.

DATA ANALYSIS

The visualization of morpho-species by a distance tree was done using NJ distance trees (Kimura K80) with MEGA 6 version 6 [27]) from the *trn*LF and *ITS*2 alignments. This makes it possible to check the consistency of the data: the sequences producing long branches are inspected with particular attention. The questionable footage systematically blasted NCBI to identify environmental contaminations.

DNA barcoding

DNA barcoding requires setting an a priori nucleotide distance threshold below which specimens are considered to belong to the same species and above which they are considered to belong to different species (intraspecific and interspecific variations) [10, 14, 40, 6]. It allows the identification of individuals of already known species and the discovery of new species to reference databases. These variations consider that individuals of a given species have more molecular similarities than individuals of different species. By plotting the distribution of distances between pairs of sequences in a dataset, when this distribution reveals an obvious gap (called a Barcode gap), a threshold placed in this space can be used to delineate the species [19].

Sequence analysis and partitioning with ABGD software

The method, called Automatic Barcode Gag"p Discovery (ABGD) is a method of dividing a set of sequences in an alignment into groups, considered to be molecular hypotheses of species. ABGD allows a change in the group delimitation threshold [30]. The parameters used are P = 0.01 and X = 0.5 with P (the maximum intraspecific limit distance) and X (the minimum value of the barcode gap). Geneious's "Species Delimitation" plugin was used to obtain summary information on the species hypotheses (ABGD groups): maximum intraspecific distance, interspecies distance from the nearest monophyly group.

RESULTS AND DISCUSSION

Transfer of morpho-species hypotheses to a distance tree: With the tree obtained with the *trn*L-F sequences without those of Genbank, we confirm our groups of primary hypotheses of morpho-species (FIG. 1). Regarding the *ITS*2 alignment, given the low number of sequences, only two groups of more than one individual could be recognized ((FIG. 1). Samples CR13E64, CR13E44 and CR11615 (*R. sp* with scales black) form and CR11528 and CR11564 (*R. vulcanicola*) each form a clade within which the distances are very close, *R.sp.5* (maximum intra specific distance 0.006 and minimum interspecific distance 0.027), *R. vulcanicola* (maximum intra specific distance specific 0.002 and minimum inter-specific distance 0.054) Other morpho-species are not represented only by a sample but which appear genetically distant from the others (interspecific distance with the closest morpho-species *R. sp4* d = 0.086). By count morpho-species are dispersed within two clades *R. atropurpurea*. The specimens CR13E50, CR13E51, CR13E55 and CR13E57 form a clade. This clade which is a little distant (d = 0.007) from the second clade CR13E11A and CR13E14 which are sample s placed in the same hypothesis. Most morpho-species are recognized by ABGD for trnL-F except *R. congoana* which is partitioned into three (group 2.8 and 9) (FIG. 3). For ITS2, a certain number of morphospecies are recognized despite the low number of sequences obtained. However, the group circled in gray (FIG. 2) shows a mixture of specimens belonging to several morpho-species.

The synthesis provided by the "Species Delimitation" plugin for trnL-F indicates an average minimum interspecific distance d = 0.0504 (Table 3). This table is not presented for ITS2, given the small number of sequences and individuals per group.

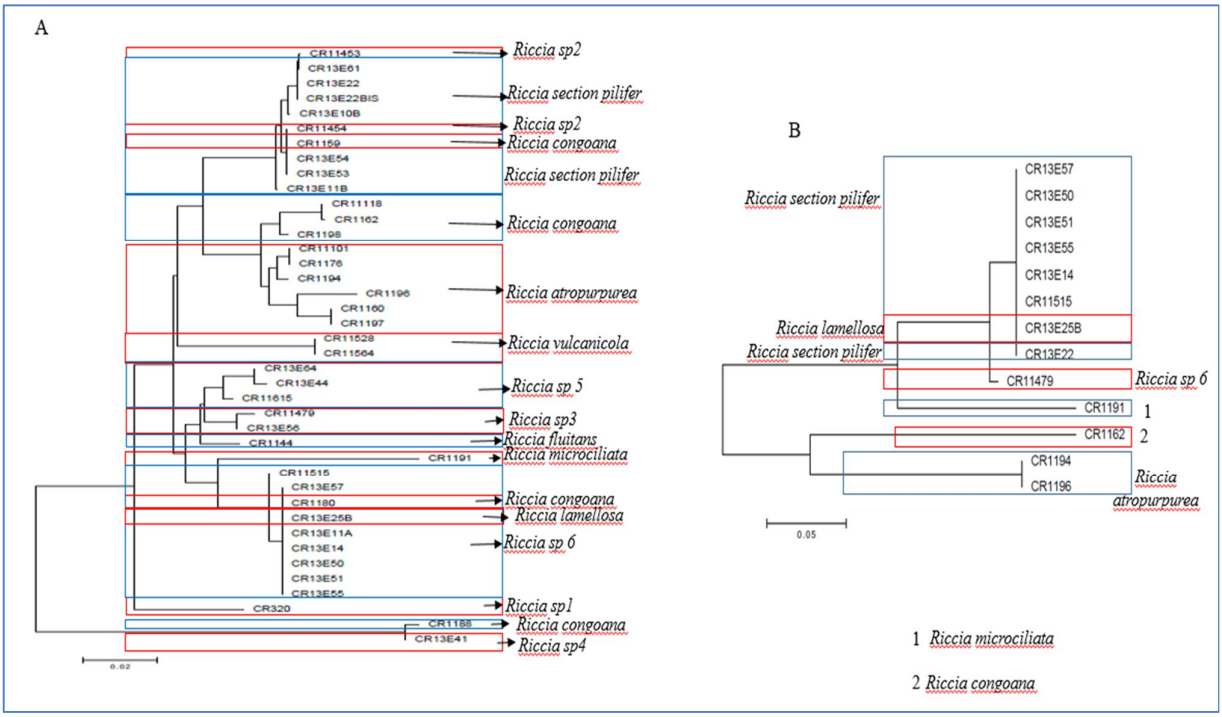

Fig-1

The inclusion of GenBank sequences was carried out as a second step, because the number of specimens per morpho-species became so low that ABGD in general can provide biased assumptions in this case [30]. We had to modify the parameters of P and choose P = 0.05 in order to find the previously delimited groups as well as the specimens identified on GenBank as different morphospecies. The interest of this last analysis is to compare the sequences of specimens identified with the Ethiopian and Malagasy specimens of our study. Only *Riccia lamellosa* shows a low genetic distance (intra-specific distance = 0.002) with CR13E25B, making it possible to confirm that these two specimens belong to the same species. The other specimens included are either in independent groups or grouped within the same group (group 4: group of *Riccia section pilifer*).

Figure 1: Distance tree (*Neighbor-Joining test*) resulting from the analysis of the *Trn*L-F (A) and *ITS*2 (B) sequences. The colors (blue or red) denote the morphological hypotheses (morpho-species) developed during the sample identification phase

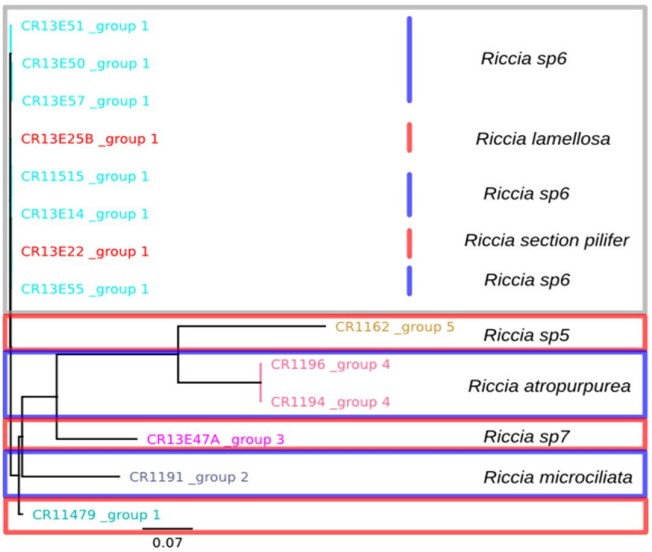

Fig-2

Figure 2: Boundary tree produced by ABGD for trnL-F with the parameters Pmin = 0.001; Pmax = 0.01, X (relative gaps with) = 0.5, step = 10 and Kimura (K80). Each ABGD group is colored in a specific color. Each blue or red rectangle represents a morpho-species whose name is shown on the right.

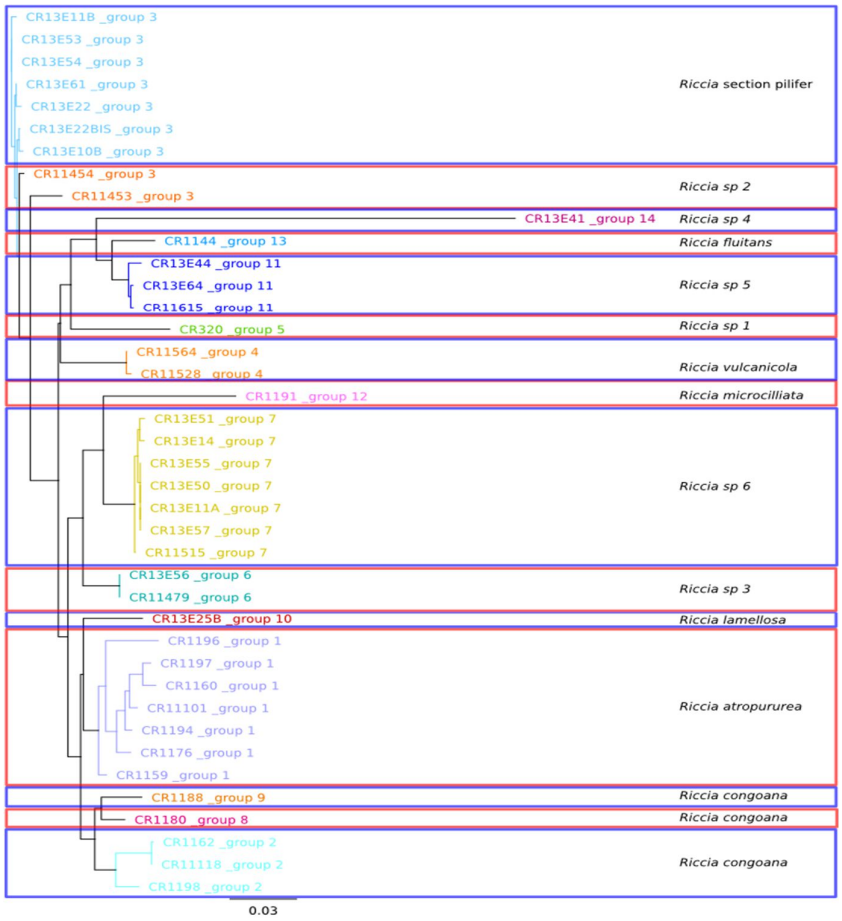

Fig-3

Figure 3: Tree of primary molecular hypotheses produced by ABGD for *ITS*2 with parameters Pmin = 0.001; Pmax = 0.01, X (relative gap width) = 0.5; Step = 10 and Kimura (K80) TS / TV. Each ABGD group is colored in a specific color. Each blue or red rectangle represents a morpho-species whose name is shown on the right. The gray rectangle indicates a mixture of morphological hypotheses.

Table-3: The inter and intra specific distances between the closest morphospecies.

Species	Espèce la plus proche	Monophylie?	Intra Dist	Inter Dist - + proche esp.
1: pilifer	2: sp4	no	0.004	0.014
2: sp2	1: pilifer	no	0.021	0.014
3: fluitans	4: sp5	oui	0	0.03
4: sp5	3: fluitans	oui	0.006	0.03
5: sp1	1: pilifer	oui	0	0.07
6: vulcanicola	1: pilifer	oui	0.002	0.052
7: microcilliata	8: sp6	oui	0	0.075
8: sp6	9: sp3	oui	0.003	0.042
9: sp3	8: sp6	oui	0	0.042
10: lamellosa	13: congoana	oui	0	0.047
11: atropurpurea	13: congoana	oui	0.023	0.045
12: congoana	13: congoana	oui	0	0.028
13: congoana	12: congoana	oui	0	0.028
14: congoana	13: congoana	oui	0.018	0.037
15: sp4	4: sp5	oui	0	0.203

DISCUSSION

This study allows us to propose 9 secondary hypotheses of species, confirmed by morphoanatomy and molecular delineation of species by a chloroplastic *trn*LF and nuclear gene *ITS*2: *Riccia sp1*, *Riccia sp2*, *Riccia sp3*, *Riccia sp*4, *Riccia sp5*, *Riccia atropurpurea*, *Riccia fluitans*, *Riccia microcilliata* and *Riccia vulcanicola*.

However, the incongruences noted for *Riccia congoana* (3 groups ABGD with *trn*LF, *Riccia section pilifer*, *Riccia lamellosa* (identical sequences for ITS2 with *Riccia sp6*) are not sufficient to definitively eliminate these species. Indeed, for *Riccia* section *pilifer* and *Riccia lamellosa* there is concordance between *trn*LF and the morphospecies (supported by the GenBank sequence for *Riccia lamellosa*.). It is probable that: (1) the low number of specimens in *ITS*2 could have led to a bias for the group; (2) or more likely that there were errors in the sequencing of the two specimens CR13E25B and CR13E22. Based on the results of the analysis, there is no congruence (correspondence) between the morpho- hypotheses. Species collected between the two study sites on either side of the Mozambique Channel, namely in Madagascar and Ethiopia. By account, there are species common between Ethiopia and continental African species like *Riccia vulcanicola* or *Riccia lamellosa* which has already been described. For Madagascar there is also correspondence between studied species and species already described in the African continental zone (*Riccia atropurpurea*, *Riccia congoana* and *Riccia microciliata*) even sometimes European (*Riccia fluitans*). *Riccia sp1*, *Riccia* sp*2*, *Riccia sp4*, *Riccia sp5*, *Riccia sp6* could not be identified requiring further study: species already described or new species for science.

The results highlight the diversity of *Riccia*. The *trn*L-F sequences show a certain divergence with the sequences deposited on NCBI (except *Riccia lamellosa*), which reveals that all our sequences belong to species not yet sequenced or not yet deposited. ABGD detects the deviation from the barcodes it uses for data partitioning. For this difference to be as correct as possible, the number of specimens per sample must be at least greater than 3 [30]. In conclusion ABGD is fast, it is a simple method to divide a set of sequences into molecular species that must be supplemented by other evidence in an integrative taxonomic approach.

CONCLUSION

This study is an approach to the little-known diversity of complex-thallus liverworts of these genera *Riccia* from tropical areas, both to discover a group that we rarely have the opportunity to approach and to measure the extent of the work remaining. The diversity of liverworts, and in particular of these genera, is unknown, and under-studied in these countries with the exception of some citations. These results showed that this group represents a particularly interesting case of study for taxonomists: solving an integrative taxonomy is a challenge that cannot be met with a large number of specimens and the right appropriate markers plus a good in-depth study of the morphology. The various results obtained during this study not only enrich our knowledge of biodiversity, but also provide essential material for analyzing the processes that are at the origin of this biodiversity. A sampling effort is needed for all countries in mainland Africa and countries in the Indian Ocean.

Conflict of Interest

The authors hereby declare that there is no conflict of interest.

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Appendices

Table-1: Morphospecies assumptions established during sample identification

Morpho-species	Specimens	Port of the thallus	Color dorsal surface	Size	Shape of branches	Color of scales	Appearance of dorsal surface	Spores characters	Other Remark
Riccia fluitans L.	CR1144	In a rosette with long, filamentous tufts	Yellow green	5-10mm long, 1-3mm wide	Long straps	No scales			Dichotomous branching and no scales
Riccia congoana	CR1180 CR1162 CR1188 CR1198 CR11118	Partially irregular rosette	Green yellow Blue green	4-10mm long, 2-5mm wide	Large ovals	Peripheral black scales	Reticulated dorsal surface	Spores yellowish-brown, wingless and strong triradial mark	Narrow scalloped border
Riccia atropuupurea	CR1176 CR1160 CR1196 CR1194 CR1197 CR11101 CR1159	incomplete or isolated thalli	blue gray, blue-green	15 mm long max, branches 2 to 3 mm wide	linear to weakly oval	typical: black then purple passing to white at the margin		Black brown spores	Clear white margin when dry, the edges of the thallus often touching above the dorsal surface
Riccia section pilifer	CR3E61 CR13E22B CR13E22A CR13E53 CR13E54 CR13E10B CR13E11B	Thallus in rosettes	Green yellow	4-10mm long, 2-5mm wide	Ovals	Black scales on the sides and top	Spongy-looking thallus		Thalli with dorsal face with white green papillae giving it a velvety appearance
Riccia sp 1	CR320	Thallus in rosettes	Gray green	4-10mm long, 2-5mm wide	Oval and wide	No scales			Gutter inconspicuous
Riccia microciliata OH Volk & Perold	CR1191	Thalles in rosette	Gray green	6-18mm long, 4-8mm wide	Oval and wide thalli	No scales	Thallus a little smooth	Triangular globular spores and reticulate ornamentation	Thallus with hyaline log cilia in several crowded rows at the apex, arched and channeled
Riccia sp2	CR11453 CR11454	Thallus in rosettes	Green white	3 to 10 mm long, 1 to 5 mm wide	Oval thalli	White colored scales	Thallus with a rather smooth appearance		

Riccia sp3	CR13E56 CR11479	Thalles in rosette	Green yellow	4-10mm long, 2-5mm wide	Oval thalli	No scales	Spongy-looking thallus		
Riccia sp4	CR13E41	Thalles in rosette	Green-white	4-10mm long, 1-5mm wide	Oval fronds				
Riccia vulcanicola	CR11528 CR11564	Thalle in tender-textured rosettes	Gray green Green yellow	2 to 13 mm long, 6 to 10 mm wide	Thallus in rosettes		Spongy-looking thallus	Yellow-brown to light brown triangular globular spores, thin wing	Dorsal surface with distinct vesicles and pores or gaps in the epidermis
Riccia sp 6 (yellow green)	CR13E14 CR11515 CR13E51 CR13E50 CR13E55 CR13E57 CR13E11A	Thallus in rosette	Green-white Green yellow	3 to 15 mm long, 2 to 4 mm wide	Oval thalli	White peripheral scales	Spongy-looking thallus	Whitish border covered with white scales	
Riccia sp5 (black scales)	CR13E41 CR13E64 CR11615	Thallus in rosettes	Green yellow	3 to 15 mm long, 2 to 4 mm wide		Shiny peripheral black scales			Black scales forming a continuous layer on the edges of the thallus
Riccia lamellosa	CR13B25B	Thalles in rosette	Green yellow	6 to 20 mm long, 6 to 10 mm wide		No scales	Smooth thallus	Bifurcated fronds	Concave thalli with smooth surface, inconspicuous gutter

Table-2: Primer pairs used.

Uncomfortable	**Region**	**Sequence**		**Taq**	**Temperature**
		Primer F	**Primer R**		
trnL cf	Chloroplastic	CGAAATTGGTAGACGCTACG	ATTTGAACTGGTGACACGAG	TaqPol	55 ° - 58.1 °
trnL cd		CGAAATTGGTAGACGCTGCG	GGTTCAAGTCCCTCYAYCC	TaqPol	55 ° - 58.1 °
trnL ef		GGTTCAAGTCCCTCCACCCC	ATTTGAACTGGTGACACGAG	TaqPol	55 ° - 58.1 °
ITS2 5.8F & 25R	Nuclear	GCAACGATGAACGCAGC	TCCTCCGCTTAGTGATATGC	QbioTaq	58 ° - 59.6 °