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Research Article
Phylogeny of *Alpinia coriandriodora* D. Fang and Implications for Character Evolution and Conservation

[1]Xuan Duong Vu, [2]Chi Toan Le, [2]Thi Bich Do, [1]Phi Bang Cao, [3]Quoc Binh Nguyen, [3]Tien Chinh Vu, [4]Trong Luong Dang, [5,6]Van Du Nguyen and [7]Bing Liu

[1]Hung Vuong University, Nong Trang, Viet Tri, Phu Tho, Vietnam
[2]Ha Noi Pedagogical University No. 2, 32 Nguyen Van Linh, Xuan Hoa, Phuc Yen, Vinh Phuc 15000, Vietnam
[3]Vietnam National Museum of Nature, Vietnam Academy of Science and Technology (VAST), 18 Hoang Quoc Viet, Hanoi, Vietnam
[4]Agricultural Genetics Institute (AGI), Pham Van Dong, Hanoi, Vietnam
[5]Institute of Ecology and Biological Resources (IEBR), Vietnam Academy of Science and Technology (VAST),
Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
[6]Graduate University of Science and Technology, Vietnam Academy of Science and Technology (VAST),
Hoang Quoc Viet, Cau Giay, Hanoi, Vietnam
[7]State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

Abstract

Background and Objective: *Alpinia*, the largest genus of Zingiberaceae, includes ca. 250 species. The *A. coriandriodora* D. Fang was recognized for Vietnamese flora. However, the systematic position of this species within *Alpinia* genus was unclear. The study aimed to understand the phylogenetic placement of *A. coriandriodora* based on the molecular data and interpret evolution of the key morphological characters. **Materials and Methods:** The phylogenetic analysis were conducted by using the combined dataset of two DNA regions by both Maximum Likelihood (ML) and Bayesian Inference (BI) methods. Seven morphological characters were selected for morphological character evolution and the analysis was performed in Mesquite. **Results:** *Alpinia coriandriodora* was supported closely related to southern Chinese species of *Alpinia*. Morphological character optimizations suggest that the presence/absence of tomentum in leaf, inflorescence rachis and ovary is an important character for the taxonomy of *Alpinia*. The character evolution analyses indicated that panicle is ancestral character in *Alpinia*. The *A. coriandriodora* shares different evolutionary histories based on our character re-construction to most members of Southeast Asian *Alpinia*. The presence of filament is supposed to be an adaptation to the pollination by insects for species of *Alpinia*. **Conclusion:** The present study revealed the molecular phylogenetic relationship of *A. coriandriodora* within *Alpinia*. The presence of filament could be an adaptation to the pollination by insects for species of *Alpinia*. Some reasonable conservation strategies are proposed to protect the species including maintenance of the plant's natural habitats, seeds or seedlings collection for germplasm storage and artificial breeding using biotechnology.

Key words: Zingiberaceae, *Alpinia coriandriodora*, molecular phylogeny, monophyly, morphology, evolution, conservation

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Corresponding Author: Chi Toan Le, Ha Noi Pedagogical University No. 2, 32 Nguyen Van Linh, Xuan Hoa, Phuc Yen, Vinh Phuc 15000, Vietnam

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alpinia Roxb. is the largest and most widespread genus in Zingiberaceae with ca. 250 species, which is distributed in tropical and subtropical Asia, Australia and Pacific Islands[1,2]. Several species of *Alpinia* are important for their ornamental, medicine and economic values. Additionally, *Alpinia* also plays an important ecological role in the understory vegetation of tropical and subtropical forests[1]. The genus is characterized by their horizontal or pendulous, broad labellum and filament usually shorter than corolla or labellum.

The phylogenetic relationship among *Alpinia* species has not been clearly understood yet due to missing data and poor sampling. Rangsiruji *et al.*[3] recognized nine clades of *Alpinia* based on a dataset from 47 species of *Alpinia*. They demonstrated significant statistical support for several monophyletic groups of species of *Alpinia* and suggested that this genus may not be monophyletic.

Kress *et al.*[4] addressed the relationships among genera in the Zingiberaceae by using data from both ITS and matK regions. The study showed well-resolved phylogenetic relationships among the genera and provided a new classification of the Zingiberaceae with four subfamilies and four tribes. Kress *et al.*[4] recognized four major clades of *Alpinia*, demonstrated that a number of the larger genera in the family such as *Amomum*, *Alpinia*, *Etlingera*, *Boesenbergia* and *Curcuma* could be non-monophyletic as well as suggested that more extensive sampling is necessary for these genera.

Kress *et al.*[1] conducted the molecular phylogeny of *Alpinia* based on 72 taxa of *Alpinia* and using two molecular makers matK and ITS. The results of this study showed that *Alpinia* is polyphyletic, six clades within *Alpinia* were recognized. A new classification of the tribe Alpinieae was also provided by this study. However, the monophyly and phylogenetic position of a number of *Alpinia* species have not yet been established.

Alpinia coriandriodora D. Fang was described by Fang[5] and recognized as an endemic species in Guangxi, China. The *A. coriandriodora* species is of cultural importance, which was used as food and local medicines for hundreds of years. Vu *et al.*[2] reported *A. coriandriodora* from Vietnam as a new record for the country. The authors suggested that the morphology of *A. coriandriodora* is similar to *A. bambusifolia* and only differs in having an elliptic-lanceolate leaf blade, light yellow labellum with red-brown stripes and triangular anther crest. However, the phylogenetic relationship of *A. coriandriodora* within *Alpinia* has not been investigated by using molecular data. Furthermore, the status of *A. coriandriodora* species is endangered and needs to be preserved under the exploitation of people. The present study aims to conduct molecular analyses to understand the phylogenetic position of *A. coriandriodora* within *Alpinia* as well as the relationship among other *Alpinia* species and interpret evolution of the key morphological characters and provide some recommendations for the conservation of the species.

MATERIALS AND METHODS

Study area: The study was carried out at National Key Laboratory of Gen Technology-Institute of Biotechnology, Herbarium (HN) of Institute of Ecology and Biological Resources under the Vietnam Academy of Science and Technology, Faculty of Natural Sciences-Hung Vuong University, Department of Botany, Ha Noi Pedagogical University No. 2 from May, 2019-July, 2020.

Taxon sampling, DNA extraction, amplification and sequencing: Total genomic DNA was isolated from silica gel-dried leaves of the collections GD01LT, GD02LT, GD03LT, GD01XD, GD02XD, GD03XD. The Vouchers of the collections were deposited in the Herbarium (HN) Institute of Ecology and Biological Resources, Vietnam Academy of Science and Technology. Total 12 new sequences were generated for two markers matK and ITS. The primers and protocol for PCR and sequencing in this study followed by Kress *et al.*[1].

It was successfully extracted total DNA and conducted a PCR reaction for 06 samples with the two markers matK and ITS. The total DNA without color and soluted in TE. The PCR products have high quality and clear in an electrophoresis gel, sequencing was performed using the primers designed by Kress *et al.*[1]. The sequences were aligned in Geneious v.8.0.5[6].

Molecular phylogenetic analysis: We added the new sequences into a dataset with a global sampling scheme of *Alpinia* from previous study[1]. The combined dataset was partitioned into two subsets corresponding to two gene regions and was analyzed with the Maximum Likelihood (ML)[7] and Bayesian Inference (BI) methods[8]. The ML analysis was conducted in RAxML 8.2.10[7], applying 1,000 bootstrap replicates with the GTR+I+G substitution model selected in jModeltest 2.1.6[9]. The Bayesian analysis was conducted in MrBayes 3.2.6[8] as implemented on the CIPRES Science Gateway Portal[10] based on the same model as in the ML analysis. The Markov chain Monte Carlo (MCMC) algorithm was run for 5,000,000 generations with a total of four chains, starting from a random tree and trees were sampled every

Table 1: List of the 7 morphological characters and character states scored for the morphological character evolution analysis of *Alpinia*

Leaf tomentum: Glabrous (0), sparsely to densely hairy (1)
Inflorescence type: Raceme (0), panicle (1), spike (2)
Inflorescence rachis tomentum: Glabrous (0), sparsely to densely hairy (1)
Ovary tomentum: Glabrous (0), sparsely to densely hairy (1)
Filament: Absent (0), present (1)
Bract: Absent (0), present (1)
Staminodes: Absent (0), present (1)

Table 2: Morphological matrix of *Alpinia* and outgroups

Species	01	02	03	04	05	06	07
Alpinia coriandriodora-1	1	2	1	1	1	1	1
Alpinia coriandriodora-2	1	2	1	1	1	1	1
Alpinia guangdongensis	1	1	1	0	1	1	1
Alpinia stachyodes	0	2	1	1	1	1	?
Alpinia japonica	1	0	1	1	1	1	1
Alpinia guinanensis	1	1	1	1	1	1	1
Alpinia kwangsiensis	1	0	1	1	1	1	0
Alpinia calcarata	0	1	1	1	1	1	1
Alpinia officinarum	0	0	1	1	1	1	0
Alpinia glabra	0	1	0	1	1	1	0
Alpinia vulcanica	0	2	0	0	1	1	?
Alpinia modesta	0	2	0	1	1	0	?
Alpinia elegans	0	2	0	0	0	0	1
Alpinia purpurata	0	2	0	0	0	0	1
Alpinia carolinensis	0	1	0	0	0	1	1
Alpinia javanica	0	1	0	0	0	0	0
Alpinia rafflesiana	1	0	1	1	0	1	0
Alpinia nigra	0	1	1	1	1	1	1
Alpinia aquatica	0	1	0	0	0	0	0
Siliquamomum tonkinense	0	0	1	0	1	1	1
Zingiber officinale	0	2	1	?	1	1	1
Distichochlamys citrea	0	2	0	0	0	1	1
Curcuma longa	0	2	1	1	1	0	1
Siphonochilus kirkii	0	2	0	0	0	1	1

"?" represents missing data; 01: Leaf tomentum, 02: Inflorescence type, 03: Inflorescence rachis tomentum, 04: Ovary tomentum, 05: Filament, 06: Bract, 07: Staminodes

1000 generations. The program Tracer v.1.6[11] was used to check that Effective Sample Sizes (ESSs) were attained for all relevant parameters assessed (>200). With the first 25% of sampled generations discarded as burn-in, the 50% majority-rule consensus tree and Bayesian Posterior Probabilities (PP) were obtained using the remaining trees.

Morphological character evolution: We selected 23 species of *Alpinia* and outgroups for the reconstruction of ancestral characters, all selected species represented for all the sections and clades from the results of Kress *et al.*[1] and this study. Seven morphological characters concerning leaf tomentum, inflorescence type, inflorescence rachis tomentum, ovary tomentum, filament, bract and staminodes were selected for ancestral character optimization (Table 1).

The morphological description of *Alpinia* used several morphological characters, however, the seven above characters have usually been highlighted in the description and classification of Zingiberaceae and *Alpinia*[2,4,12], thus we corroborated each character stated by examining specimens and consulting published works[2,12]. The morphological dataset was presented in Table 2. Additionally, it reduced the taxa sampling in the molecular dataset as in the morphological dataset but including *Zingiber officinale* and *Curcuma longa* as out groups and two individuals of *Alpinia coriandriodora* to construct a molecular simplified tree for morphological character evolution analysis.

The evolutionary history of each of the seven characters was traced over the Bayesian 50% majority-rule tree with 24 taxa sampled, using ML approach available in Mesquite v.2.75[13]. We used the Markov k-state one-parameter model and the option "Trace Character History" to reconstruct each character.

RESULTS

Phylogenetic relationship: Twelve newly generated sequences in this study were submitted to GenBank and the

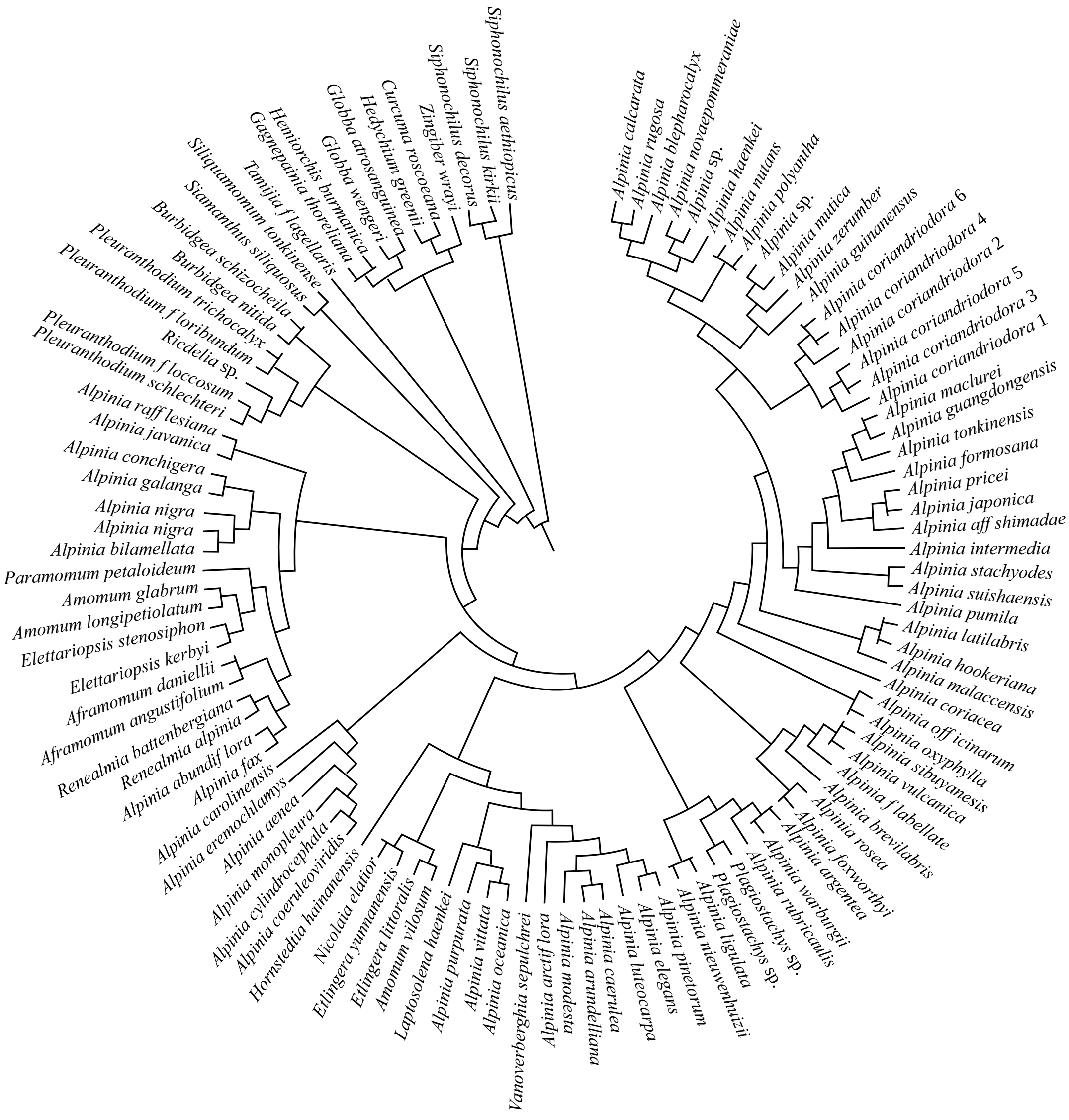

Fig. S1: Maximum Likelihood (ML) tree derived from analysis of matK gene

Table 3: Voucher information and GenBank accession numbers for DNA sequences generated in this study

Species	Country of origin	Vorcher	matK	ITS
Alpinia coriandriodora D. Fang	Vietnam	GD01LT (HN)	MN335320	MN227653
Alpinia coriandriodora D. Fang	Vietnam	GD02LT (HN)	MN335321	MN227654
Alpinia coriandriodora D. Fang	Vietnam	GD03LT (HN)	MN335322	MN227655
Alpinia coriandriodora D. Fang	Vietnam	GD01XD (HN)	MN335323	MN227656
Alpinia coriandriodora D. Fang	Vietnam	GD02XD (HN)	MN335324	MN227657
Alpinia coriandriodora D. Fang	Vietnam	GD03XD (HN)	MN335325	MN227658

voucher information and GenBank accession numbers of the sequences were presented in Table 3. The single data sets of matK and ITS have 3023 and 729 pb respectively, the combined molecular dataset with 3752 (pb) aligned positions across all taxa.

The molecular results of single makers showed lower resolution of relationships within *Alpinia* than the combined dataset (Fig. S1 and S2). Thus, we used the combined dataset (matK and ITS) to analyze the phylogenetic relationship within *Alpinia* and systematic position of *A. coriandriodora*.

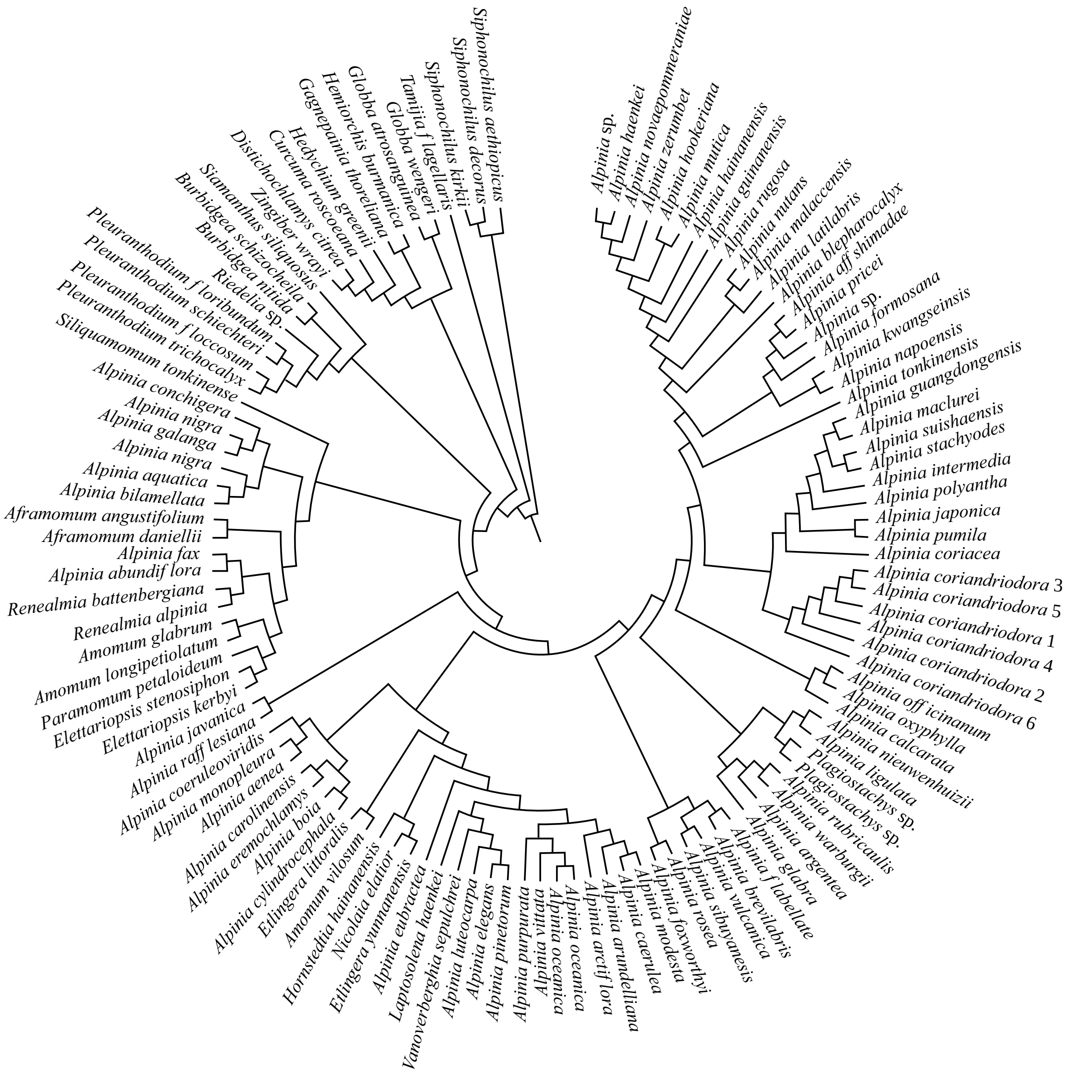

Fig. S2: Maximum Likelihood (ML) tree derived from analysis of ITS gene

The results from the combined dataset by ML and BI analyses are highly congruent and few differences have low support. Thus, we combined results in ML tree, the detailed phylogenetic relationships within *Alpinia* are presented in Fig. 1a. The molecular analysis strongly supported *Alpinia* as a non-monophyletic group (BS: 100%, PP: 1.0) (Fig. 1). Six clades were recognized within *Alpinia*. *A. coriandriodora* was supported as a member of *Alpinia* and placed into clade IV (Fig. 1b).

Character evolution: The reconstruction of the ancestral character state for *Alpinia* based on the ML method was shown in Fig. 2-5. For characters 1, 3, 4, "glabrous" was inferred to be the ancestral state in the genus *Alpinia* (Fig. 2, 3). Reconstructions of characters 1, 3, 4 indicated that the presence of hairs on leaf, inflorescence and ovary was derived more than once in *Alpinia* clade (Fig. 2, 3). While, for character 5, the presence of filament is not unique in *Alpinia* and also occurs in *Zingiber*, *Curcuma* and *Siliquamomum* (Fig. 5). Whereas reconstructions of characters 6 indicated that the presence of bracts was inferred as ancestral character, most members of *Alpinia* have bracts and only few species evolved without bracts (Fig. 5). Reconstructions of characters 2 and 7 are quite complex (Fig. 4), all three types of inflorescence were evolved multiple times in *Alpinia*, that are the similarity of absent staminodes in character 7. Additionally, some approaches for *ex situ* conservation of *A. coriandriodora* were shown in Fig. 6.

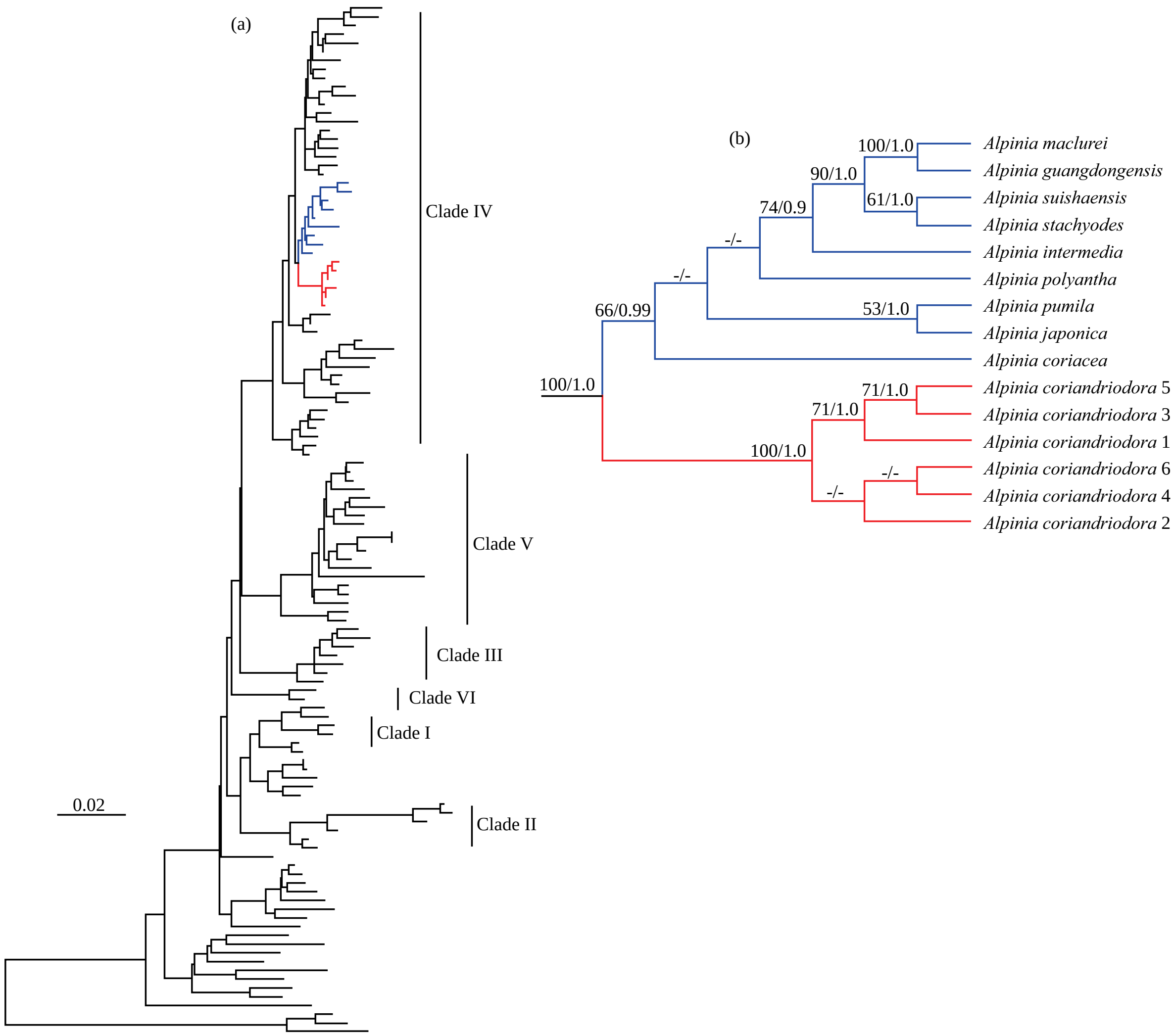

Fig. 1(a-b): Maximum Likelihood (ML) tree derived from analysis of combined dataset matK and ITS, (a) A phylogram overview and (b) Clade including *Alpinia coriandriodora*

ML bootstrap values and Posterior Probabilities (PP) of the BI analysis are presented above the branches."–" indicates the support values less than 50%

DISCUSSION

Results of the present study indicated that suggested *Alpinia coriandriodora* is close to southern China *Alpinia*. Morphological character significance suggested that *Alpinia* adapt to the pollination by insects and need an approach to protect and increase the population. Our molecular results are congruent with Kress *et al.*[1] and *Alpinia coriandriodora* was strongly supported as a member of clade IV. Fang[5] suggested that *A. coriandriodora* is similar to *A. warburgii* K. Schum. and *A. stachyodes* Hance. *A. coriandriodora* and *A. warburgii* are similar by their caudate leaf apex, appendage on connective but *A. coriandriodora* differs from *A. warburgii* by the spike inflorescence, ternate flowers, presence of bracts and bracteoles, smaller calyx, short corolla lobes with pubescent hairs. While, *A. coriandriodora* and *A. stachyoides* are similar by their spike inflorescence and ternate flowers but *A. coriandriodora* differs from *A. stachyodes* by petiolate leaf, caudate leaf apex, non-obvious bracts and bracteoles, larger flowers, sinuate margin of labellum, absence of appendage on connective. The two species *A. warburgii* and *A. stachyodes* were recognized as members of clade IV by both Kress *et al.*[1] and the present study (Fig. 1, S1 and S2). Moreover, our molecular results indicated that *A. coriandriodora* was supported as sister to *A. stachyodes* (Fig. 1, S1 and S2), while *A. warburgii* was placed far from the clade including *A. coriandriodora* and *A. stachyodes*.

Additionally, Vu *et al.*[2] suggested that *Alpinia coriandriodora* is similar to *A. bambusifolia*, the endemic species from Guangxi and Guizhou, China. However,

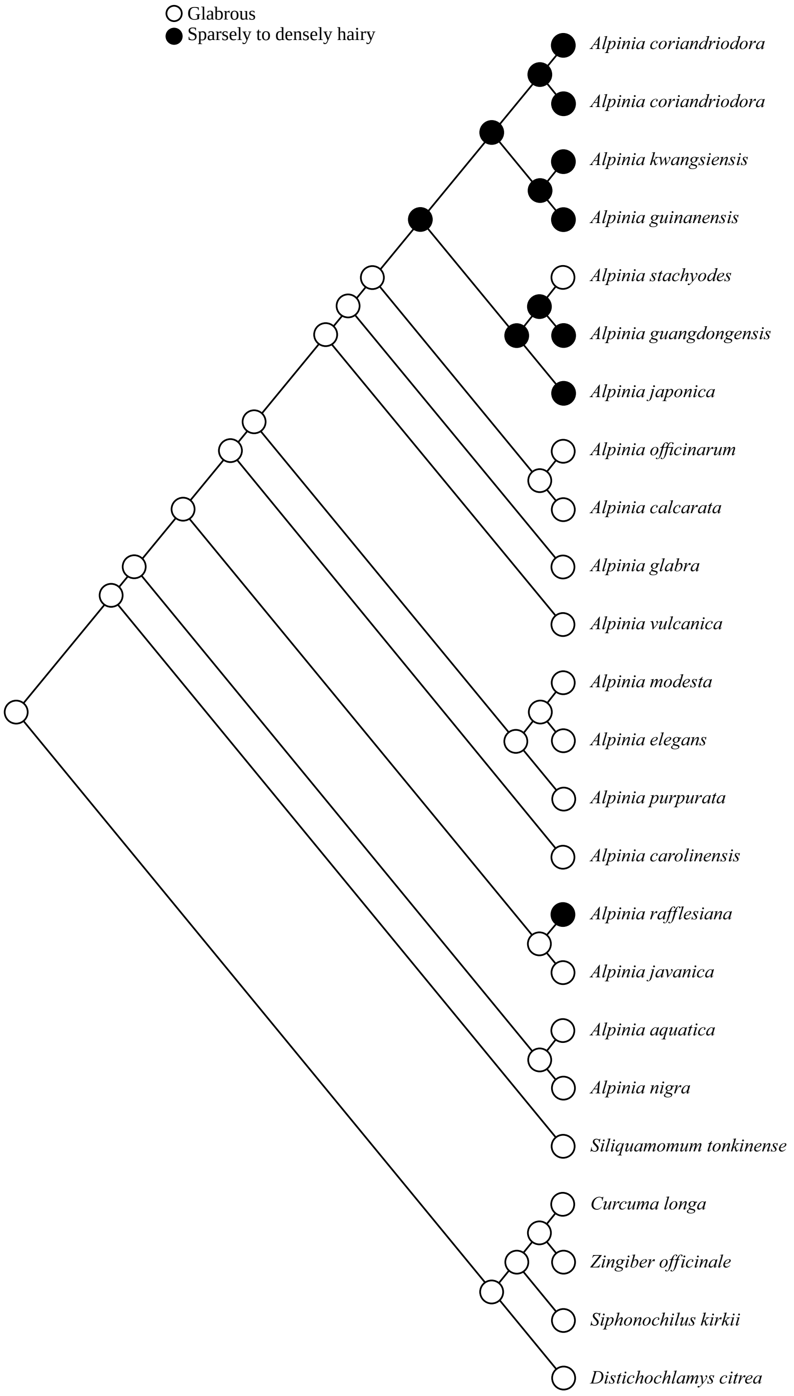

Fig. 2: Character optimization for leaf tomentum (character 1) inferred on a Bayesian majority-rule tree for 24 taxa, based on the maximum likelihood method

A. coriandriodora can be easily distinguished with *A. bambusifolia* by elliptic-lanceolate leaf blade (vs. narrowly lanceolate in *A. bambusifolia*), yellow calyx from base to middle and purple-red from the middle to the apex (vs. pale purple-red in *A. bambusifolia*), yellow labellum with red-brown stripes (vs. white with red stripes in *A. bambusifolia*), triangular and entire anther crest (vs. absent in *A. bambusifolia*) and globose fruits (vs. cylindrical in *A. bambusifolia*). However, the molecular data of *A. bambusifolia* is not available in both previous studies

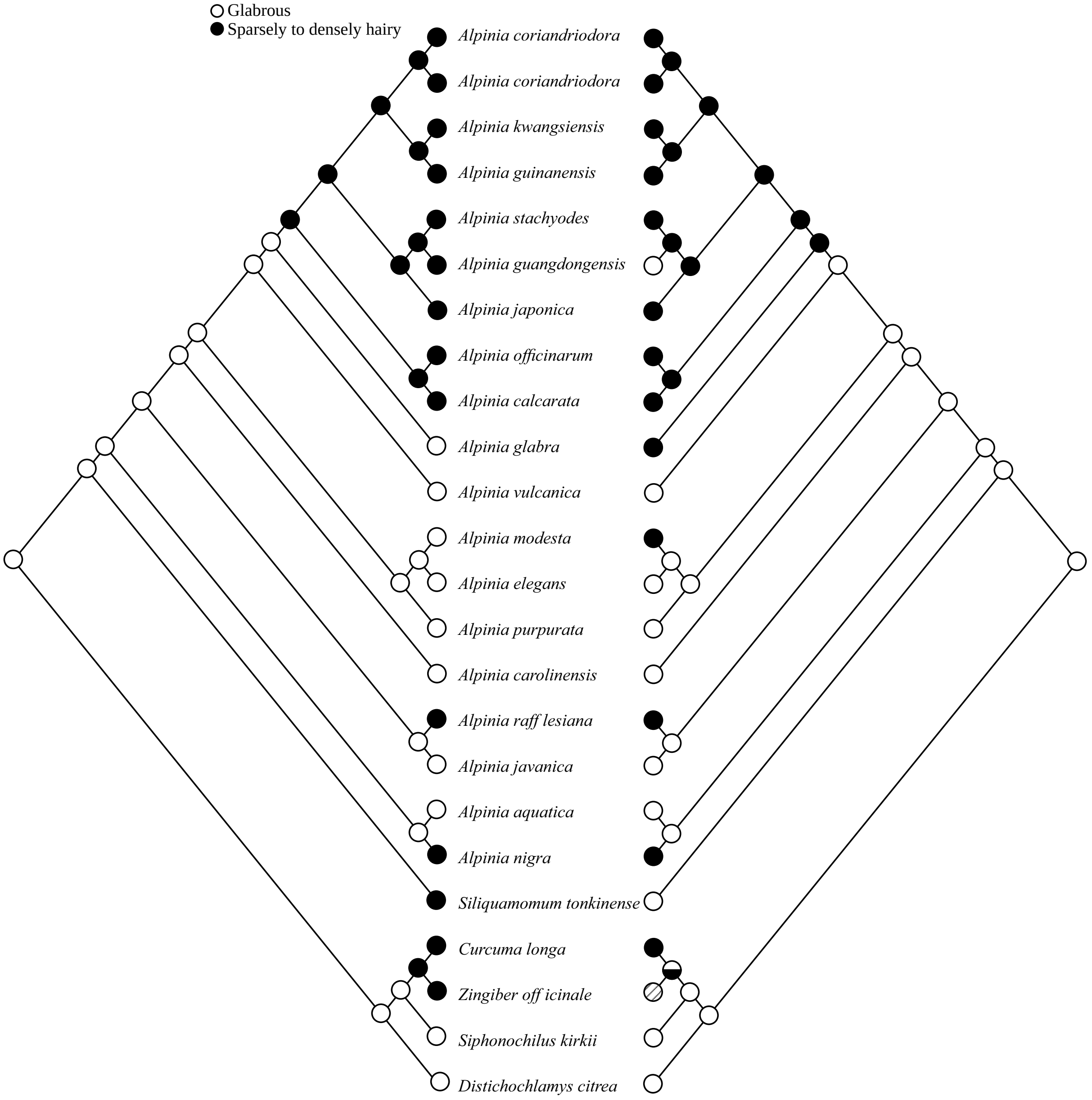

Fig. 3: Character optimization for inflorescence rachis tomentum (character 3) and Ovary tomentum (character 4) inferred on a Bayesian majority-rule tree for 24 taxa, based on the maximum likelihood method

and this study, thus the future study including data of *A. bambusifolia* should be conducted to determine the phylogenetic relationship of these related species within *Alpinia*.

The phylogenetic results reported here are an advancement over previous analyses[1,4] in terms of taxon and gene sampling. *Alpinia coriandriodora* is closely related to southern Chinese *Alpinia* species (Guangdong, Guangxi, Hainan and Yunnan) such as *A. japonica*, *A. coriacea*, *A. warburgii*, *A. stachyodes* and *A. guangdongensis* with strong support (BS: 100%, PP: 1.0) (Fig. 1). Moreover, the molecular results supported *A. coriandriodora* as sister to a subclade within clade IV (including some species above plus others). Furthermore, *Alpinia* was distributed in whole Vietnam including subtropical and tropical regions[2,12,14]. Luu *et al.*[14] and Vu *et al.*[2] recorded 34 species of *Alpinia* for flora of Vietnam. The molecular analyses indicated that 19 of 34 species of Vietnamese *Alpinia* were placed in clade IV (Fig. 1, S1 and S2). Thus present result suggested that *A. coriandriodora* also is closely related to Vietnamese *Alpinia*.

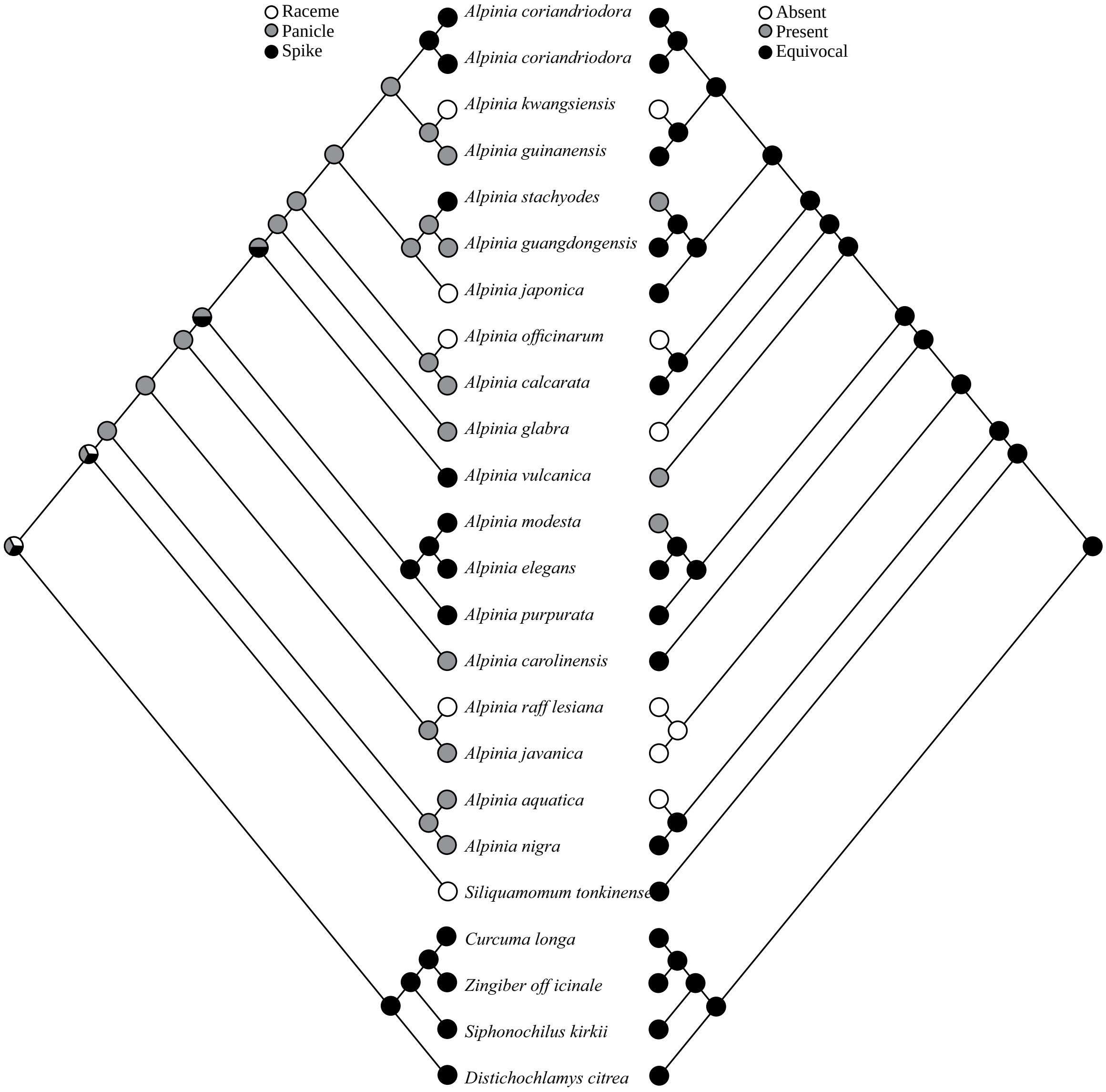

Fig. 4: Character optimization for inflorescence type (character 2) and staminodes (character 7) inferred on a Bayesian majority-rule tree for 24 taxa, based on the maximum likelihood method

In fact, *Alpinia coriandriodora* shares some similar morphological characters with other *Alpinia* in southern China and northern Vietnam such as inflorescence a panicle, raceme or spike, bracteoles flat or concave, bracts present. Thus, the molecular result of this study supported the treatments of Fang[5] and Vu *et al.*[2]. Moreover, the results suggested that *A. coriandriodora* shares the ancestor with southern Chinese *Alpinia*. Thus future study on divergence time of *Alpinia* is necessary to clarify the evolutionary history of this genus.

Additionally, phylogenetic results strongly supported Vu *et al.*[2] to recognized the former *Zingiber* member “Gùng đá” to *Alpinia coriandriodora* for Vietnamese flora.

The result of character optimizations suggested that the tomentum of leaf (character 1) evolved twice in *Alpinia* (Fig. 2) but appears in most species from Southeast Asia including *A. coriandriodora*. While, tomentum of inflorescence and ovary (characters 3, 4) seems to have appeared multiple times in *Alpinia* (Fig. 3). Character 1 thus can be used to distinguish the Southeast Asia *Alpinia* including *A. coriandriodora* from other members. The inflorescence type has been used in both the intergeneric and infrageneric classification of *Alpinia* and *A. coriandriodora*[1,2].

To evaluate its taxonomic significance within the genus *Alpinia*, we optimized the inflorescence type with three character states onto a Bayesian majority-rule tree (Fig. 4)

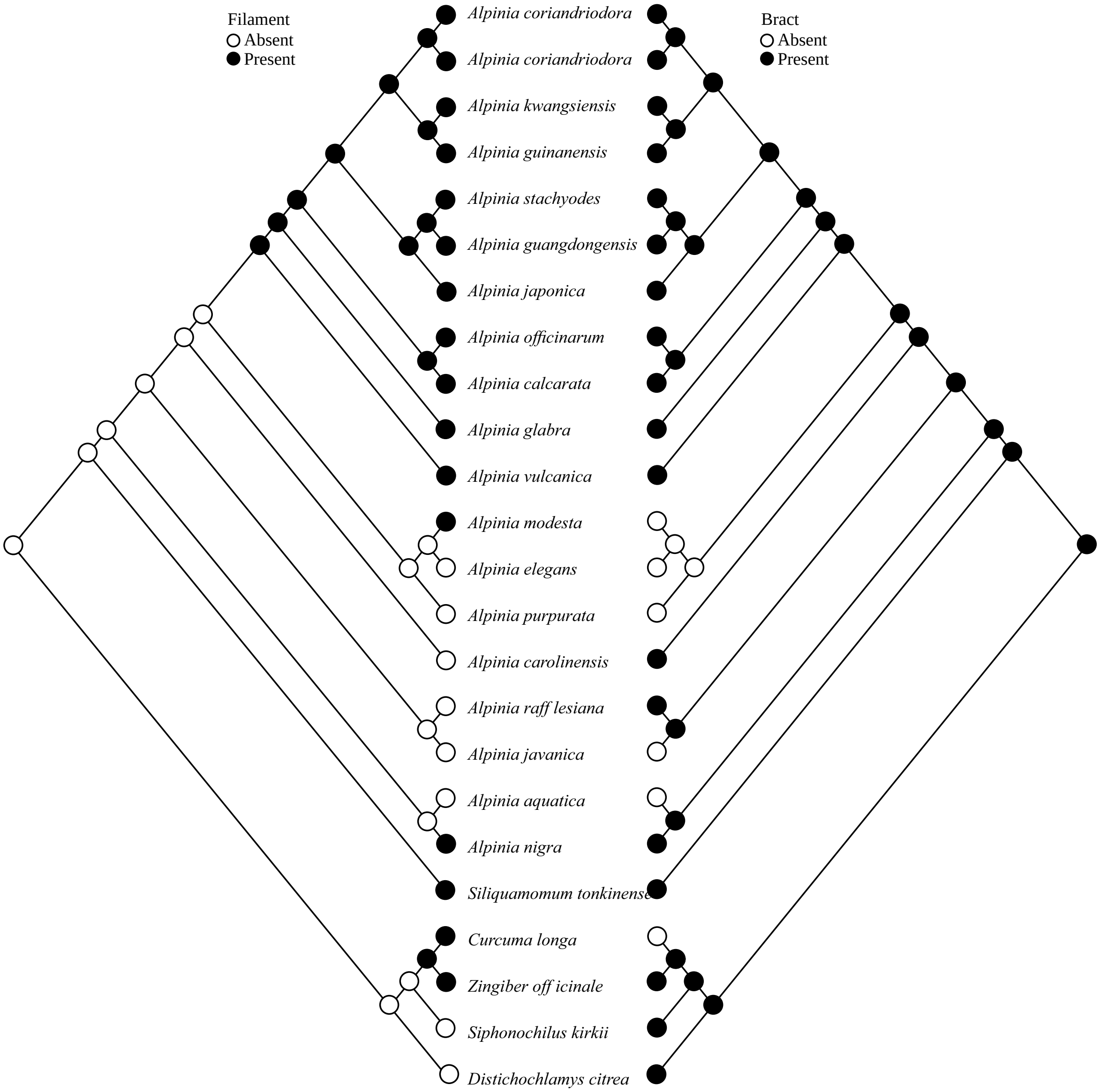

Fig. 5: Character optimization for filament (character 5) and bract (character 6) inferred on a Bayesian majority-rule tree for 24 taxa, based on the maximum likelihood method

which was not presented in previous studies. Our reconstruction result suggested that the state of panicle is ancestral in *Alpinia*. However, raceme and spike have evolved several times within *Alpinia*. *A. coriandriodora* shares different evolutionary history based on our character reconstruction to most Southeast Asian *Alpinia* (Fig. 4). Additionally, our result indicated that the presence of staminodes is an ancestral character of *Alpinia* and some groups and species have evolved to reduct this part.

Alpinia often has beautiful and colorful flowers[12], however, stamens have short or very short filament or sometimes even absent. Our reconstruction of character 5 indicated that the absence of filament is an ancestral character of *Alpinia* but the presence of filament evolved multiple times and especially in Southeast Asian members of *Alpinia* (Fig. 5). This character state appears to be an adaptation to the pollination by insects. In addition, almost all the species of *Alpinia* have bracts, however, some species have evolved to reduct this part (Fig. 5).

The results from molecular analysis of the endangered species *Alpinia coriandriodora* provided valuable insight for the conservation and management of this species. Our results also suggest that *A. coriandriodora* might have great evolutionary potentiality and can adapt to varied

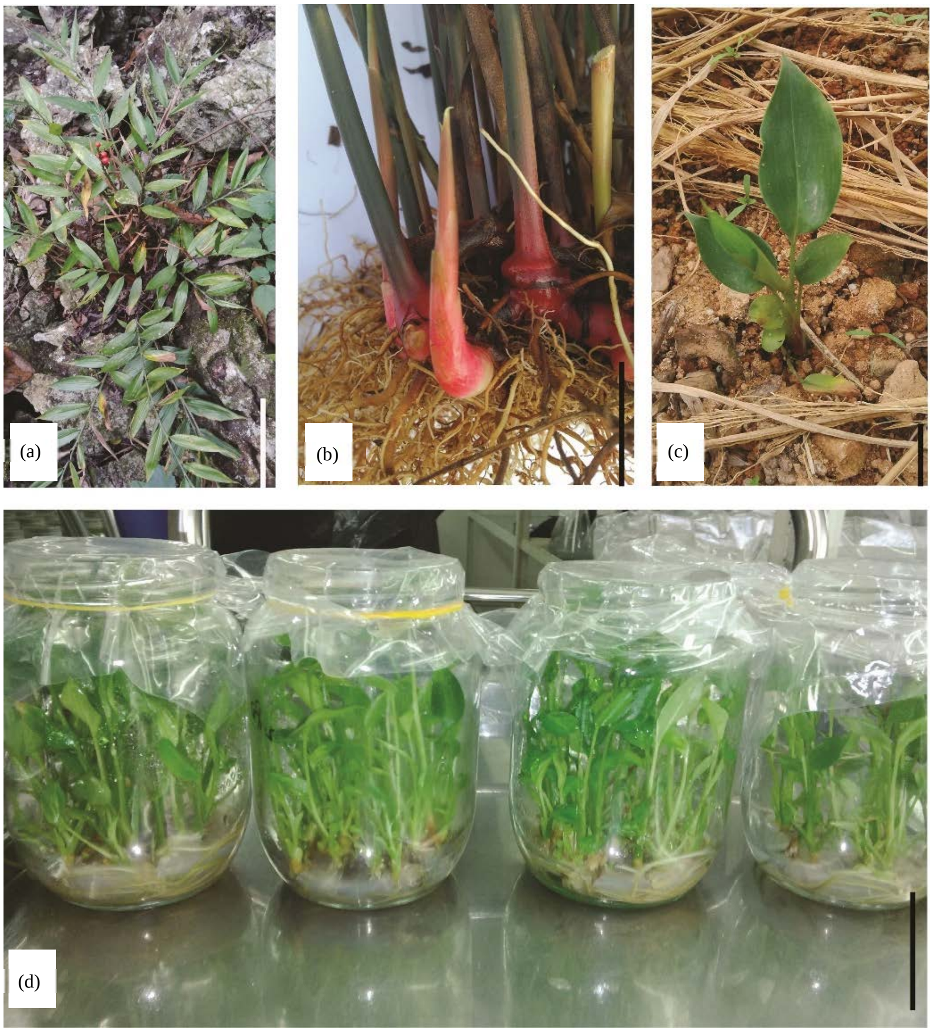

Fig. 6(a-d): (a) Habitat with fruits, (b) Root, stem and bud, (c) Young tree from micropropagation of *Alpinia coriandriodora* and (d) Micropropagation of *Alpinia coriandriodora* in laboratory
Scale bars are 5 cm

environmental conditions[2,15]. Based on the results of this study and investigations with local people during our field trips which were inferred that genetic factors could not be a reason for the population decline of *A. coriandriodora* but due to human-related factors, such as habitat degradation and fragmentation and overexploitation and land reclamation, may have contributed to the endangered status of the species. *A. coriandriodora* is perennial herbs with small population size and mostly distributed in the border between Vietnam and China (Bac Kan and Guangxi provinces)[2,12]. Nybom[16] suggested that the species with small population size and narrow geographic ranges might face a growing risk of genetic drift and inbreeding recession. Therefore, to protect and increase viable populations of *A. coriandriodora*, we propose both *in situ* and ex situ conservation and restoration strategy. *In situ* conservation, expanding the existing protected areas and therefore the reclamation and restoration of habitats destroyed by local people for farmland expansion are most important[15], which may maintain an appropriate, effective population size of *A. coriandriodora*. For *ex situ* conservation, seeds or seedlings should be collected for germplasm storage and to maximize the protection of existing genetic diversity. In addition, artificial breeding using biotechnology (splitting buds, micropropagation) should be encouraged for regression and population reconstruction (Fig. 6). Finally, strengthening public outreach and conservation education for people should be enabled to protect the species and ecological system. Our sampling has confirmed identification and systematic position of *A. coriandriodora*. The present study has clarified phylogeny of *A. coriandriodora*, however, future work with comprehensive taxon sampling should focus on the phylogeny of the tribe Alpinieae and evolutionary history of *Alpinia*.

CONCLUSION

In this study, our molecular analyses supported the recognization of *Alpinia coriandriodora* for Vietnamese flora. This species has a close genetic relationship with some other *Alipinia* members that share the same distribution in southern China and northern Vietnam. Character optimizations suggest that the presence of filament could be an adaptation to the pollination by insects for species of *Alpinia*. Some reasonable conservation strategies are proposed to protect the species.

SIGNIFICANCE STATEMENT

The present study shows the molecular phylogenetic relationship of *A. coriandriodora* within *Alpinia*. Character optimizations indicate evolutionary of morphology and adaptation to the pollination by insects for species of *Alpinia*. This study provides conservation strategies to protect the species including maintenance of the plant's natural habitats, seeds or seedlings collection for germplasm storage and artificial breeding using biotechnology.

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