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PHYLOGENETIC RELATIONSHIPS OF GENUS *ERIOBOTRYA* LINDL. (ROSACEAE), BASED ON NUCLEAR RIBOSOMAL DNA (ITS) SEQUENCE

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Abstract

The phylogenetic relationships of genus *Eriobotrya* Lindl. (Rosaceae) was evaluated based on the nuclear ribosomal internal transcribed spacers (ITS) sequence using 22 wild species of *Eriobotrya*, and 4 closest genera were used as an outgroup (*Mespilus germanica* L., *Malus sieboldii* (Regel) Rehder, *Photinia beauverdiana* C.K Schneid. and *Rhaphiolepis indica* (L.) Lindl.). Our results supported the view that the *Eriobotrya* species were monophyletic and suggested that *E. condaoensis*, *E. henryi* and *E. seguinii* as the most primitive group of *Eriobotrya.* Our results also suggest that *E. grandiflora* considered as a valid species and have close relationship to *E. fragran*s. We reported here three more taxa of the genus for the first time, *E. hookeriana* have close relationship with *E. petiolata*; *E. bengalensis* f. *contracta*have close relationship with *E. malipoensis* and *E. bengalensis* var. *intermedia* have close relationship to *E. salwinensis* and *E. tengyehensis*. Furthermore, it was also resolved that *E. malipoensis* have close relationship to *E. bengalensis* var. *contracta*. In addition, *E. cavaleriei* and *E. fragrans* were found to be distantly related to each other.

Key words: *Eriobotrya*, ITS sequence, Phylogenetic relationship, China.

Introduction

The genus *Eriobotrya* Lindl., belongs to the tribe Pyreae (subtribe Pyrinae, subfamily Spiraeoideae) of the family Rosaceae (Campbell *et al.*, 2007; Potter *et al.*, 2007). The genus is mainly distributed in the Guangxi Province, in the Yangtze River Valley, Dadu River Valley, and Yunnan Province, China and the area is considered as the centre of diversity (Zhang *et al.*, 1996; Yang, 2005; Yang *et al.*, 2005). Other distribution centers include: the Himalayas (Bhutan, Nepal and Sikkim); southern Japan; Taiwan and southeast Asia, mainly Cambodia, Indonesia, Laos, Myanmar, Thailand, and Vietnam (Vidal, 1965; Vidal, 1968; Pham, 2000; Yang *et al*., 2005; Wong & van der Ent, 2014; Idrees *et al*., 2018). The medicinal importance of *E. Japonica* has been documented in many studies (Liu *et al*., 2016).

Molecular makers are the most important tools for estimating phylogenetic studies, species and cultivars identification, genetic mapping, detection of mutant gene, and population studies (Hartl & Jones, 2005; Shinwari *et al.*, 2014; Zahra *et al.*, 2016; Shinwari *et al.*, 2018). They are independent of environmental conditions and show high polymorphism. In previous studies, the phylogenetic relationships of *Eriobotrya* were analyzed by various molecular markers, such as ISSR (Xie *et al*., 2007), RAPD and AFLP (Yang *et al*., 2007), AFLP (Yang *et al*., 2009). Li *et al*. (2009) was the first to evaluate the phylogenetic relationship of *Eriobotrya* using ITS sequences and showed the genus *Eriobotrya* to be monophyletic and further suggested that *E. cavaleriei* could be treated as a variety of *E. fragrans.* Zhao *et al*., (2011) evaluated the genetic relationships among loquat cultivars and wild species of the genus *Eriobotrya* based on ITS sequences. They revealed that *E. malipoensis* and *E. seguinii* could be the most primitive species of the genus *Eriobotrya.* Yang *et al*., (2012) supported the previous molecular studies (Yang, 2005; Li *et al*., 2009) and suggested that *E. malipoensis* was genetically separated from others and further studies should be conducted to confirm its relationship. However, most of these studies have been conducted in *E. japonica* cultivars and a few wild species. The phylogenetic relationships of the genus *Eriobotrya* and complete classification within the genus is still unclear. The available little information is insufficient to evaluate the whole genus of *Eriobotrya*.

The current study aims to investigate the phylogenetic relationship of wild *Eriobotrya* species by using ITS sequences and to confirm the previous suggestions on the species relationships.

Materials and Methods

Taxon sampling: A total of 19 *Eriobotrya* species were obtained from herbarium specimens at the China National Herbarium (PE), Kunming Institute of Botany (KUN), South China Botanical Garden (IBSC), corresponding to more than half of accepted species in the genus.

According to previous studies, four genera of Roseaceae (*Mespilus*, *Malus*, *Photinia* and *Rhaphiolepis*) are closely related to the genus *Eriobotrya* (Campbell *et al*., 1995; Campbell *et al*., 2007). Therefore, *Mespilus germanica* L., *Malus sieboldii* (Regel) Rehder, *Photinia beauverdiana* C.K Schneid. and *Rhaphiolepis indica* (L.) Lindl., were chosen as outgroup. All species with detailed information are given in Table 1.

DNA extraction and PCR amplifications: Total genomic DNA was extracted from 20-30 mg of dried herbarium specimens, using the Tiangen Plant Genomic DNA Extraction Kit (Beijing), according to instructions from the manufacturer. DNA quality was visually checked on 0.8% of agarose gel electrophoresis.

The nrDNA ITS region was amplified by using the primers 'ITSF' and 'ITSR' of Li *et al*., (2009) and Yang *et al*., (2011). Polymerase chain reactions (PCR) were performed in a 25 µl volume containing 2.5 µl of 10 X EasyTaq buffer with $MgCl_2$, 10 mM of 0.5 µl of dNTP Mix, 1 µl of each forward and reverse primer (10 mmol/L), 5 U/µl of EasyTaq DNA polymerase and 1 µl of genomic DNA (20-100 ng)and18 µlsterile water. PCR amplification reactions were performed using SimpLiAmp thermo cycler (Applied Biosystem, Life technology). Conditions for amplification of the region consisted of initial denaturation at 94°C for 5 min, then 35 cycles at 94°C for 1 min (denaturation), annealing temperature at 58°C for 1 min, 72°C for 1 min and 30 sec (extension), with the final step of extension at 72°C for 7 mint., followed by maintaining temperature at 4°C. PCR products were electrophoresed on 1% Agarose TAE gel and were sequenced by Tsingke Biological Technology Co., Ltd. (Chengdu, China).

Data analysis

A total of 19 generated sequences together with additional ITS sequences of the three species of *Eriobotrya* were retrieved from GenBank and included in final data set. Sequence alignment was performed using ClustalW (Larkin *et al*., 2007) with default parameters as implemented in MEGA 6 (Tamura *et al.,* 2013). Alignments were then verified and modified manually using Bioedit sequence alignment editor v 7.0.5.3 (Hall, 1999). The GC content was also analyzed by Bioedit (v 7.0.5.3). Phylogenetic analysis was conducted using Maximum Likelihood (ML) method. The K2+G model was selected asthe best model for phylogenetic analysis. ML analysis was performed using MEGA 6 (Tamura *et al.,* 2013), with gaps treated as missing data. Support values were assessed using the bootstrap option with 1000 replicates.

Table 1. List of species, locality and GenBank accession numbers used in the present study.

Species name	Locality	Vouchers	Herbarium	Genbank accession No.
				ITS
E. bengalensis (Roxb.) Hook. f.	China	0298946	IBSC	MH246941[a]
E. bengalensis var. *angustifolia* Card.	China, Yunnan	0298949	IBSC	MH246942[a]
E. bengalensis var.*intermedia* Vidal	China,Yunnan	0772338	KUN	MG938047[a]
E. bengalensis f. *contracta* Vidal	China, Yunnan	0704812	IBSC	MH246943[a]
E. cavaleriei (H.Lévl.) Rehd.	China, Guangxi	0299011	PE	MH246944[a]
E. condaoensis X.F. Gao, M. Idrees & T.V. Do	Vietnam	VNMN_CN 633	CDBI	MG938050[a]
E. × *daduheensis* Liao et al.	China, Sichuan	00004578	PE	MG938045[a]
E. deflexa (Hemsl.) Nakai	China, Taiwan	01568202	PE	MG938042[a]
E. deflexa var. *buisanensis* (Hay.) Kah. & Sas.	China, Taiwan	0299109	IBSC	MG938043[a]
E. fragrans Champ. ex Benth.	China, Guandong	0299116	IBSC	MH246945[a]
E. grandiflora Rehder & E.H.Wilson	China	00799225	PE	MH246946[a]
E. henryi Nakai	GenBank		-	KJ170777[b]
E. hookeriana Decne.	Bhutan	1575791	PE	MG938046[a]
E. japonica (Thunb.) Lindl.	China, Sichuan	00799571	PE	MG938044[a]
E. malipoensis K.C.Kuan	China, Yunnan	0299390	IBSC	MH246947[a]
E. obovata W.W.Sm.	China, Yunnan		PE	MH246948[a]
E. petiolata Hook. f.	Bhutan	01639921	PE	MH246949[a]
E. prinoides Rehder&E.W.Wilson	China, Yunnan	0299340	IBSC	MH246950[a]
E. salwinensis Hand.-Mazz.	China, Yunnan	607631	KUN	MG938048[a]
E. seguinii (H.Lév.) Card. ex Guill.	China, Yunnan		-	FJ571507[b]
E. serrata Vidal	China, Yunnan	1227861	KUN	MG938049[a]
E. tengyuehensis W.W.Sm.	China, Yunnan		-	FJ796915[b]
Mespilus germanica L.	USA, Chicago	M645-80	-	EF127040[ab]
Photinia beauverdiana C.K Schneid.	GenBank	1733-80A	-	JQ392492[ab]
Rhaphiolepis indica (L.) Lindl.	GenBank		-	KP093148[ab]
Malus sieboldii (Regel) Rehder	Japan, C. & E. China	East Malling A1406	-	AF186505[ab]

[a] stands for sequences used in present study, [b] stands for sequences from GenBank, [ab] stands for sequences used as an outgroup, - stands for no information about the specimen

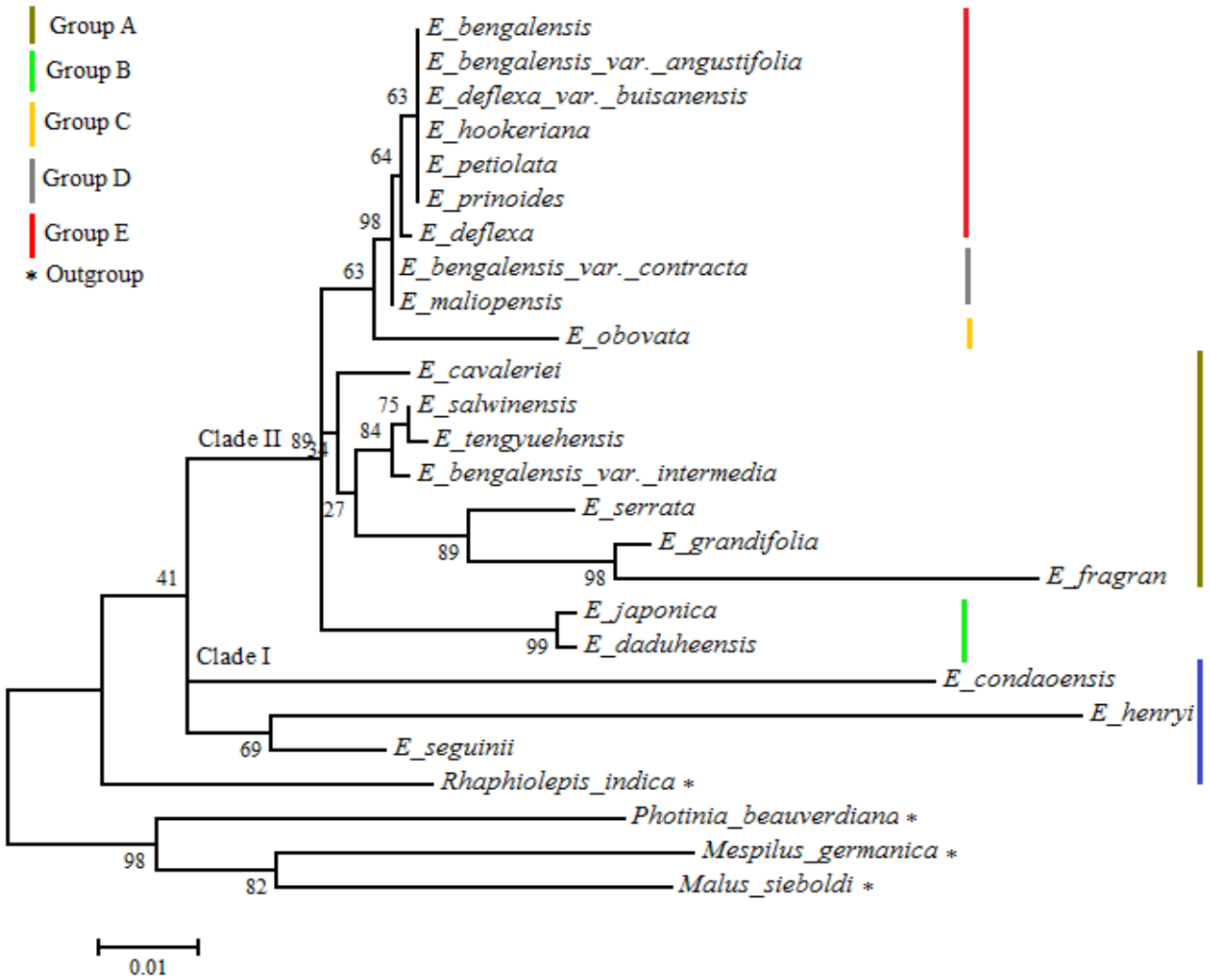

Fig. 1. Maximum Likelihood strict tree illustrating the phylogeny of the genus *Eriobotrya* based on nrDNA ITS datasets. Maximum Likelihood bootstrap values are shown above branches.

Results

The nrDNA data set had a total of 22 sequences, including 6 sequences reported here for the first time. The final data set consisted of 596 aligned DNA characters, of which 414 were constant, 181 variable sites and 79 informative sites. The GC content ranged from 62.99% to 66.95%. The genus *Eriobotrya* was resolved as monophyletic group with a bootstrap support value 95%. The best tree from the phylogenetic analysis of ITS DNA sequences of 22 taxa including species, varieties and one hybrid species, and *Mespilus germanica* L., *Malus sieboldii* (Regel) Rehder, *Photinia beauverdiana* C.K Schneid. and *Rhaphiolepis indica* (L.) Lindl., as an outgroup species is shown in Figure 1. The phylogenetic tree divided the genus into three main clades. *E. henryi* and *E. seguinii* formed a clade I, which might be the most primitive taxa in the genus *Eriobotrya*. *E. condaoensis* formed a distinct clade II, whereas others clustered into the clade III, which was further divided into 5 groups. Group A can be subdivided into 3 subgroups, *E. cavaleriei* formed subgroup I, *E. serrata*, *E. grandifolia*, *E. fragrans* formed subgroup II, *E. tengyuehensis*, *E. salwinensis* and *E. bengalensis* var. *intermedia*, formed subgroup III. Group B consisted of *E. japonica* and *E. daduheensis*. *E. obovata* formed a monophyletic group C. Group D consisted of *E. maliopensis* and *E. bengalensis* var. *contracta* with bootstrap 96 %, while Group E consisted of *E. bengalensis*, *E. bengalensis* var. *angustifolia*, *E. deflexa*, *E. deflexa* var. *busianensis*, *E. petiolata*, *E. prinoides*, *E. hookeriana*, with bootstrap 64%.

Discussion

Phylogenetic information of ITS sequences: The length of internal transcribed spacers (ITS) region in different *Eriobotrya* species ranged from 638 bp in *E. japonica* to 652 bp in *E. serrrata* and *E. condaoensis*. Our results agreed with the previously reported finding in *Eriobotrya* sequences (Li *et al*., 2009, Yang *et al*., 2011) and with others fruits plants, including *Dimocarpus*, *Mespilus*, *Paeonia* (Lo *et al*., 2007, Jiang *et al*., 2008, Zhao *et al*., 2008) which showed that the length of the nrDNA ITS sequences of angiosperms was more conserved, because of its faster evolution rate (Wendel *et al*., 1995). ITS regions in many angiosperms have a higher GC content, normally ranged from 50% to 75% (Baldwin *et al*., 1995). In the present study, the GC content ranged from 62.99%

to 66.95%, which was close to the report of Yang *et al*., (2011) and other plants of Rosaceae (Lo *et al*., 2007). Variable nucleotide character in our result has higher (181) than those reported by Yang *et al*., (2011), that consisted of 132 variable sites. The parsimony-infomatives in our result had lower (79), than those reported by Li *et al*., (2009) that consisted of 154 variable sites and 132 informative sites.

Phylogeny and taxonomy of the *Eriobotrya*: Internal transcribed spacer (ITS) sequences can be used for phylogenetic analysis and evolutionary studies. The nrDNA ITS sequence was used to study the phylogenetic relationships of closely related taxa, including Rosaceae (Eriksson *et al*., 1998; Guo *et al*., 2011; Li *et al*., 2012; Zhu *et al*., 2015). Several attempts have been made to resolve the phylogenetic and interspecies relationships in the genus *Eriobotrya*. However, only a limited number of wild species of *Eriobotrya* have been included in the previous phylogenetic analysis. In the present study, more than half of accepted *Eriobotrya* (22 wild species), were included alongwith *Mespilus germanica*, *Malus sieboldii*, *Photinia beauverdiana* and *Rhaphiolepis indica* as an outgroup to construct the phylogenetic tree by nrDNA ITS sequence. The phylogenetic tree was resolved as monophyletic group and consistent with those presented by Li *et al*., (2009). In his analysis, suggested that *E. henyri* and *E. seguinii* might be the most primitive taxa in *Eriobotrya*. We also agreed with their analysis, but the addition of more taxa and new data improved the relationships within the genus, *E. condaeoensis* collected from Con Dao National Park (Vietnam) reported here for the first time supported by ITS sequence data. This newly described species formed a small clade II and had close relationship with *E. henryi*, *E. seguinii*and with the rest of the species. Hereafter we confirmed that *E. condaoensis*, *E. henryi* and *E. seguinii* are the most primitive taxa in genus *Eriobotrya*.

Yang (2005) concluded that *E. cavaleriei* morphologically closely resembled to *E. fragrans*, and could not delimited on the basis of leaf size under some ecological condition, and suggested that one of them should be reduced to a varietal level. Later, Li *et al*., (2009) based on ITS sequences data showed that *E. cavaleriei* and *E. fragrans*formed a group with bootstrap value (93%). The pairwise divergence was determined to be 0.003, and was suggested to be treated *E. cavaleriei* as a variety of *E. fragrans*. Based on our ITS analysis, we disagree with both author suggestions, and found that *E. cavaleriei* and *E. fragrans* were distantly related to each other. *E. fragrans* formed a clade with *E. serrata*, *E. grandifolia*, whereas *E. cavaleriei* formed a monophyletic subgroup. The pairwise divergence of *E. cavaleriei* and *E. fragrans* was determined to 0.075. Our results confirmed the position of both species and should be treated as distinct species. Morphologically, *E. cavaleriei* and *E. fragrans* have some common morphological characters and can be mainly distinguished from each other by the shape, margins of leaves, pairs of lateral veins, petals shape, styles number, indumentum of ovary and fruit size. *E. cavaleriei* has oblong, oblong-lanceolate leaves with slightly sharply serrate margins, 10–14 pairs of lateral veins, obovate petals, 2 (–3) styles, glabrous ovary and subglobulose elliptic fruits, 1.5 cm in diameter. In contrast, *E. fragrans* has oblong-elliptic or elliptic leaves with remotely inconspicuously apically serrate margins, elliptic or broadly ovate petals, 4 or 5 styles, pubescent apically ovary and globose, rounded fruits, 1.5–2 cm in diameter. Our results confirmed that both taxa should be treated as distinct species.

E. grandiflora was originally described by Rehder & Wilson (1913). Later it was reduced to the variety as *E. deflexa* var. *grandiflora* (Nakai 1924), and also as conspecific with *E. cavaleriei* (Gu Cuizhi & Spongberg, 2003). Although Huxley (1992) treated it as distinct species. Our results showed that *E. grandiflora* formed a subclade with *E. fragrans* and *E. serrata* with bootstrap value above 90 %, while *E. cavaleriei* and *deflexa* formed a different clade with others species. Thus we confirmed the phylogenetic positions and relationships with other species of the genus. Hereafter, *E. grandiflora* considered here as a distinct species as described by Rehder & Wilson (1913). We observed that most of the habit foliage and morphological characters of *E. grandiflora* has close resemblance to *E. deflexa* and *E. cavaleriei*, but distinguished from the former by having elliptic-oblong, rarely oblong-oblanceolate leaves, blades 10–19 × 3–5.5 cm, cuspidate or obtuse apex, remotely appressedly serrated margins, inflorescence 10–13 cm long, flowers larger, 2–2.5 cm and ovoid to oblong-ovoid fruits, 0.8–1.5 cm. In contrast, *E. deflexa* has oblong, oblong-lanceolate or elliptic leaves, blades 11–25 × 3–7 cm, shortly caudate or acute apex, coarsely obtusely serrate or remotely irregularly incurved-serrate or crenate margins, inflorescence short, 5.5–10 cm long, flowers shorter, 1.5–1.8 cm in diameter and ellipsoid or subglobose fruits, 1–2 cm in diameter. Comparison from the latter, *E. grandiflora* is 6–10 m high tree, elliptic-oblong, rarely oblong-oblanceolate leaves, blades 10–19 × 3–5.5 cm, cuspidate or obtuse apex, remotely appressedly serrated margins, inflorescence 10–13 cm long, pedicel 6–10 mm, filament 2–3 mm long, orbiculate to obovate-orbiculate petals, 7–9 × 4–8 mm, and ovoid to oblong-ovoid fruits, red-orange. In contrast, *E. cavaleriei* is 4–5 cm high tree, oblong, oblong-oblanceolate leaves, blades 10–18.5 × 3–6 cm, acuminate apex, slightly sharply serrate margins, and entire near base, inflorescence short, 9–12 cm long, filament short, 1 mm long, obovate petals, 8–10 mm, and subglobulose elliptic fruits, yellowish-red, dark when mature.

E. bengalensis var. *intermedia* reported here for the first time supported by ITS sequence data, and formed a subclade III with bootstrap 88%, and with close relationships to *E. tengyuehensis* and *E. salwinensis*. Yang *et al*., (2012) demonstrated that *E. maliopensis* formed a monophyletic clade and was separated from the others. They also concluded that further studies were needed for getting clear picture of its phylogenetic relationships. Our results confirmed the phylogenetic relationships of *E. maliopensis* and formed a clade with *E. bengalensis* f. *contracta* with bootstrap value of 96%. *E. hookeriana* and *E. petiolata* are also reported here for the first time by ITS sequence data and formed a clade with *E. petiolata* and *E. prinoides* and with other species of the genus with bootstrap value 64%, and consistent with previous studies those of Yang *et al*., (2017), which revealed that *E. petiolata* formed a clade with *E. prinoides* and *E. x daduheensis*.

Conclusions

This study revealed the phylogenetic relationships in the genus *Eriobotrya* based on nrDNA ITS sequence. A total of 22 *Eriobotrya* species and 4 closely related genera were used to construct the phylogenetic tree which resolved as monophyletic. Most of the phylogenetic relationships are essentially in agreement with previous *Eriobotrya* studies those of Yang *et al*., 2005; Li *et al*., 2009 and Yang *et al*., 2011, such as *E. japonica* and *E. x daduheensis* formed a clade and were close to each others. *E. deflexa*, *E. deflexa* var. *busianensis* were close to each other's, *E. bengalensis* and *E. benaglensis* var. *angusifolia* were also close. Following conclusion was drawn, (1) *E. condaoensis*, *E. henryi* and *E. seguinii* might be the most primitive taxa in genus *Eriobotrya*. (2) The position of *E. fragrans*, and have close relationship to *E. serrata* and *E. grandiflora*. (3) The position of *E. cavaleriei* was confirmed with others species (4) *E. grandiflora* is examined for the first time using molecular ITS data, suggested as a distinct species and have a close relationship to *E. fragran*s (5) *E. hookeriana* is also reported here for first time and have a close relationship with *E. petiolata*. (6) *E. bengalensis* forma *contracta* is also reported here for the first time and have a close relationship with *E. malipoensis*. (7) *E. bengalensis* var. *intermedia* is also reported here for the first time and have a close relationship to *E. salwinensis* and *E. tengyehensis*. However, the complete phylogeny and relationship is still unclear, further research with taxa from Mynmar is needed to resolve the whole phylogenetic and systematic positions and evolutionwithin the genus of *Eriobotrya*.

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