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Original research article

Genetic Relationship Assessment and Identification of Orchids in the Genus *Eria* Using HAT-RAPD Markers

Jutamas Jiemjuejun[1] , Somchit Damrianant[1] , Theerachai Thanananta[1]
[1]*Department of Biotechnology, Faculty of Science and Technology*
Thammasat University, Pathum Thani 12120, Thailand

Narumol Thanananta[2,*]
[2]*Faculty of Science and Technology,*
Valaya Alongkorn Rajabhat University under Royal Patronage, Pathum Thani 13180, Thailand

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ABSTRACT

High Annealing Temperature-Random Amplified Polymorphic DNA (HAT-RAPD) markers were used to study the genetic relationship among 15 samples of orchids in the genus *Eria*. DNA amplification of 31 random primers out of 72 random primers was successful. In this study, we selected 21 random primers with clear amplified products for further genetic analysis. The result showed significant differences among all samples and also showed specific bands in each sample. Moreover, we found 21 random primers which tended to be DNA markers for further identification. Based on polymorphic bands, a dendrogram was constructed. Genetic similarities among 15 samples of *Eria* species were demonstrated, and the samples could be classified into 3 groups with similarity coefficients ranging from 0.36 to 0.85.

Keywords: *Eria*; Orchids; HAT-RAPD markers; Genetic relationships

1. Introduction

Orchids are commercially important in Thailand. Export value is expected to increase further because of a wider product range, longer shelf life, and ability to export flowers year round. The orchid family is probably one of the most important plant families from a horticultural point of view. Orchidaceae is one of the largest families among angiosperms. The family includes

*Corresponding author: narumolpla@yahoo.com **doi**: 10.14456/scitechasia.2017.15

800 genera and 25,000 species [1]. Orchids are distributed in all regions of the world except Antarctica and are found growing in many different habitats and elevation gradients [2]. They are well known for their economic importance and widely cultivated for ornamental purposes and cosmopolitan in distribution [3]. The genus *Eria* is one of the largest and most polymorphous groups of orchids, comprising about 370 taxa [4]. *Eria* plants are among the most prevalent orchids for commercial production of cut flowers and potted plants and the most popular genus in horticultural industries [4]. The genetic relationships and identification of the *Eria* genus is not well known.

Orchid classification has been poorly understood because of the lack of fossil records, the large number of species, and a historical emphasis on characters related to floral morphology. Circum-scription of genera, subtribes, tribes, and subfamilies and the relationships among them were unclear because of homoplasy in morphological and anatomical features [5]. There has been no explicit morphological cladistic analysis of Orchidaceae [6]. Phylogenetic research using DNA sequences is now unraveling the complex picture at every taxonomic level. Assessment of genetic variability is important for the use of genetic resources and for determining the uniqueness of genotypes to exploit heterosis [7]. Genetic relationships can be inferred from genetic similarity or distance estimates determined using phenotypic variation and molecular markers.

Molecular markers are not subject to environmental influence, and because of that, they are considered superior to morphological markers [8]. Several types of molecular markers are available and have been used in assessment of orchid genetic relationships [9], whereas a dendrogram from inter simple sequence repeat (ISSR) was constructed for orchid [10] used the intersimple sequence repeat markers to evaluate genetic relationships and identification among 13 *Dendrobium* species and Tansa-nga *et al.*, [11] determined the genetic relationships among 13 species of *Aerides* species using random amplified polymorphic DNA markers.

The random amplification of polymorphic DNA is relatively simple way of creating genomic DNA 'fingerprints' and can be used successfully to identify orchids. RAPD markers can be useful for the identification and differentiation of species. Furthermore, high annealing temperature (HAT-RAPD) has been shown to increase the reproducibility of the technique and is used successfully for genetic character-ization of numerous plant. Studies on detection of genetic relationship and identification of orchids in the genus *Eria* have not been reported from Thailand. Hence, the present study was undertaken to differentiate *Eria* by using HAT-RAPD markers.

2. Materials and Methods

2.1 Plant Materials

A total of 15 *Eria* species was examined. A list of the *Eria* accessions are provided in Table 1.

Table 1. List of *Eria* species.

No.	Name
1	*E. javanica* (SW.) Blume
2	*E. bractescens* Lindl.
3	*E. lasiopetala* (Willd.) Ormerod
4	*E. ornata* (Blume) Lindl.
5	*E. densa* Ridl.
6	*E. sarcophylla* Schltr.
7	*E. biflora* (Griff.) Lindl.
8	*E. sutepensis* Rolfe ex Downie
9	*E. cristata* Rolfe
10	*E. xanthocheila* Ridl.
11	*E. robusta* (Blume) Lindl.
12	*E. globulifera* Seidenf.
13	*E. acervata* Lindl.
14	*E. discolor* Lindl.
15	*E. truncata* Lindl.

2.2 DNA extraction

The genomic DNA of each *Eria* species was extracted from young leaves using the cationic hexadecyl trimethyl ammonium bromide (CTAB) by modified method from Doyle and Doyle [12], and the method from Thanananta *et al.*, [13]. The quality and quantity of extracted DNA samples were assessed by electrophoresis on 0.8 % agarose gel electrophoresis followed by UV spectrophotometric analysis [14]. The concentration of extracted DNA was adjusted to 100 ng/ µ l and samples were stored at -20 °C.

2.3 HAT-RAPD analysis

Screening of 72 HAT-RAPD primers (Wako Company, Japan) was performed using 15 samples of orchids in the genus *Eria*, only 21 HAT-RAPD primers (A22, A23, A24, A27, A29, A30, A31, A32, B21, B22, B23, B25, B27, B32, C22, C24, C29, C31, D31, E23 and E31) showing strong amplification and good reproducibility were selected. The PCR was performed in a volume of 20 µl containing 100 ng of DNA template, PCR buffer (50 mM KCl, 10 mM Tris-HCl (pH 9 .1), 0 .1 % Triton™ X-1 0 0 and 0.25 mM MgCl2) , 200 µM of dNTPs (dATP, dCTP, dGTP and dTTP), 250 nM of HAT-RAPD primers and *Taq* DNA polymerase (Vivantis technologies Sdn Bhd., Malaysia) 1 U (Unit) [13].

The amplification protocol follows 3 steps; (1) 1 cycle of 94°C for 3 minutes (2) 40 cycles of 94°C for 30 seconds, 46°C for 30 seconds and 72°C for 1 minute and then a final cycle (3) 1 cycle of 72°C for 5 minutes. After the thermal cycling program was complete, stored at 4°C prior to the electrophoresis analysis. The amplification reaction products were at least three PCR amplifications that performed for each sample with HAT-RAPD primers to evaluate the reproducibility of the obtained bands. Amplifications were performed in a thermocycler and PCR products were separated on 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Twenty-one primers were firstly screened with individual of 15 samples of orchids in the genus *Eria*. Primers with reproducible patterns and clear cut polymorphisms were chosen to use for analysis of 15 DNA samples. HAT-RAPD profiles were manually scored as 1 for presence or 0 for absence of a band. The scores were entered into a database program (NTSYS-pc version 2.0) and a similarity coefficient matrix was constructed. The unweighted pair group method using arithmetic averages (UPGMA) cluster analysis was performed on genetic similarity matrixes, and relationships among species were visualized as a dendrogram using the NTSYS-pc Exeter Software version 2.0 [15].

3. Results and Discussion

The aim of the present study was to produce HAT-RAPD markers for identification *Eria* species. A total of 72 primers was taken initially and utilized for the amplification from all studied species. Twenty-one HAT-RAPD markers (A22, A23, A24, A27, A29, A30, A31, A32, B21, B22, B23, B25, B27, B32, C22, C24, C29, C31, D31, E23 and E31) were analyzed by PCR. The sequences of these primers are given in Table 2. HAT-RAPD marker was optimized for *Eria* species and produced amplified fragments varying from 100 to 3,000 base pairs (bp) in size for the different primers to be scored across 15 species of orchids in the genus *Eria*. Each primer yielded a wide array of strong and weak bands (Fig. 1). However, only the data of the 21 primers that gave reproducible product formation were included in the statistical analysis.

Table 2. The sequences of primers for HAT-RAPD analysis.

Primers	Sequences
A22	GCCTGCCTCACG
A23	ACTGACCTAGTT
A24	CTCCTGCTGTTG
A27	ATCGCGGAATAT
A29	GGTTCGGGAATG
A30	GACCTGCGATCT
A31	AAGGCGCGAACG
A32	TTGCCGGGACCA
B21	AAGCCTATACCA
B22	GGTGACTGGTGG

B23	GGTGCCGGAGCA
B25	AGCACTGAATCT

Table 2. The sequences of primers for HAT-RAPD analysis. (Continued)

Primers	Sequences
B27	**GGCGGTTATGAA**
C22	**GGTCACCGATCC**
C24	**CCTTGGCATCGG**
C29	**GTCGCCTTACCA**
C31	**TCTGCTGACCGG**
D31	**GGAGGTCGACCA**
E23	**AGGTACGCCGCA**
E31	**GAGGACAGCAA**

The genetic similarity coefficients for the 15 species of orchids in the genus *Eria* ranged from 0.36 to 0.65 (Fig. 3). The UPGMA analysis made it possible to cluster all of the species. The dendrogram (Fig. 2) showed a clear distinction into 3 major groups.

The first group was *E. discolor* Lindl. The second group included *E. bractescens* Lindl. and *E. truncata* Lindl. And the third group could be divided into three subgroups. The first subgroup was composed of *E. javanica* (SW.) Blume, *E. densa* Ridl. and *E. sarcophylla* Schltr. The second subgroup contained *E. lasiopetala* (Willd.) Ormerod, *E. ornata* (Blume) Lindl., *E. cristata* Rolfe, *E. sutepensis* Rolfe ex Downie and *E. biflora* (Griff.) Lindl. Within this second subgroup, the *Eria* genus named *E. lasiopetala* (Willd.) Ormerod paired with *E. ornata* (Blume) Lindl. at the closest genetic similarity coefficients of 0.65. And the third subgroup was composed of *E. xanthocheila* Ridl., *E. robusta* (Blume) Lindl., *E. globulifera* Seidenf. and *E. acervata* Lindl.

From the results, *E. lasiopetala* (Willd.) Ormerod is most closely related to *E. ornata* (Blume) Lindl. (Fig. 2) and appears indistinguishable from it externally. Both taxa share similar features such as slenderly clavate stems topped by a few leaves, biflorous inflorescences with relatively small floral bracts, pubescent flowers with a prominent subglobose mentum, same color, and a more or less subquadrate lip [16]. Not only the HAT-RAPD markers those are effective to identify the *Eria* species but they can also be used to detect other types of orchids such as, *Coelogyne* (Orchidaceae) [17], *Aerides* [18], *Dendrobium* Section Nigrohirsutae [19], *Bulbophyllum* Section Sestochilus [20] and *Dendrobium* spp. (Ueang Sai group) [21]. Furthermore, The HAT-RAPD approach for genetic characterization is highly sensitive and even scare amount of DNA template are sufficient for analysis. HAT-RAPD markers have been successfully used for identification of various plant species such as, banana [22], Lychee [23], *Ficus* spp. [24], *Brassica* spp. [25] and *Curcuma* [26].

4. Conclusion

This paper, study for the characterization of genetic relationship among 15 species of orchids in the genus *Eria* based on HAT-RAPD markers. The genetic similarity coefficients for the 15 species ranged from 0.36 to 0.65. The dendrogram showed a clear distinction within the 3 major groups. This study shows that the molecular markers could be utilized for the comparison of genetic relationship in orchid species, which their morphological descriptions are most similar. It also indicates that the HAT-RAPD markers may be considered as an effective and convenient method to classify *Eria* species. In conclusion, this research has demonstrated the HAT-RAPD

markers as a useful tool for DNA polymorphisms detection, to examine genetic relationship of orchids in the genus *Eria*. The present study highlights the importance of HAT-RAPD markers. It has been widely used as a genetic screening method [27] because it is rapid, relatively simple to be performed, and required only a small amount of genomic DNA without genome sequence information prior to analysis [28]. Furthermore, it can also be used for the study of genetic diversity of orchid species.

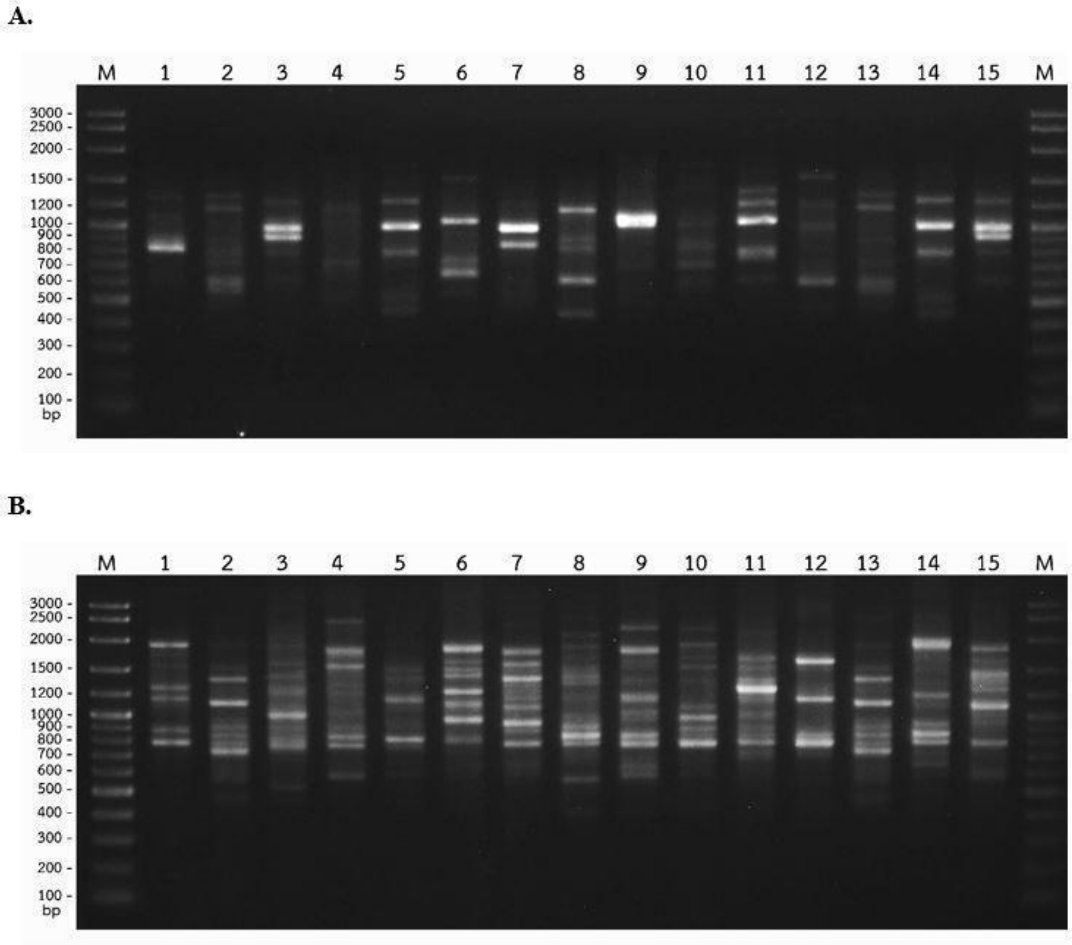

Fig. 1 Examples of HAT-RAPD patterns amplified A32 (A) and B23 (B) primers of the *Eria* 15 species. [Lane M is molecular weight marker 100 bp plus DNA Ladder (Vivantis, Vivantis technologies Sdn. Bhd., Malaysia), Lanes 1-15, (1) *E. javanica* (SW.) Blume (2) *E. bractescens* Lindl. (3) *E. lasiopetala* (Willd.) Ormerod (4) *E. ornata* (Blume) Lindl. (5) *E. densa* Ridl. (6) *E. sarcophylla* Schltr. (7) *E. biflora* (Griff.) Lindl. (8) *E. sutepensis* Rolfe ex Downie (9) *E. cristata* Rolfe (10) *E. xanthocheila* Ridl. (11) *E. robusta* (Blume) Lindl. (12) *E. globulifera* Seidenf. (13) *E. acervata* Lindl. (14) *E. discolor* Lindl. and (15) *E. truncata* Lindl.]

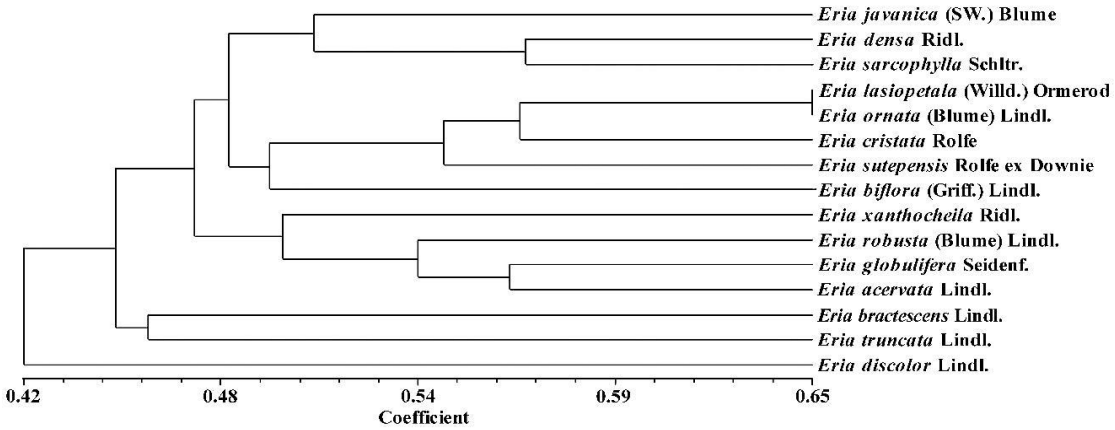

Fig. 2 Dendrogram constructed by cluster analysis (UPGMA) of HAT- RAPD data of the 15 *Eria* species with 21 primers.

Eria javanica (SW.) Blume	1.000														
Eria bractescens Lindl.	0.463	1.000													
Eria lasiopetala (Willd.) Ormerod	0.487	0.540	1.000												
Eria ornata (Blume) Lindl.	0.492	0.460	0.652	1.000											
Eria densa Ridl.	0.507	0.400	0.485	0.438	1.000										
Eria sarcophylla Schltr.	0.502	0.400	0.506	0.510	0.567	1.000									
Eria biflora (Griff.) Lindl.	0.451	0.427	0.531	0.442	0.448	0.532	1.000								
Eria sutepensis Rolfe ex Downie	0.465	0.451	0.528	0.559	0.494	0.513	0.486	1.000							
Eria cristata Rolfe	0.423	0.458	0.567	0.564	0.420	0.532	0.510	0.543	1.000						
Eria xanthocheila Ridl.	0.474	0.438	0.465	0.502	0.433	0.431	0.412	0.506	0.508	1.000					
Eria robusta (Blume) Lindl.	0.514	0.413	0.500	0.512	0.474	0.471	0.458	0.500	0.494	0.480	1.000				
Eria globulifera Seidenf.	0.464	0.439	0.465	0.461	0.407	0.494	0.496	0.518	0.498	0.485	0.556	1.000			
Eria acervata Lindl.	0.408	0.429	0.384	0.444	0.462	0.450	0.438	0.506	0.484	0.523	0.516	0.563	1.000		
Eria discolor Lindl.	0.413	0.358	0.397	0.457	0.495	0.382	0.409	0.403	0.424	0.382	0.466	0.472	0.427	1.000	
Eria truncata Lindl.	0.454	0.456	0.548	0.414	0.389	0.475	0.486	0.480	0.451	0.441	0.435	0.442	0.381	0.387	1.000
	Eria javanica (SW.) Blume	*Eria bractescens* Lindl.	*Eria lasiopetala* (Willd.) Ormerod	*Eria ornata* (Blume) Lindl.	*Eria densa* Ridl.	*Eria sarcophylla* Schltr.	*Eria biflora* (Griff.) Lindl.	*Eria sutepensis* Rolfe ex Downie	*Eria cristata* Rolfe	*Eria xanthocheila* Ridl.	*Eria robusta* (Blume) Lindl.	*Eria globulifera* Seidenf.	*Eria acervata* Lindl.	*Eria discolor* Lindl.	*Eria truncata* Lindl.

Fig. 3 The similarity coefficients of HAT- RAPD data of the 15 *Eria* species with 21 primers.

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