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PHYLOGENETIC STUDY OF *ERIOBOTRYA* (ROSACEAE) BASED ON COMBINED CPDNA PSBA-TRNH AND ATPB-RBCL MARKERS

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The phylogenetic relationships of *Eriobotrya* were examined based on cpDNA psbA-trnH and atpB-rbcL markers using 21 names with *M. germanica*, *P. beauverdiana* and *R. indica* as an outgroup. The results revealed that the sequencing analysis contained around 841 bp aligned DNA characters, where 81 bp were variables and 42 bp were informative polymorphic sites. Further analysis showed that the phylogeny tree divided the species of *Eriobotrya* into 4 clades. *Eriobotrya bengalensis*, *E. bengaensis* var. *angustifolia* and *E. obovata* were closely related and formed clade A. *E. deflexa*, *E. fragrans* and *E. seguinii* clustered into clade B. *E. japonica*, *E. malipoensis* and *E.* × *daduheensis* clustered into clade C and *E. fusca*, *E. cavaleriei*, *E. tengyuehensis*, *E. condaoensis*, *E. serrata*, *E. hookeriana*, *E. petiolata*, *E. salwinensis* and *E. elliptica* were clustered into clade D. The present study concluded that future research with more taxa and the use of more DNA barcoding markers may be needed to resolve the complete phylogeny and evolution of *Eriobotrya*.

Keywords: *Eriobotrya*, classification, DNA barcoding markers, sequence analysis, polymorphic sites

INTRODUCTION

The plant genus *Eriobotrya* Lindley (Lindley 1821), belongs to the family Rosaceae (Potter et al. 2007), tribe Maleae and subtribe Malinaeals (Sun et al. 2018). The genus includes more than 30 names, of these 21 names were recorded in China (Yang et al. 2005), whereas the remaining names were record in southeast Asia (Laos, Malaysia, Myanmar and Thailand), southern Japan, and the Himalayas (Bhutan, India and Nepal) (Vidal 1965). Further research work is necessary to evaluate the evolution and origin of *Eriobotrya* and to further exploit *Eriobotrya* species for pharmaceutical and industrial purposes.

The generic circumscription of *Eriobotrya* species were primarily discussed based on morphological characters, such as leaf blade abaxially tomentose and without tomentose (Yu 1974), autumn-flowering group and winter-flowering group (Zhang et al. 1990), leaf size, style number and stamens (Yang et al. 2007, Yang et al. 2017) and numerical taxonomy (Zhang et al. 2017).

The systematic treatments of *Eriobotrya* based on molecular analysis were reported from broader studies on the family Rosaceae (Potter et al. 2007). In a previous study, Xie et al. (2007) analysed the phylogenetic relationships based on ISSR markers and concluded that *E. japonica* was closely related to *E. prinoides* var. *daduheensis*, *E. maliopensis*. *E. deflexa*, and *E. deflexa* var. *koshunensis*. *E. elliptica* was genetically distant and made a separate clade. *E. henryi*, *E. serrata* and *E. seguinii* were closely similar to each other. In addition, they further concluded that *E. kwangsiensis* was a distinct species. Yang et al. (2009), examined the phylogenetic relationships of *Eriobotrya* species using amplified fragment length polymorphism (AFLP) markers revealed that the species were grouped into 3 main clusters. Cluster I consisted of *E. henryi* and *E. seguinii,* Cluster II consisted of *E. elliptica* and *E. serrata,* and Cluster III consisted of *E. japonica* and *E. maliopensis*. Furthermore, *E. prinoides* and *E. prinoides* var. *daduheensis*, *E. cavaleriei* and *E. fragrans* were closely related,

and *E. deflexa*, *E. deflexa* var. *koshunenis* were closely related and indicating that substantial genetic variations were found in *Eriobotrya*. Li et al. (2009) evaluated the phylogenetic study of *Eriobotrya* species based on nrDNA ITS sequence revealed that the genus *Eriobotrya* formed a monophyletic group and genetically close to *Rhaphiolepis indica* than *Photinia serrulata* and concluded that *E. cavaleriei* treated to be a variety of *E. fragrans* as previously reported by Yang et al. (2005). Li et al. (2011) examined the preliminary phylogenetic study based on chloroplast rbcL and trnL-trnF sequences revealed that *E. seguinii* was the primitive taxa in the genus *Eriobotrya* and concluded that cpDNA sequences could not resolve certain relationships of *Eriobotrya* species due to the limitations in evolution. Yang et al. (2012) analysed the phylogenetic study on the genus *Eriobotrya* based on nrDNA *adh* sequences revealed that *E. japonica*, *E. prinoides*, *E. prinoides* var. *daduheensis* and *E. elliptica* were closely related to each other but *E. malipoensis* was genetically separated from other species. Furthermore, they concluded that *E. cavaleriei* could be classified as a variety of *E. fragrans*.

Taxon relationships and classification of the genus *Eriobotrya* are debatable and unclear. Several early treatments for species relationships and classification were based on cultivars and few wild species of *Eriobotrya*. Thus, the present study aimed to evaluate the phylogenetic relationships of the genus *Eriobotrya* based on combined cpDNA psbA-trnH and atpB-rbcL markers from southeast Asia and Himalaya regions.

MATERIALS AND METHODS

Plant materials

A total of 21 names including eighteen taxa of *Eriobotrya* while *Mespilus germanica*, *Rhaphiolepis indica* and *Photinia beauverdiana* were selected as an outgroup in the present study (Table 1).

Genomic DNA extraction and PCR amplifications

Plant genomic DNA extraction kit was used to extract the genomic DNA from dried specimens. The two chloroplast regions; psbA-trnH and atpB-rbcL (Savolainen et al. 1994, Sang et al. 1997) were used for amplification. Polymerase chain reactions (PCR) were carried out in a 25 µl tubes containing 1 µl of genomic DNA (20–100 ng), 1 µl of each primer pair (10 mM), 0.5 µl of dNTP Mix (10 mM), 2.5 µl of 10X $MgCl_2$ buffer, 5 U μl^{-1} of DNA polymerase and sterile water. A thermo cycler was used to performed PCR and amplification of the region. The process consisted of initial denaturation for 5 min at 94 °C and at 35 cycles for 1 min at 94 °C and followed by annealing temperature for psbA-trnH at 58 °C for 1 min and for atpB-rbcL at 72 °C for 1.5 min. The final extension step was conducted at 72 °C for 7 min and later maintained at 4 °C. The PCR product was gel-separated by 1 % Agarose TAE buffers and the sample was finally sequenced.

Sequences information and alignment

Closet sequences and others published sequences of *Eriobotrya* names were retrieved from National Center for Biotechnology Information and included in the final datasets for phylogenetic analysis (Table 1). *Mespilus germanica, Rhaphiolepis indica* and *Photinia beauverdiana* were designated as an outgroup for rooting purposes following Campbell et al. (2007). ClustalW software was used for sequences alignment (Larkin et al. 2007) and manually edited using Bioedit sequence alignment editor v 7.0.5.3 (Hall 1999).

Data analysis

The best-fit DNA substitution model for each dataset with default setting was performed using MEGA 6 software and the parameter values were determined by Alkaike Information Criterion. Phylogenetic analysis was conducted using Maximum Likelihood (ML) method and selected T92 model with gaps were treated as missing data using MEGA 6 software (Tamura et al. 2013). Support values were assessed using the bootstrap option with 1000 replicates.

RESULTS

Analysis of cpDNA markers

The combined cpDNA datasets were composed of 21 names and the sequencing analysis comprised 841 bp aligned DNA characters, of these 81 bp were variables and 42 bp were informative polymorphic sites.

Table 1 List of taxa, locality, vouchers, herbaria and GenBank accession numbers used in the present study

Taxon	Locality	Vouchers	Herbarium	GenBank Accession No.
				PsbA-trnH atpB-rbcL
E. bengalensis (Roxb.) Hook. f.	China	0298946	IBSC	Present study
E. bengalensis var. angustifolia Card.	Yunnan, China	0298949	IBSC	Present study
E. cavaleriei (H.Lévl.) Rehd.	Guangxi, China	0299011	PE	Present study
E. condaoensis X.F.Gao, M.Idrees & T.V.Do	Vietnam	VNMN_CN 633	CDBI	Present study
E. × *daduheensis* Liao et al.	Sichuan, China	00004578	PE	Present study
E. deflexa (Hemsl.) Nakai	Taiwan	01568202	PE	Present study
E. elliptica Lindl.	Nepal	0652620	KUN	Present study
E. fragrans Champ. ex Benth.	Guangdong, China	0299116	IBSC	Present study
E. fusca K.Kuan	Yunnan, China	00799408	PE	Present study
E. hookeriana Decne.	Bhutan	1575791	PE	Present study
E. japonica (Thunb.) Lindl.	Sichuan, China	00799571	PE	Present study
E. malipoensis K.C.Kuan	Yunnan, China	0299390	IBSC	Present study
E. obovata W.W.Sm.	Yunnan, China	–	PE	Present study
E. petiolata Hook. f.	Bhutan	01639921	PE	Present study
E. salwinensis Hand.-Mazz.	Yunnan, China	607631	KUN	Present study
E. seguinii (H.Lév.) Card. ex Guill.	Yunnan, China	–	–	FJ571507 [c]
E. serrata Vidal	Yunnan, China	1227861	KUN	Present study
E. tengyuehensis W.W.Sm.	Yunnan, China	–	–	FJ796915 [c]
Mespilus germanica L.	Chicago, USA	M645-80	–	[ab] HQ427046.1[a] [ab] HQ427046.1[b]
Photinia beauverdiana C.K Schneid.	GenBank	1733-80A	–	[ab] HQ427047.1[a] [ab] HQ427046.1[b]
Rhaphiolepis indica (L.) Lindl.	GenBank		–	[ab] HQ427046.1[a] [ab] HQ427046.1[b]

[a] = sequences from GenBank psbA-trnH, [b] = sequences from GenBank atpB-rbcL,
[c] = sequence from GenBank, [ab] = sequences used as an outgroup,
– = no information about the specimen

Cluster analysis

The phylogenetic tree based on combined chloroplast DNA (cpDNA) and sequence of 18 *Eriobotrya* names with *M. germanica*, *R. indica* and *P. beauverdiana* as an outgroup was constructed by the ML method. The results revealed that the *Eriobotrya* phylogenetic tree could be divided into 4 groups (Figure 1). *E. bengalensis*, *E. bengaensis* var. *angustifolia* and *E. obovata* were clustered into clade A. *E. deflexa*, *E. fragrans* and *E. seguinii* formed clade B. *E. japonica*, *E. malipoensis* and *E.* × *daduheensis* clustered into clade C. Whereas the remaining names clustered into clade D. Clade D was further divided into 4 subclades. Subclade I included *E. fusca*, *E. cavaleriei* and *E. tengyuehensis*. Subclades II included only *E. condaoensis*. Subclade III included *E. serrata* and *E. hookeriana*. Subclade IV included *E. petiolata*, *E. salwinensis* and *E. elliptica*.

DISCUSSION

In the past, a variety of molecular markers such as nrDNA ITS, *adh* sequences and genome-wide RAD sequence were used to evaluate the species relationship of *Eriobotrya* (Yang et al. 2017). The chloroplast rbcL and trnL-trnF sequences were used to evaluate the phylogenetic property of *Eriobotrya* (Li et al. 2011), but the results could

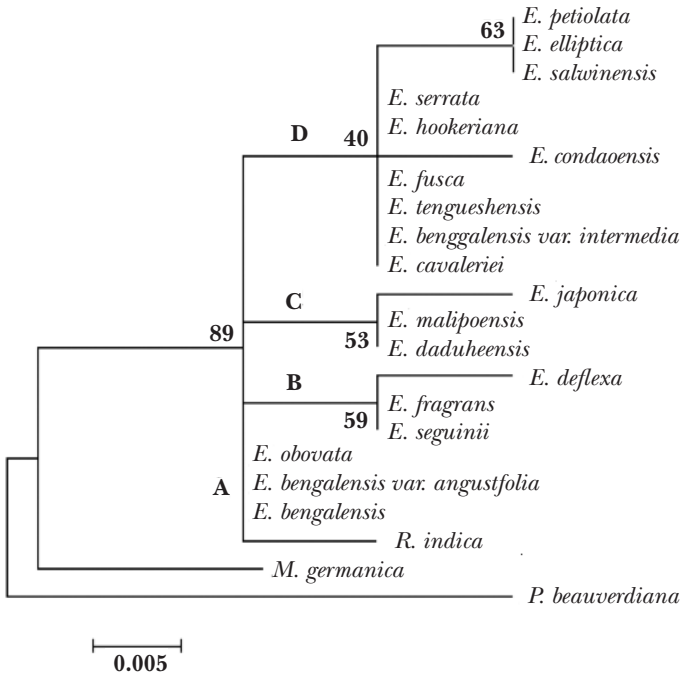

Figure 1 Maximum likelihood tree illustrating the phylogeny of the genus *Eriobotrya* based on cpDNA psbA-trnH and atpB-rbcL datasets.

not resolve the species relationships. The non-coding region between psbA-trnH and atpB-rbcL was used in phylogenetic studies in other important plant species such as *Cyathea* spp. and *Aquilaria hirta* (Balkrishna et al. 2020, Mohd Syafik et al. 2020) and recommended the use of the non-coding region as a promising plant DNA barcoding marker for various plant comparative studies (China Plant BOL Group 2011).

In the current study, we assessed the utility of two chloroplast DNA (psbA-trnH and atpB-rbcL) for identifying 21 *Eriobotrya* names, with *M. germanica*, *R. indica,* and *P. beauverdiana* as an outgroup using the maximum likelihood method. The phylogenetic tree produced, divided the *Eriobotrya* names into four clades. *E. bengalensis*, *E. bengalensis* var. *angustifolia* and *E. obovata* formed a group, which is largely consistent with previous studies (Yang et al. 2017, Zhang et al. 2017). Previous studies reported that *E. maliopensis* formed a separate clade and was genetically different from the rest of the species, and concluded that further studies should be needed to confirm its phylogenetic relationships (Yang et al. 2012). Findings from this study confirms the position of *E. maliopensis* and close relationship with *E. japonica*, *E. malipoensis* and *E.* × *daduheensis,* which is consistent with earlier studies on *Eriobotrya* (Yang et al. 2012, Zhang et al. 2017). Li et al. (2009) and Yang et al. (2012) revealed that *E. cavaleriei* was classified to be a variety of *E. fragrans*. The present study showed that *E. fragrans* had close relationships with *E. deflexa* and *E. seguinii* to formed a clade, whereas *E. cavaleriei* formed a clade and close relationship with *E. tengyuehensis* and *E. fusca*. Our results confirmed the position of both species and treated to be distinct species, as reported in our previous nrDNA ITS analysis (Idrees et al. 2020a). Some *Eriobotrya* species were not previously reported and their close relationships with other species were unknown. *E. petiolata* formed a clade with *E. elliptica* and *E. salwinensis* and had close relationships with each other. *E. hookeriana* was closely related to *E. serrata*. *E. bengalensis* var. *intermedia* had a close relationship with *E. cavaleriei*, *E. tengyuehensis,* and *E. fusca*. *E. condaoensis* formed a long branch and made a distinct clade, which was consistent with our previous analysis on nrITS sequence (Idrees et al. 2020a).

The phylogenomic analysis by Liu et al. (2020) revealed that *Rhaphiolepis* and *Eriobotrya* strongly supported the paraphyly of *Eriobotrya*, with *Rhaphiolepis* nested within it and *Eriobotrya* was embedded in *Rhaphiolepis*. Shaw (2020) conserved the name *Eriobotrya* against *Rhaphiolepis* and concluded that the epithet *japonica* was

preoccupied and the name of the panglobal loquat changes both in its genus and specific epithet. The replacement name *Rhaphiolepis loquata* (Liu et al. 2020) was superfluous and thus inadmissible because the cited synonym *Crataegus bibas* (Loureiro 1790), provided a prior available epithet that was unoccupied. Furthermore, new species continue to be described in *Eriobotrya* including *E. condaoensis* (Idrees et al. 2018), *E. capitata* (Averyanov 2019), *E. laoshanica* (Chen et al. 2020) and *E. fusca* (Idrees et al. 2020b). Presently, *Eriobotrya* and *Rhaphiolepis* are widely accepted globally in flora and horticulture references.

The two genera can be distinguished from each other by the following morphological characters; the primary lateral veins reaching at the leaf margin, often in a tooth (Craspedodromous) in *Eriobotrya*, whereas the primary veins consistently end without reaching the margins (Camptodromous) in *Rhaphiolepis*; the inflorescence are paniculate in *Eriobotrya* and racemose in *Rhaphiolepis*; flower white and carpels (2 or) 3–5 in *Eriobotrya,* whereas flower pink and carpels) (–1) 2 in *Rhaphiolepis*; the sepals are persistent on the fruits in *Eriobotrya,* while in *Rhaphiolepis*, the sepals are early deciduous, leaving an annular ring at the summit of the fruit (Gu & Spongberg 2003).

CONCLUSIONS

The present finding based on combined chloroplast DNA markers, psbA-trnH and atpB-rcbcL produced similar results obtained in previous analysis of phylogenetic relationships of *Eriobotrya* based on ITS sequences. However, the complete phylogeny and the interspecies relationship is still unclear and difficult to determine, due to the lack of specimens from Southeast Asia including Vietnam, Malaysia and Myanmar. Further research will be necessary to include more taxa of *Eriobotrya* and the use of more DNA barcode markers to provide a more comprehensive and complete phylogeny and evolution of *Eriobotrya.*

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