Morphological and molecular data reveal a new species of *Alocasia* (Araceae) from Vietnam

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Abstract:

Alocasia rivularis **(Araceae) is proposed as a new species from central Vietnam. The new species is morphologically similar to those of the Cuprea Group of *Alocasia*, but according to results of the *trn*L-*trn*F IGS and *mat*K sequences, and based on morphological differences, it is clearly different from the closest congeners. Phylogenetic analysis, descriptions, and illustrations are provided.**

Keywords: ***Alocasia rivularis, Araceae, central Vietnam, new species, phylogeny.***

Classification number: ***3.5***

Introduction

Alocasia was first used as a section of *Colocasia* (Araceae) by Schott (1832) [1] and was raised to generic rank by Don in Sweet (1839) [2]. It includes more than 100 species occurring primarily in the subtropical eastern Himalaya as throughout subtropical and tropical Asia into the tropical western Pacific and eastern Australia [3-5]. In Vietnam, seven species of *Alocasia* have been recorded, including *A. cucullata, A. evrardii, A. lecomtei, A. longiloba, A. macrorrhizos, A. Odora,* and *A. Vietnamensis,* plus another three species with doubtful distribution in Vietnam, namely *A. hainanica*, *A. Acuminata,* and *A. navicularis* [6-8]. In general, Vietnam is one of the countries where the genus is least understood [9].

In November of 2015, we conducted a field trip to Nui Dau Mountain, Nghia Hanh District, in Quang Ngai Province, and encountered a population of an aroid species superficially resembling either *Alocasia* or *Colocasia* (Araceae), which have distinguished peltate leaf blades and fruiting spathes. A set of specimens were then collected (Luu 1110, SGN). Our subsequent surveys collected up until 2016 of the areas did not yield inflorescences. Our examination of the collected specimens determined that the fruit were 6-8 mm in diameter, had 1 to 2 mature seeds of 5-6 mm in diameter, and had 12 to 13 unfertilised and reduced seeds with basal placentation. These facts would suggest placing the species (which is hereafter referred to as *A. rivularis*) in *Acolosia* rather than in *Colocasia* as the latter has < 3 mm fruits with many small seeds and ovaries with parietal placentation [5, 10]. *A. rivularis* seems to resemble those of the Cuprea Group (such as *A. beccarii* Engl.*, A. cuprea* K.Koch*, A. perakensis* Hemsl., and *A. peltata* M.Hotta), which typically has nearly completely peltate adult leaves alternating with long cataphylls and the staminate zone of the spadix being mostly to completely within the lower spathe [4]. However, in our collections, no cataphylls alternating with adult leaves were found, and the position of the staminate zone within the spathe was not known. Besides that, the number of ovules per ovary, which is possibly 13-14 as drawn from the examined fruits, is a few more than the range of 6 to 10 found in known species of *Alocasia,* but lower than that (i.e. very numerous) found in those of *Colocasia* [3-5, 9, 11]. Due to the morphological divergence of *A. rivularis*, we have used molecular data to ascertain its phylogenetic relationship to known species of *Alocasia*, especially those of the Cuprea Group, and other genera of the tribe Colocasieae, applying the methods employed similarly in previous phylogenetic studies of the Araceae [12-16].

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Table 1. Specimens of sixteen taxa of Araceae species sequenced in this study.

Sequenced voucher number	Taxon	Sequenced voucher number	Taxon
H.T. Van 99	*Acorus verus*	H.T. Van 54	*Homalonema cochinchinensis*
H.T. Van 69	*Aglaonema simplex*	H.T. Van 56	*Lasia spinosa*
Luu 1110	*Alocasia rivularis*	H.T. Van 102	*Pistia stratiotes*
H.T. Van 119	*Alocasia odora*	H.T. Van 61	*Pothos chinensis*
H.T. Van 03	*Amorphophallus scaber*	H.T. Van 70	*Rhaphidophora chevalieri*
H.T. Van 79	*Arisaema roxburghii*	H.T. Van 60	*Schismatoglottis harmandii*
H.T. Van 98	*Cryptocoryne ciliata*	H.T. Van 104	*Scindapsus officinalis*
H.T. Van 67	*Epipremnum giganteum*	H.T. Van 42	*Typhonium trilobatum*

Table 2. Sequences from the GenBank database used in the study [14].

Taxon	GenBank accession no. (*trn*L-*trn*F/*mat*K)
Alocasia beccarii	JQ238731/JQ238817
Alocasia cuprea	JQ238740/JQ238826
Alocasia gageana	JQ238742/EU886580
Alocasia grandis	JQ238743/JQ238828
Alocasia heterophylla	JQ238744/JQ238829
Alocasia hollrungii	JQ238745/JQ238830
Alocasia peltata	JQ238766/JQ238850
Alocasia perakensis	JQ238767/JQ238851
Ariopsis protanthera	AY248947/EU886587
Colocasia esculenta	JQ238804/JQ238890
Englerarum hupnosum	JQ238746/JQ238831
Leucocasia gigantea	JQ238807/JQ238893
Protarum sechellarum	JQ238810/KC466580
Remusatia vivipara	JQ238812/JQ238897
Steudnera assamica	EF517214/JQ238898

Table 3. Primers used in the present study.

Primers (*)	Region	Sequence (5’-3’)	References
C (F)	*trn*L-*trn*F IGS	GGTTCAAGTCCCTCTATCCC	[18]
D (R)	*trn*L-*trn*F IGS	ATTTGAACTGGTGACACGAG	[18]
MF (F)	*mat*K	ACCCAGTCCATCTGGAAATCTTGGTTC	[19]
MR (R)	*mat*K	CGTACAGTACTTTTGTGTTTACGAG	[19]

(*) Direction of primer, F = forward, R = reverse.

Materials and methods

DNA samples taken from fresh leaves of the collected specimen (Luu 1110) and fifteen other taxa of Araceae collected from southern regions of Vietnam were used in this study (Table 1). All respective vouchered specimens were collected following the Kew Botanic Garden protocols [17] and deposited at SGN. Sequences of *trn*L-*trn*F IGS and *mat*K regions of additional species representing typical genera of the tribe Colocasieae from GenBank were also used (Table 2).

The total genomic DNA was extracted from fresh leaf tissues using a Genomic DNA Purification Mini Kit (Thermo, USA). The *trn*L-*trn*F IGS and *mat*K chloroplast DNA regions were amplified using the polymerase chain reaction (PCR). A list of primers is shown in Table 3. The PCR reactions were observed in an Eppendorf Mastercycler Gradient using a volume of 25 µl reaction mixture: 12.5 µl go taq green master mix (Promega, USA), 1.25 µl of each forward and reverse primers (10 µM), 9.5 µl HPLC water, and 0.5 µl DNA template (25 ng). PCR cycles consisted of an initial denaturation for five minutes at 95°C; 35 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 50°C) and extension (1:30 minutes at 72°C); and a final extension at 72°C for 10 minutes. The PCR products were visualised in a 1.5% agarose gel and sent for purification and direct sequencing at Nam Khoa Biotek Company Ltd. (Vietnam) using an ABI 3130 XL Sequencer.

For multiple alignments, the Clustal W [20] was used to recognise the homology between sequences. Phylogenetic analysis was carried out with the software PAUP*4.0a146 [21], using the maximum parsimony and neighbor-joining methods of *Acorus* *verus* (Acoraceae) as the outgroup, following Cabrera, et al. [12], Cusimano, et al. [13], and Nauheimer, et al. [15]. The maximum parsimony trees were calculated based on chloroplast sequence data using gaps treated as missing data and heuristic search algorithms [22] with the following parameters: 1,000 random addition sequence replicates, tree bisection and reconnection (TBR) branch swapping, and 10 parsimonious trees held after each replicate [23, 24]; and all characters were equally weighted and treated as unordered [25]. The fit of characters to the trees was also tested by calculating the consistency index (CI), the retention index (RI), and the rescaled consistency index (RC) [26, 27]. The neighbor-joining tree was constructed based on the matrix of pairwise distances between species [28]. The statistical support for phylogenetic trees was carried out using the bootstrap method [29] with 1,000 replicates. The bootstrap values of more than 50% were performed in the discussed trees. The pairwise genetic distances [30] were calculated using the software MEGA6 [31].

Besides that, morphological characteristics were used in the comparison of the new species and its close congeners.

Results

The length of the combined *trn*L-*trn*F IGS and the *mat*K data sets of studied species ranged from 1,160 to 1,179 bp. The entire aligned length of the two regions was 1,459 bp. The phylogenetic analysis of the combined data sets resulted in a parsimonious tree (length: 859 steps, CI: 0.80, RI: 0.67, RC: 0.53) and the neighbor-joining tree (Fig. 1, 2).

In the neighbor-joining tree, *A. rivularis* and other *Alocasia* species were grouped within one clade with a

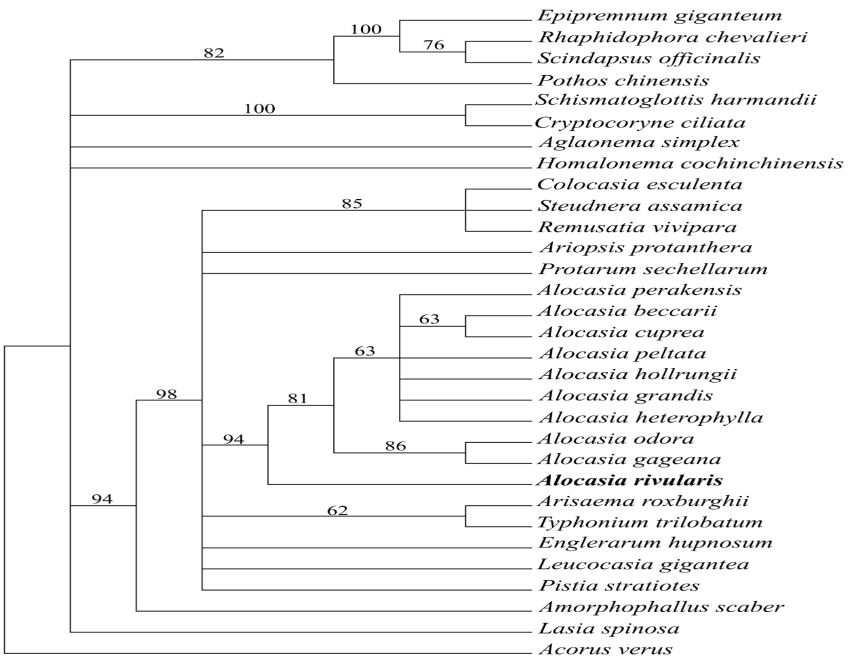

Fig. 1. One of the largest parsimonious trees obtained based on the combined trnL-trnF IGS and matK data sets. Gaps were treated as missing data. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes. Tree length = 859 steps, CI: 0.80, RI: 0.67, RC: 0.53.

Fig. 2. The neighbor-joining tree obtained from the combined trnL-trnF IGS and matK data sets. The bootstrap values of 50% or more than from 1,000 replicates are shown above the nodes.

bootstrap value = 58%. Meanwhile, a very high bootstrap value (94%) was found in the parsimony tree which showed *A. rivularis* as sister to the remaining *Alocasia* species.

Comparing the *trn*L-*trn*F IGS and *mat*K sequences of *A. rivularis* with those of the Cuprea Group studied (i.e. *A. beccarii*, *A. cuprea, A. peltata* and *A. perakensis* which have *trn*L-*trn*F IGS and *mat*K sequences available in the GenBank) after alignment, we found 11 and 8 variable positions among 408 and 730 positions of the *trn*L-*trn*F IGS and *mat*K sequences, respectively. The number of different substitutions was 5, 6, 7, and 5 in the *trn*L-*trn*F IGS region and 5, 4, 3, and 6 in the *mat*K regions, respectively (Table 4, 5). As results showed, the pairwise genetic distances of *A. rivularis* to any of the *A. beccarii, A. cuprea, A. peltata,* and *A. perakensis* ranged from 0.008 to 0.009 while those found between each pair of the latter four species range from 0.003 to 0.009 (Table 6).

Table 4. Variable nucleotide positions of the trnL-trnF IGS region among Alocasia species.

	43	89	184	221	257	258	259	260	305	335	371
A. rivularis	G	C	A	G	-	-	-	-	A	C	G
A. beccarii	A	T	A	G	-	-	T	T	C	C	G
A. cuprea	A	T	C	G	-	-	-	T	C	A	G
A. peltata	A	T	A	C	T	T	T	T	A	C	G
A. perakensis	A	T	A	G	-	-	T	T	A	C	A

Table 5. Variable nucleotide positions of the matK region among Alocasia species.

	29	97	277	305	373	405	508	711
A. rivularis	C	G	T	C	C	T	A	A
A. beccarii	T	G	T	T	C	C	C	-
A. cuprea	C	G	A	T	C	C	A	-
A. peltata	C	G	T	T	C	C	A	-
A. perakensis	C	T	T	T	T	-	-	-

Discussions

In both analyses, the order of the genera and species are in agreement with those in the previous studies [12-16].

The shown phylogenetical trees obviously indicate that *A. rivularis* is closer to the species of *Alocasia* than to those of the other genera in this study, including *Colocasia esculenta*. As a result, the study species should be placed in *Alocasia* rather than *Colocasia*; which is supported by its morphological

Table 6. Mean pairwise genetic distances among species of the tribe Colocasieae based on the combined trnL-trnF IGS and matK data sets.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. *Alocasia odora*																
2. *Alocasia gageana*	0.003															
3. *Colocasia esculenta*	0.012	0.012														
4. *Steudnera assamica*	0.013	0.013	0.001													
5. *Remusatia vivipara*	0.016	0.016	0.004	0.005												
6. *Ariopsis protanthera*	0.023	0.023	0.019	0.020	0.023											
7. *Protarum sechellarum*	0.013	0.013	0.012	0.013	0.011	0.023										
8. *Leucocasia gigantea*	0.012	0.012	0.011	0.012	0.012	0.022	0.009									
9. *Englerarum hypnosum*	0.009	0.007	0.008	0.009	0.011	0.019	0.008	0.007								
10. *Alocasia rivularis*	0.004	0.004	0.011	0.012	0.015	0.022	0.012	0.011	0.008							
11. *Alocasia heterophylla*	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005						
12. *Alocasia grandis*	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005	0.003					
13. *Alocasia beccarii*	0.005	0.005	0.015	0.016	0.016	0.026	0.013	0.015	0.012	0.007	0.004	0.004				
14. *Alocasia cuprea*	0.008	0.008	0.018	0.019	0.019	0.026	0.016	0.018	0.015	0.009	0.007	0.007	0.005			
15. *Alocasia hollrungii*	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005	0.003	0.003	0.004	0.007		
16. *Alocasia peltata*	0.004	0.004	0.013	0.015	0.018	0.024	0.015	0.013	0.011	0.005	0.003	0.003	0.004	0.007	0.003	
17. *Alocasia perakensis*	0.007	0.007	0.016	0.018	0.020	0.027	0.018	0.016	0.013	0.008	0.005	0.005	0.007	0.009	0.005	0.005

characteristics, i.e. 1-loculed and 1- to 2-seeded fruits of 6-8 mm in diameter and ovaries with basal placentation.

The numbers of different substitutions in the *trn*L-*trn*F IGS and *mat*K sequences between *A. rivularis,* and each species of the Cuprea Group (i.e. *A. beccarii*, *A. cuprea, A. peltata,* and *A. perakensis*) are equal to or higher than those found between each pair of the latter four species (ranging from 1 to 5 in the *trn*L-*trn*F IGS and 1 to 3 in the *mat*K region). Consequently, the pairwise genetic distance of *A. rivularis* to any of each *A. beccarii, A. cuprea*, *A. peltata,* and *A. perakensis,* is equal to or higher than that calculated for each pair of the four latter. Similarly, the genetic distance between *A. rivularis* to any of the other studied *Alocasia* species (i.e. *A. heterophylla* (C.Presl) Merr., *A. grandis* Clemenc. and *A. hollrungii* Engl.) is higher than that found in at least one pair of them (Table 6). Therefore, the newly found species is well phylogenetically separated from all the other mentioned *Alocasia* species.

Morphologically, *A. rivularis* closely resembles *A. peltata,* which is distributed in Borneo, in scattered localities in Sarawak, Brunei, and central Kalimantan. Both species share similar shapes and dimensions of leaves, fruits and seeds as well as the diameter of stem. However, *A. peltata* is readily distinguishable from the Vietnamese congener due to its height of ca. 30 cm, fewer leaves, symmetric leaf blades, an anterior lobe 2-3 times as long as the combined posterior lobes, 5-6 ovuled ovaries, an inflorescence per axil, and obovoid fruiting spathes [4, 11]. Further, *A. peltata* is distributed in the mossy forest floor on ridges at ca. 1,200 m altitude, while our species are found in the dipterocarp-dominated lowland tropical evergreen forest at around 80 m altitude.

The shape of the leaf blades in *A. rivularis* also looks similar to that of the *A. minuscula* A.Hay from Sarawak, but the latter has a smaller size (height of 10-20 cm and stem of ca. 1 cm diameter, very thick and smaller leaf blades (8-13x2-3 cm), more primary lateral veins (8-10 on each side of midrib), striate secondary venation, and shorter petioles (5-10 cm long) with shorter petiole sheaths (ca. 1/7 of petiole length) [4].

Three other *Alocasia* species that have leaf blades similar to those in *A. rivularis* are *A. beccarii* from northwestern Borneo are *A. perakensis* from Peninsula Malaysia and Thailand, and *A. kerinciensis* A.Hay from Sumatra (Indonesia) and but these three latter, like the two compared just above, are readily distinguishable from our new species by their cataphylls interspersed with leaves and symmetric leaf blades. In addition to that, *A. beccarii* and *A. kerinciensis* are different from *A. rivularis* because *A. beccarii* has a small habit (12-28 cm tall), a slender stem that is 5-10 mm in diameter, very long internodes, ca. 2 cm long, and ovoid fruiting spathes, while *A. kerinciensis* has a smaller stem (1 cm diameter, and up to ca. 40 cm long) with internodes that are 5 cm long, an anterior costa with 2-3 primary lateral veins on each side, and smaller ovoid fruiting spathes (ca. 2 cm long) [4, 9]. *A. perakensis* is further distinct from *A. rivularis* in having thick leaf blades, ovoid fruiting spathes, ellipsoid, and red fruits [9].

Taxonomic treatment

Alocasia rivularis Luu, Nguyen-phi & H.T. Van (Fig. 3).

Diagnosis: The new species is morphologically similar to *A. peltata*, *A. minuscula*, *A. beccarii*, *A. kerinciensis,* and *A. perakensis,* and differs in having no cataphylls interspersed with leaves, asymmetric leaf blades with the anterior lobe 5-6 times as long as combined posterior lobes, two inflorescences per axil, oblong ellipsoid, and longer fruiting spathes.

Type: VIETNAM. Quang Ngai Province, Nghia Hanh District, Hanh Tin Dong Commune, Nui Dau Mountain, 14°52’23.63”N, 108°48’49.48”E, around 80 m in elevation, 14 November 2015, *Hong Truong Luu & Hoang Minh Duc Luu 1110* (holotype, SGN; isotypes, SGN & VNMN).

Herbs to 70 cm tall have the following characteristics: *Stem* rhizomatous, elongate, slender, unbranched, and 20-26 mm in diameter, with internodes nearly as wide as long, as well as decumbent and then erect, and often completely exposed. Also having *leaves* ranging from several to 11, clustered at the tips of stems, peltate, and drying brown; *petioles* pale green, glabrous, and 25-35 cm long; petiolar sheath 8-15 cm long, mostly persistent; *blades* narrowly lanceolate to oblong-ovate, asymmetric, sized 16-24x7-8.5 cm, shining, and thinly coriaceous on both surfaces, adaxially dark green, and abaxially pale green; midribs adaxially impressed, and abaxially prominent; *anterior lobes* 12-20 cm long, with 1 cm long acuminate tip, which are 5-6 times as long as the combined posterior lobes; anterior costae with four adaxially impressed, abaxially prominent primary lateral veins on each side (subopposite) diverging at ca. 45-60° which run straight or upcurved into a conspicuous intramarginal vein 1-1.5 mm from the margin; secondary venations inconspicuous to be invisible; *posterior lobes* completely united except for a 1.5-2 mm incision at the extreme base of the leaf; and combined posterior lobes 3-4 cm long, which are widest at the petiole insertion. Also, infructescences two, peduncle 18-24 cm, 6-8 mm in diameter; fruiting spathes which are dark green, oblong ellipsoid,

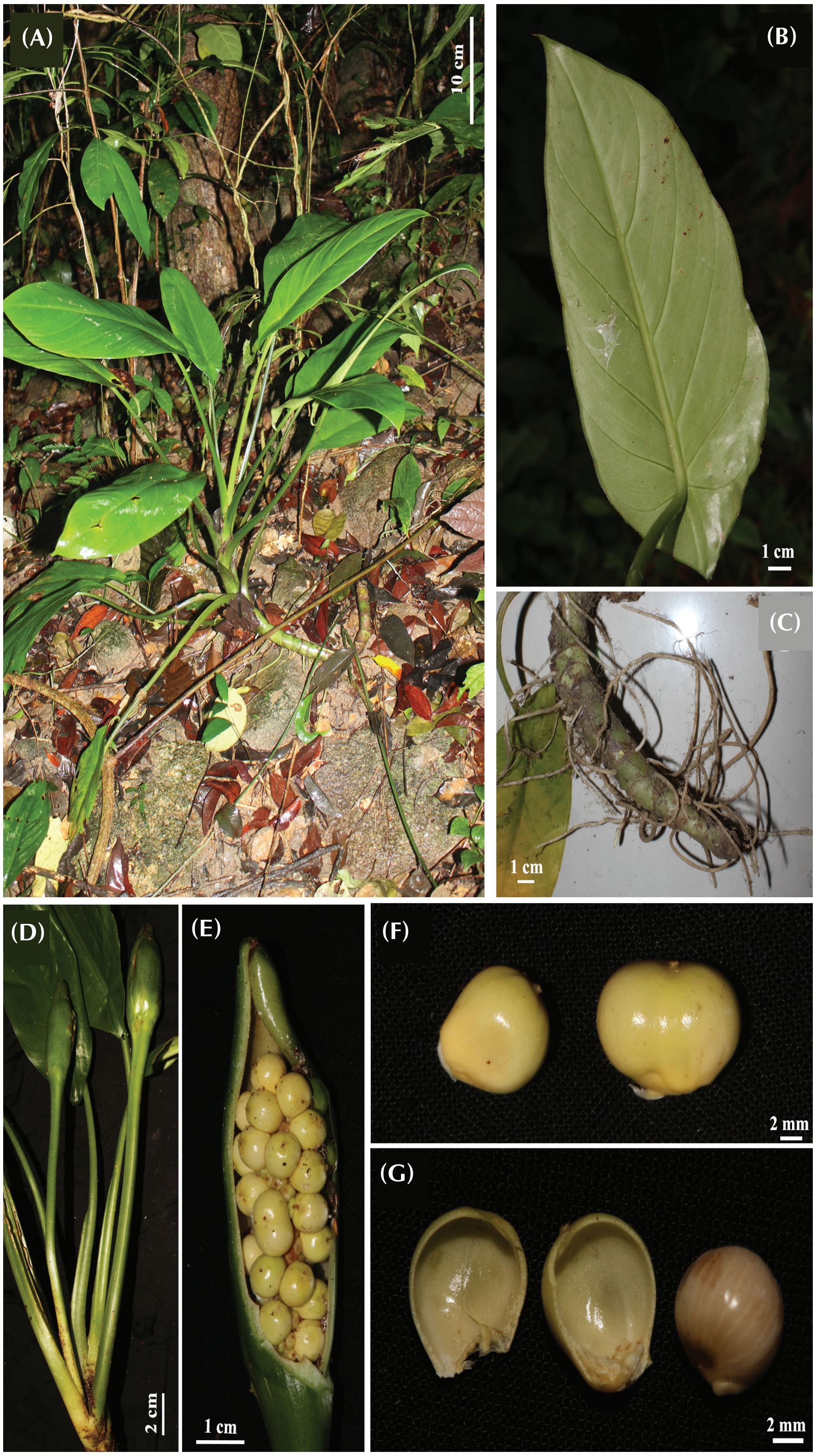

Fig. 3. Alocasia rivularis Luu, Nguyen-phi & H.T. Van. **(A)** The species in the habitat, **(B)** Leaf blade, abaxial surface, **(C)** Rhizome, **(D)** Infructescences, **(E)** Fruiting spike, **(F)** Fruits, and **(G)** Opened berry showing one seed with basal placentation.

6.5-7.5 cm long, ca. 2 cm in diameter, and opened at the middle; fruits obovoid, pale greenish yellow, and usually 7 mm in diameter; and seeds 1 and rarely 2, round, pale pinkish to brownish white, with longitudinal white veins, which are 5-6 mm in diameter.

Habitat: *A. rivularis* is found in the lowland evergreen tropical forests on fertile soils along small streams and ponds; fruits were seen in November.

Distribution: The new species has been found only from the type location.

Etymology: Named to reflect the typical habitat of the species which grows along small streams and ponds.

Based on the available literature [3-10] and the authors' field observations, the known species of *Alocasia* in Vietnam (except those doubtful) can be distinguished by the following key:

1A. Plant epiphytic; spadix with staminodes at the base of the female portion..2

1B. Plant geophytic; spadix without staminodes at the base of the female portion..3

2A. Stem tuberous, subglobose .... *A. evrardii*

2B. Stem rhizomatous, elongate .... *A. vietnamensis*

3A. Leaf blade with lateral veins originating almost from base; basal lobes not distinct ....*A. cucullata*

3B. Leaf blade with lateral veins originating from midrib; basal lobes clearly distinct.....................................4

4A. Adult leaves asymmetric; posterior lobes almost completely united .... *A. rivularis*

4B. Adult leaves partially peltate or not peltate, symmetric; posterior lobes partially united at base or not.............5

5A. Mature plants massive, at least 2 m tall..6

5B. Mature plants not as above............7

6A. Stem erect to decumbent; leaves peltate ....*A. odora*

6B. Stem erect; leaves not peltate .... *A. macrorrhizos*

7A. Leaf blade sagittate; posterior lobes triangular.............................*A. longiloba*

7B. Leaf blade ovate to obovate; posterior lobes rounded.......................*A. lecomtei*

Conclusions

The morphological and molecular data as presented above indicate that *A. rivularis* from Quang Ngai Province, Vietnam should be treated as a new good species of *Alocasia*.

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