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A Preliminarily Phylogeny Study of the *Eriobotrya* Based on the nrDNA *Adh* Sequences

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Abstract

Phylogenetic relationships of the genus *Eriobotrya* Lindl. were examined based on the nrDNA *Adh* sequences. A phylogenetic tree of 14 loquat accessions (species, varieties and forma) was generated by using *Photinieae serrulaia* L. as an outgroup and *Rhaphiolepis indica* (L.) Lindl. as an ingroup, which represent the two closest genera of *Eriobotyra*. The results showed that these loquat accessions were divided into two main clades in the consensus tree. Clade I included *E. seguinii Card* and group A formed by *E. henryi* Nakai, *E.bengalensis* Hook.f., and forma *angustifolia* Vidal. Clade II is composed of the other taxas which included three groups. *E. cavaleriei* Rehd and *E. fragrans* Champ formed group B; group C consisted of *E. prinoides* Rehd. & Wils. *var. dadunensis* H.Z.Zhang, and *E. japonica* Lindl.; and group D included *E. deflexa* Nakai and *E. deflexa* Nakai *Var.buisanensis* NaKai. Since *E. deflexa* Nakai, *E. deflexa* Nakai *Var.buisanensis* NaKai and *E. kwangsiensis* Chun, were closer in the phylogenetic tree; while *E. prinoides* Rehd. & Wils. *var. dadunensis* H.Z.Zhang, *E. japonica* Lindl, *E. prinoides* Rehd & Wils and *E.elliptica* Lindl. were close with each other, they may be locataed at a similar place of the phylogenetic stage. However, *E. malipoensis Kuan* need further studies on its phylogenetis relationship for it was separated from the others. Results further support the theory that *E. cavaleriei* Rehd could be a variety of *E. fragrans* Champ.

Keywords: *Adh* gene, *Eriobotrya*, loquat, phylogenesis

Introduction

Eriobotrya Lindl. belongs to the family Rosaceae, subfamily Maloideae (Lindley, 1822). The genus *Eriobotrya* Lindl. comprises 21 species including varieties and forms, with Yunnan Province in China the centre of diversity (Yang, 2005; Yang *et al.*, 2005). Further research on the origin and evolution of *Eriobotrya* is important to establish a solid foundation to achieve improvement of cultivated species such as loquat (*Eriobotrya japonica* Lindl.) and to further exploit species of *Eriobotrya* plants for industrial and pharmaceutical purposes.

In previous studies, the interspecific relationship, origin and evolutionary development of *Eriobotrya* were studied by morphology and molecular markers (Cheng *et al.*, 2003; Vilanova *et al.*, 2001). Li *et al.* investigated the molecular phylogeny of *Eriobotrya* by ITS sequence and suggested that the taxonomic treatment of *E. cavaleriei* Rehd as a variety of *E. fragrans* Champ(Li *et al.*, 2009). However, results from different studies were not always consistent.

Previous studies have demonstrated that fast evolving introns of low-copy nuclear genes could provide more essential phylogenetic information than chloroplast or nuclear genes to better define interspecific relationships. In addition, *Adh* gene structure is a useful tool for improving the robustness of phylogenetic reconstruction at all taxonomic levels (Small and Wendel, 2000). Moreover, *Adh* is also an acetaldehyde-metabolic related gene and exists in both animals and plant. Thus, the *Adh* gene was selected to study the relationship and evolution of *Eriobotrya*.

Materials and methods

Sixteen accessions were selected as experimental materials, including 14 *Eriobotrya* and two relative genera. All of them were preserved *ex situ* for the future research in the Loquat Germplasm Repository of Horticultural College, South China Agricultural University, P. R. China. The details of these materials, including their original locations are presented in Tab. 1.

Studies of Campbell *et al.* (1995; 2007) showed that *Eriobotrya* was close to other Maloideae species including *Rhaphiolepis indica* (L.) Lindl., *Osteomeles anthyllidifolia* Lindl., *Sorbus scopulina* Hedl., *Malus prunifolia* (Willd.) Barkh, *Pyrus pyrifolia* (Burm.) Nakai. In this study *Rhaphiolepis indica* was designated as an ingroup while *Photinieae serrulata* L. was designated as an outgroup.

Tab. 1. Some information of the materials

Code	Taxon	Original location	Voucher
1	*E.elliptica* Lindl.	Daweishan, Yunnan	(S.Q.Lin) Ee4
2	*E. prinoides* Rehd & Wils.	Shiping, Yunnan	
3	*E. deflexa* Nakai	Fenghuangshan, Guangdong	(S.Q.Lin) Ed2
4	*E. deflexa* Nakai var.*buisanensis* Nakai	Henchun, Taiwan	(S.Q.Lin) Edk1
5	*E. malipoensis* Kuan	Malipo, Yunnan	(S.Q.Lin) Em1
6	*E. henryi* Nakai	Chengjiang, Yunnan	(S.Q.Lin) Eh1
7	*Photinieae serrulata* L.*	Guilin, Guangxi	(S.Q.Lin) Ps1
8	*E. japonica* Lindl.	Nanling, Guangdong	(S.Q.Lin) Ej1
9	*E. kwangsiensis* Chun	Dayaoshan, Guangxi	(S.Q.Lin) Ek3
10	*E. prinoides* Rehd. & Wils. var. *dadunensis* H.Z.Zhang	Hanyuan, Sichuan	(S.Q.Lin) Ed1
11	*E.bengalensis* Hook.f.	Gaoli-Gongshan, Yunnan	(S.Q.Lin) Eb2
12	*forma angustifolia* Vidal	Kunming, Yunnan	(S.Q.Lin) Eba1
13	*E. fragrans* Champ	Nanling, Guangdong	(S.Q.Lin) Ef1
14	*E. cavaleriei* Rehd	Nanling, Guangdong	(S.Q.Lin) Ec1
15	*E. seguinii* Card	Lingle, Guangxi	(S.Q.Lin) Ese1
16	*Rhaphiolepis indica(*L.*)* Lindl. **	Xinyi, Guangdong	(S.Q.Lin) Ri1

*outgroup; **ingroup

Genomic DNAs were isolated from leaf tissue by a modified method (Liu *et al.,* 2005) according to Doyle and Doyle (1987). PCR amplification by MJ Research thermocyclers, were carried out with *Adh*F2 and *Adh*R2 primers (Lin *et al*., 2004) in a 50 μL reaction volume including 2.5 unit *Taq* polymerase (Promega), 10×PCR buffer (Promega), 200 μM each dNTP, 2.0 mM $MgCl_2$, 10 pmol each primer, and 2 μL template DNA (ca. 10-100 ng).

PCR programs were performed with the following temperature profile: a pre-denaturation step of 4 min at 94°C followed by 5 cycles of denaturation 1 min at 94°C, an annealing at 56°C for 45 sec, and an extension at 72°C for 1.5 min, and then followed by 30 cycles of a denaturation at 94°C for 20 sec, an annealing at 55°C for 20 sec, and an extension at 72°C for 1.5 min, with a final extension for 10 min at 72°C.

The 1300bp specific *Adh* sequences were obtained by using *Adh*F2 and *Adh*R2 primers and these PCR products were purified and cloned into the Easy Vector of PGEM@-TSystem II (Promega) according to the manufacturer's instructions, and then transferred into *E. coli* TOP10. The recombinant plasmids were obtained after screened by the blue-white method and identified by restriction endonuclease analysis and PCR.

Individual *Adh*-containing plasmids were isolated and sequenced on an ABI Prism automated DNA sequencer at Shanghai Invitrogen biotechnology Co., Ltd.. Then these sequences were compared with the corresponding DNA sequences of other species reported in GenBank by BLAST software, and finally, the nucleotide homology was found.

The DNA sequences obtained were edited, aligned with ClustalX software (Thompson *et al.*, 1997) and adjusted manually where necessary. Phylogenetic analyses by PAUP4.0b10 (Swofford, 2003), and the construction of the maximum-parsimony (MP) were performed with the heuristic search.

In phylogenetic analysis, ambiguous sites were excluded from the matrix. Gaps were treated as missing information while the inferred indels of clear alignment were recorded as unordered separated characters. All clear characters and character-state transformations were given an equal weight.

A heuristic search was performed for each data set, with RANDOM stepwise data addition (1000 replications with a start seed of 1) and TBR branch-swapping algorithm options. To assess the relative support for each clade, bootstrap values were calculated from 1000 replicate analyses with the heuristic search strategy and simple addition sequence of the taxa. The amount of phylogenetic information in the MP analysis was constructed with the consistency index (CI) and retention index (RI). Maximum parsimony trees were constructed using PAUP4.0b10 program (Swofford, 2003). Cladistic analysis of the phylogenetic relationship was conducted by using Wagner parsimony and applying heuristic search with tree bisection reconnection (TBS) branch-swapping and simple stepwise taxon application of 1000 replications.

Results

Specific primer polymerase chain reaction on Adh gene of Eriobotyra Lindl

Fourteen *Eriobotrya* plants and two relatives were amplified with the universal primers, *Adh*F2 and *Adh*R2, and the 1300bp specific amplified strands were clearly visualized. Fig. 1 shows the result of PCR amplification.

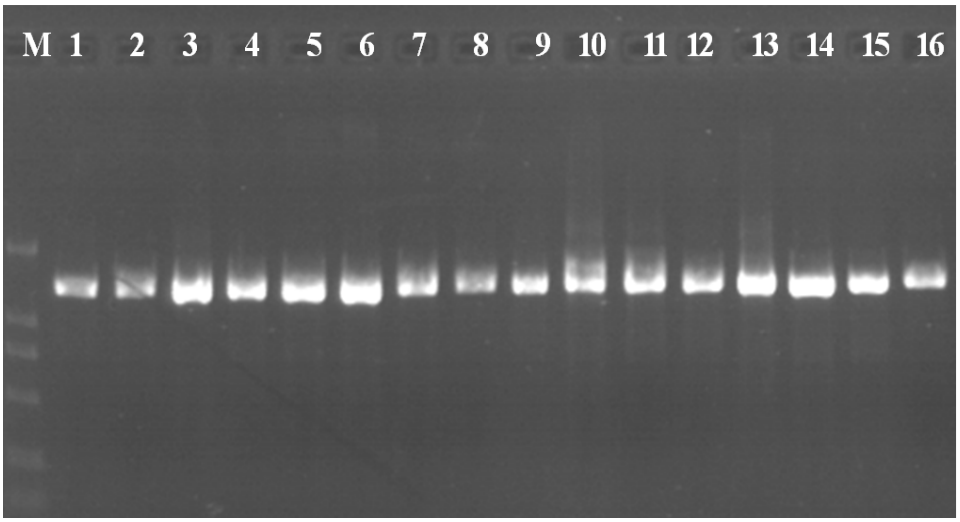

Fig. 1. Products of PCR amplified on *Adh* gene of samples on 14 *Eriobotrya* and outgroup of *Rhaphiolepis indica*. and ingroup of *Photinieae serrulaia* From left to right: M: DL2000; 1. *E. elliptica*; 2. *E. prinoides*; 3. *E. deflexa*; 4. *E. deflexa* var. *buisanensis*; 5. *E. malipoensis*; 6. *E. henryi*; 7. *Photinieae serrulaia*; 8. *E. japonica*; 9. *E. kwangsiensis*; 10. *E. prinoides* var. *dadunensis*; 11. *E .bengalensis*; 12. *E. bengalensis forma angustifolia*; 13. *E. fragrans*; 14. *E. cavaleriei*; 15. *E. seguini*; 16. *Rhaphiolepis indica*

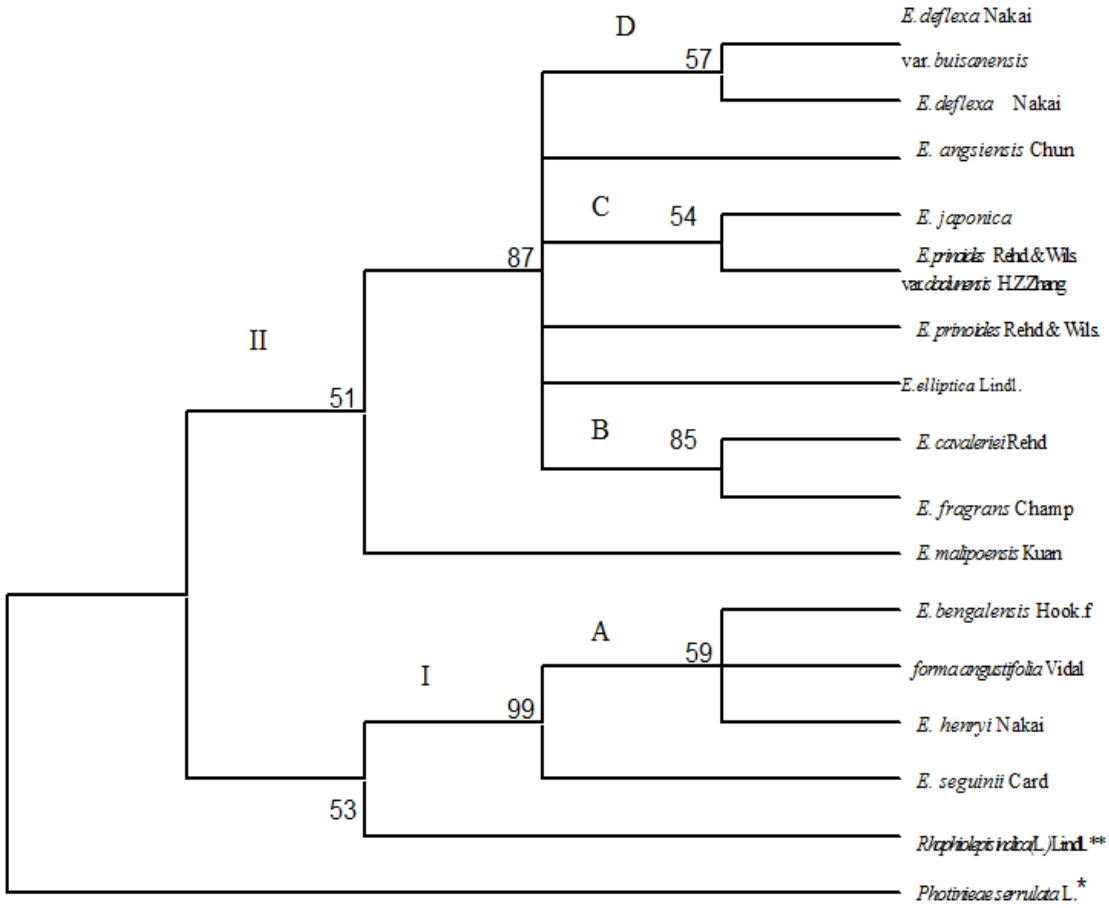

Fig. 2. Majority-rule consensus trees based on *Adh* sequences (Tree length=1001, Consistency index=0.9371), Retention index=0.9709). Numbers in the branches are bootstrap percentages above 50% and by 1000 replications;* indicate outgroup;** indicate ingroup

Phylogenetic analysis of Eriobotrya plants base on Adh gene

Sequencing analysis showed that after arrangement, the matrix is 1332 bp long. The 469 bp were the conserved motifs and 727 bp were the informative polymorphic sites. This result means that the *Adh* gene sequence have high genetic diversity in *Eriobotrya*.

The phylogenetic tree based on the sequence of 14 *Eriobotrya* plants with *Rhaphiolepis indica* as the ingroup and *Photinieae* serrulata as outgroup was constructed by the MP method. The phylogenetic tree had 1001 steps, with a consistency index (CI) 0.9371 and a retention index (RI) 0.9709. Fig. 2 shows the majority-rule consensus trees. The result shows the phylogenic tree could be divided into two clades. *E. henryi*, *E.bengalensis* and *E.bengalensis forma angustifolia* clustered into Group A (Bootstrap value = 59%), and then join together with *E. seguinii* for Clade I (Bootstrap Value = 99%). All the rest clustered into the Clade II. There are four groups in the Clade II, including Group B with *E. cavaleriei* and *E. fragrans* (Bootstrap value = 85%), Group C with *E. prinoides*. var. *dadunensis* and *E. japonica* (Bootstrap value = 54%), Group D with *E. deflexa* and *E. deflexa* var.*buisanensis* (Bootstrap value = 57%). *E. henryi* has the closest relation with the outgroup *Photinieae serrulaia* in Clade I. In Clade II, *E.elliptica*, *E. prinoides,* and *E. angsiensis* formed monophyletic group, respectively, while *E.malipoensis* was at the base of Clade II (Bootstrap Value =51%). Therefore, the interspecies relationships and the phylogeny positions of *Eriobotyra* require further investigation.

Discussion

Adh gene sequences can be used for systematic analysis, suitable for studies on phylogeny relationships and interfamily or intergeneric classification. In China, the *Adh* gene sequence was used to study the phylogenetic and genetic relationships among some plants such as *Paeonia Section Moutan* DC. (Lin *et al.*, 2004), *Oryza* (Ge *et al.*, 1999), *Gossypium* (Small *et al.*, 1998). The results show that when using universal primers, a single *Adh* gene band was obtained for *Eriobotrya* species, which provide favorable conditions to evaluate phylogeny.

The phylogenetic MP tree of 14 *Eriobotrya* plants with *Photinieae serrulaia* as an outgroup and *Rhaphiolepis indica* as an ingroup, was constructed by *Adh* gene sequence analysis. We draw the following conclusions: the phylogenetic tree could be divided into two clades. *E. henryi*, *E. bengalensis,* and *E. bengalensis forma angustifolia* clustered into Group A, and then join together with *E. seguinii* into Clade I while the rest clustered into Clade II. There are four groups in Clade II, including Group B with *E. cavaleriei* and *E. fragrans*, Group C group with *E. prinoides*. var. *dadunensis* and *E. japonica*., Group D with *E. deflexa* and *E. deflexa* var. *buisanensis*, which is largely consistent with the result analyzed by ITS sequence(Li *et al*., 2009).

The relastionships among *E. kwangsiensis*, *E. deflexa,* and *E. deflexa* var. *buisanensis* were so close that showed they may have the same ancestor, which agrees with the previous study of Yang (2005). Since *E. deflexa*, *E. deflexa* var. *buisanensis* and *E. kwangsiensis* were closer in the phylogenetic tree; while *E. prinoides* var. *dadunensis*, *E. japonica*, *E. prinoides* and *E. elliptica* were close with each other, they may lain at the similar place of the phylogenetic stage, respectively. However, *E. malipoensis* need further studies on its phylogenetic relationship for it was separated from others.

In the phylogenetic tree, *E. cavaleriei* and *E. fragrans* were clustered together and formed Group B group with high bootstrap value (85%) which indicated a relatively close relationship. The results agree with the viewpoint that *E. cavaleriei* could be treated as a variety under *E. fragrans* Champ. However, the bootstrap value of some groups are not very high (<60%), which means that to clarify the exact relationships among *Eriobotrya* plants, a more intensive study with more evidence should be carried out.

Small and Wendel (2000) believed that the low resolution of subsection *Erioxylum,* gene tree *AdhA* in *Gossypium*. was caused by both gene flow and unrecognized paralogy and linkage sorting of the *AdhA* gene. Sang *et al*. (2002) also thought that lineage sorting, unrecognized paralogy and gene transformation may cause the low resolution of *Adh* gene tree in Paeonia. From the above analysis, we conclude that although the *Adh* gene sequence holds a high genetic diversity in *Eriobotrya*, some species still cannot separated. It can be suggested that the molecular evolution research of *Eriobotrya* genus is still at an initial phase. A deeper study is required to to determine if there are gene exchanges and unrecognized paralogies.

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References

Cheng YT, Zhong ZX, Guo ZX, Guo YQ, Cai YQ, Chen JY, Lu XM, Wu JS(2003). RAPD analysis of main species and cultivars in *Eriobotrya*. Acta Agric Univ Jiangxiensis. Natural Sci 25(2):259-261.

Doyle JJ, Doyle JL (1987). A rapid isolation procedure for small quantities of fresh leaf tissue. Phytochem Bul 19:11-15.

Ge S, Sang T, Lu BR, Hong DY (1999). Phylogeny of rice genomes with emphasis on origins of *allotetraploid* species. Proc National Acad Sci (USA) 96(25):14400-14405

Li P, Lin SQ, Yang XH, Hu G.B, Jang YM (2009). Molecular

Phylogeny of *Eriobotrya* Lindl.(Loquat) Inferred from Internal Transcribed Spacer sequences of Nuclear Ribosome. Pak J Bot 41(1):185-193

Lindley J (1822). Description as E. *japonica*. Trans Linn Soc 8:102.

Lin QB, Zhou ZQ, Zhao X, Pan KY, Hong DY (2004). Interspecific relationships among the wild species of *Paeonia* Sect. *Moutan* D C. based on DNA sequences of *Adh* Gene Family. Acta Hort Sinica 31(5) 627-632.

Liu YX, Yang XH, Lin SQ, Hu GB, Liu CM (2005). An improved procedure for nuclear DNA isolation from *Eriobotrya* plants and its application. J Fruit Sci 22:182-185.

Sang T (2002). Utility of low-copy nuclear gene sequences in plant phylogentics. Crit Rev Biochem Molec Biol 37(3):121-147

Small RL, Ryburn JA, Cronn RC, Seelanan T, Wendel JF (1998). The tortoise and the hare: choosing between noncoding plastome and nuclear *Adh* sequences for phylogeny reconstruction in a recently diverged plant group. Amer J Bot 85:1301-1315.

Small RL, Wendel JF (2000). Phylogeny, Duplication, and IntraspecificVariation of *Adh* Sequences in New World diploid Cottons (*Gossypium* L. *Malvaceae*). Molecular Phylogenetics and Evolution 16(1):73-84

Swofford DL (2003). PAUP. Phylogenetic Analysis Using Parsimony and Other Methods. Version 4.0b10. Sunderland, Massachusetts: Sinauer Associates.

Thompson JD, Gibson TJ, Plewinak F, Jeanmougin F, Higgins DG (1997). The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876-4882.

Vilanova S, Badenes M L, Marinez-Calvo J (2001). Analysis of loquat (*Eriobotrya japonica* Lindl) germplasm by RAPD molecular markers. Euphytica 121:25-29

Yang XH (2005). A systematic study of the genus *Eriobotrya*. Doctoral dissertation of South China Agric Univ 1-99 p.

Yang XH, Glakpe K, Lin SQ, Hu YL, He YH, Nguyen TCN, Liu YX, Hu GB, Liu CM (2005). Taxa of plants of genus *Eriobotrya* around the world and native to Southeastern Asia. J Fruit Sci 22: 55-59.