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Application of internal transcribed spacers and maturase K markers for identifying *Anoectochilus*, *Ludisia*, and *Ludochilus*

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Abstract

Internal transcribed spacer (ITS) regions and maturase K (*matK*) sequence polymorphisms provide an efficient tool for discrimination and conservation of genetic resources of *Anoectochilus* species. The objectives of this study were to develop markers specifically distinguishing *A. formosanus* Hayata from closely related *A. koshunensis* Hayata, *A. roxburghii* (Wall.) Lindl., and *Ludisia discolor* (Ker Gawl.) A. Rich. and to identify a molecular phylogenetic relationship of a new intergeneric BC_1F_1 hybrid – *Ludochilus Jin-Chai*. Specific primers for nuclear ITS regions and chloroplast *matK* sequences were designed and converted into cleaved amplified polymorphic sequence (CAPS) markers. Results show that the *matK* sequences obtained corresponded to pseudogenes and that their digestion with enzyme *Hinf*I revealed a polymorphic pattern in *A. formosanus* and *A. koshunensis*. The pedigree of *Lud. Jin-Chai*, which was derived from the cross between *Lus. discolor* and *A. formosanus*, was also confirmed based on ITS and *matK* CAPS markers.

Additional key words: chloroplast *matK* sequence, intergeneric hybrid, jewel orchids, plant phylogenetic analysis.

Introduction

The genus *Anoectochilus* (*Orchidaceae*) consists of 30 to 40 species found in Sri Lanka, Malaysia, India, Japan, southern China, Australia, and South Pacific Islands (Teuscher 1978). It belongs to a group of terrestrial orchids known as "Jewel Orchids" which have attractive foliage with intricate reticulations of silver veins on the above surface (Cavestro 1994). They are difficult to identify beyond the flowering period because of morphological similarities among species (Cheng *et al.* 1998). In Taiwanese folk remedies, hot water extracts of the whole *A. formosanus* Hayata (Fig. 1*A*) plant are used as internal medicine for treatment of chest and abdominal pain, diabetes, nephritis, fever, hypertension, and liver and spleen disorders. Du *et al.* (2003) and Fang *et al.* (2008) reported that crude extracts of *A. formosanus* contain active ingredients for hepato-protective activity and anti-hyperliposis effect. Because *A. formosanus* is a slow growing perennial herb with a high market value, other related taxa, such as *A. koshunensis* Hayata, *Goodyera schlechtendaliana* (Du *et al.* 2008), and some unidentified herbs from South Asia are often found as adulterants on herb market.

Given that the natural sources of *Anoectochilus* genus in Taiwan are nearly exhausted at present, micro-propagation of these species by tissue culture technique and artificial cross-pollination are considered as viable alternative for propagation (Shiau *et al.* 2002). Furthermore, *A. formosanus* has been shown to readily cross pollinate with *A. koshunensis* (Cheng *et al.* 1998) or *Haemaria discolor* (Chou and Chang 2004). A new intergeneric BC_1F_1 hybrid cultivar plant – *Ludochilus Jin-Chai* ('Tainung Chaijin'; Fig. 1*B*) was registered in the International Orchid Registrar at the Royal Horticultural Society and released in 2005. The BC_1F_1 hybrid, derived from the cross of *Ludisia discolor* (Ker Gawl.) A. Rich. (Fig. 1*C*) and *A. formosanus*, is hardly distinguishable

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Abbreviations: CAPS - cleaved amplified polymorphic sequence; CTAB - cetyltrimethylammonium bromide; ITS - internal transcribed spacer; *matK* - maturase K; PCR - polymerase chain reaction; PPFD - photosynthetic photon flux density; RAPD - randomly amplified polymorphic DNA; SNP - single nucleotide polymorphism.

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from the male parent – *A. formosanus* due to morphological similarities. Medical and non-medical *Curcuma* plants are often confused due to the same reason and Cao *et al.* (2001) demonstrated how to authenticate *Curcuma* species based on nuclear 18S rDNA and chloroplast maturase K (*matK*) sequence variations. In this sense, numerous reports have indicated that combining ribosomal DNA and plastid genes could be a useful tool for species identification and phylogenetic analysis in *Orchidaceae* subtribes *Aeridinae, Disinae*, and *Cypripediolideae* (Hidayat *et al.* 2005, Bytebier *et al.* 2007, Sun *et al.* 2011).

Both nuclear internal transcribed spacer (ITS) regions and a chloroplast *matK* sequence have been widely used in plant phylogenetic analysis because of their small size, highly conserved flanks, and a higher nucleotide substitution rate (Hidalgo *et al.* 2004, Tokuoka 2008, Qiu *et al.* 2012). Lahaye *et al.* (2008) analyzed thousands of orchid samples collected from Mesoamerica and southern Africa by eight molecular markers including plastid *accD*, *matK*, *ndhJ*, *rbcL*, *rpoB*, *rpoc1*, *trnH-psbA*, and *ycf5*. The results revealed the *matK* sequence alone has a sufficient sequence variation to allow the discrimination of species. As a result, the *matK* sequence was their preferred option out of the eight markers. Furthermore, although Cheng *et al.* (1998) first demonstrated identification of *A. formosanus* and *A. koshunensis* based on randomly amplified polymorphic DNA (RAPD) markers, comprehensive phylogenetic relationships among *Anoectochilus* species have yet to be identified. The objectives of this study were to provide a molecular tool for discriminating *A. formosanus* from closely related *A. koshunensis*, *A. roxburghii*, *Lus. discolor*, and *Lud. Jin-Chai* and to overcome the difficulty for distinguishing them on a traditionally used morphological basis. To achieve these objectives, both nuclear ITS regions and chloroplast *matK* sequences were analyzed and converted into cleaved amplified polymorphic sequence (CAPS) markers to analyze sequence variation.

Materials and methods

Plantlets of *Anoectochilus formosanus* Hayata, *A. koshunensis* Hayata, *A. roxburghii* (Wall.) Lindl., and *Ludisia discolor* (Ker Gawl.) A. Richwere were used as plant materials in the present study. The three *Anoectochilus* species mentioned above have dark-green or blackish-green remarkably colorated leaves with copper, silver, or gold sparking metallic veins. The *Lus. discolor* plantlet has blackish-purple leaves with pink or red veins. Furthermore, the plant materials also included anintergeneric BC_1F_1 hybrid *Ludochilus Jin-Chai* and reciprocal cross F_1 hybrids (LA and AL) of *Lus. discolor* and *A. formosanus*. All plants were grown in the Agricultural Research Institute, Taichung, Taiwan, for two years. They were maintained in a growth chamber under a photosynthetic photon flux density (PPFD) of 30 to 38 μmol m^{-2} s^{-1} provided by white fluorescent lamps, a 16-h photoperiod, a relative humidity of 90 %, and day/night temperatures of 25/20 °C. The plants were irrigated with tap water once a week.

Total genomic DNA was extracted from 1 g of fresh leaf tissues of all plant materials using the cetyltrimethylammonium bromide (CTAB) extraction method with minor modifications (Bornet and Branchard 2001). Because few *matK* sequences for *Anoectochilus* are available, *Phalaenopsis aphrodite* Rchb. f. subsp. *formosana* (accession number AY916449) was retrieved from the *GenBank* database (http://www.ncbi.nlm.nih.gov/genbank/) for primer design which was carried out with the *Primer3* program (http://workbench.sdsc.edu/). Amplification of the *matK* sequence was conducted with *matK*-F (5'-ATGTATCATTTCATAACACAAGAA-3') and *matK*-R (5'-CCGTGCTTGCAGTTTTCA-3') primers, covering the complete *matK* sequence and partial tRNA-Lys gene. The *A. formosanus* nuclear ribosomal ITS sequence (accession number AY052780) was obtained from the *GenBank* database and we directly retrieved the first 20 bp and last 15 bp to design primers ITS-F (5'-GAGAAGTCCATTGAACCTTA-3') and ITS-R (5'-CATCCCGCCCATCCT-3'), respectively. Another universal primer pair based on *Oryza sativa*, *Solanum lycopersicum*, and *Oncidium* species ITS regions was designed as ITS-UF (5'-CGTAACAAGGTTTCC-3') and ITS-UR (5'-AGTTTCTTCTCCTCC-3') (Tsai and Chou 1999). Each 0.02 cm^3 of a PCR reaction mixture consisted of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 mM primers, 0.6 unit of *Taq* polymerase, and 100 ng of genomic DNA. All amplifications were performed on a *PTC-200* thermal cycler (*MJ Research*, Waltham, MA, USA) under the following conditions: 94 °C for 5 min, followed by 35 cycles (94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min 30 s), and a final extension at 72 °C for 5 min.

For cleaved amplified polymorphic sequence (CAPS) analysis, the PCR fragments (0.01 cm^3) were incubated at 37 °C for 2 h in a volume of 0.02 cm^3 with 1 unit of a restriction enzyme and subsequently resolved in a 1 % (m/v) agarose gel. The restriction with enzymes *Hinf*I, *Mse*I, *Dpn*II, and *Cvi*QI (*New England BioLabs*, Ipswich, MA, USA) was analyzed to generate CAPS markers. Finally, *Hinf*I was the only enzyme selected for CAPS analysis. The innate PCR product was cloned into plasmid vector *pGEM-T Easy* (*Promega*, Madison, WI, USA) which was then transformed into *Escherichia coli* strain JM109. For each sample, at least three independent clones were sequenced using universal *T7* and *SP6* primers (*Protech Technology Enterprise Company*, Nangang, Taiwan).

Results and discussion

Both *A. formosanus* and *A. koshunensis* were analyzed by PCR primers ITS, ITS-U, and *matK*. The PCR products from each sample of the ITS regions and *matK* sequence showed single fragments corresponding to approximately 1.7 kb and 750 bp in length, respectively. The PCR products were digested by four different restriction endo-nucleases, *Cvi*QI, *Dpn*II, *Hinf*I, and *Mse*I and revealed polymorphic patterns between *A. formosanus* and *A. koshunensis* in the *mat*K sequence digested with *Hinf*I and *Mes*I (Fig. 2). For the sake of convenience, *Hinf*I was selected to perform the rest of the CAPS analysis. The CAPS marker of the *mat*K sequence revealed three polymorphic patterns which can be used to separate *Lus. discolor*, *A. formosanus*, *A. koshunensis*, and *A. roxburghii* (Fig. 3). Cheng *et al.* (1998) found specific RAPD markers to identify *A. formosanus* and *A. koshunensis* using decamer primers OPC-08 and OPL-07. The present study indicates that RAPD markers could be used to identify *Lus. discolor* and *A. formosanus* as well (data not shown). However, the reproducibility of RAPD markers is low. This is because reaction conditions vary in different laboratories, even though RAPD can be developed in a

Fig. 1. The appearances of a new intergeneric BC_1F_1 hybrid – *Lud. Jin-Chai* ('Tainung Chaijin') (*B*) and its female and male parents *A. formosanus* Hayata (*A*) and *Lud. discolor* [Ker Gawl.] A. Rich (*C*). *Scale bars*: 1.5 cm (*A*), 1.7 cm (*B*), and 2.1 cm (*C*).

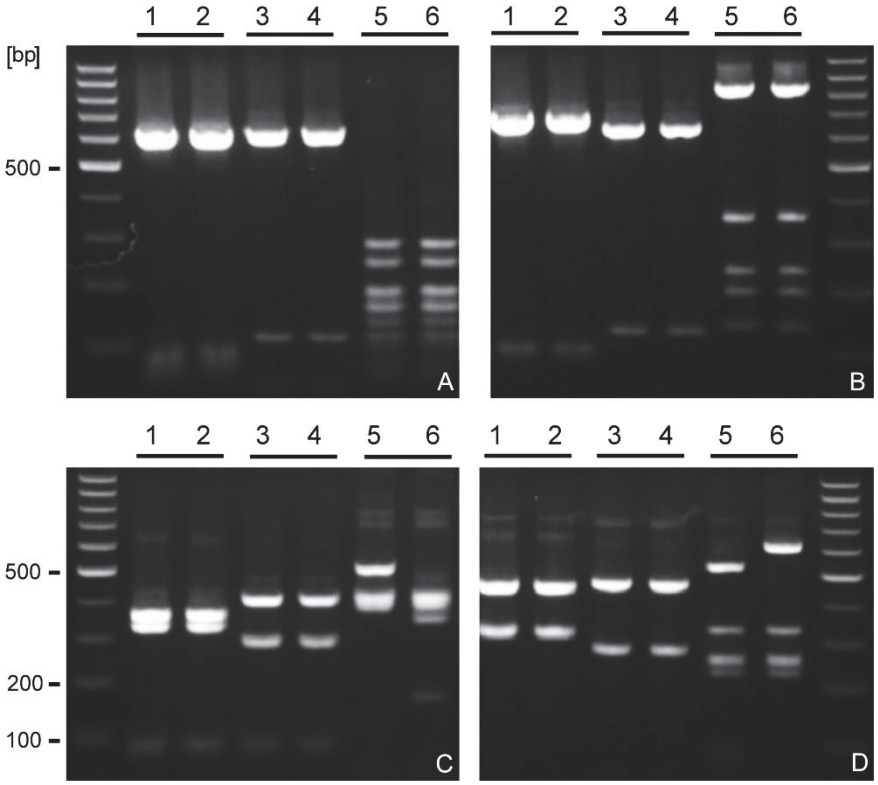

Fig. 2. CAPS analysis of ITS regions and *matK* gene of *A. formosanus* and *A. koshunensis.* PCR products amplified with primers ITS (*lanes* 1, 2), ITS-U (*lanes* 3, 4), and *matK* (*lanes* 5, 6) were digested with *Cvi*QI (*A*), *Dpn*II (*B*), *Hinf*I (*C*), and *Mse*I (*D*) to reveal polymorphisms. *Lanes* 1, 3, 5 - *A. formosanus*, *lanes* 2, 4, 6 - *A. koshunensis*.

relatively short time (Agarwal *et al.* 2008). Moreover, RAPD is not an ideal tool for the phylogenic assay of *Anoectochilus* species because it is difficult to distinguish heterozygous from homozygous progeny. These results suggest that CAPS markers derived from ITS regions and *matK* sequence might constitute efficient tools for genus and species discrimination within *Orchidacene*.

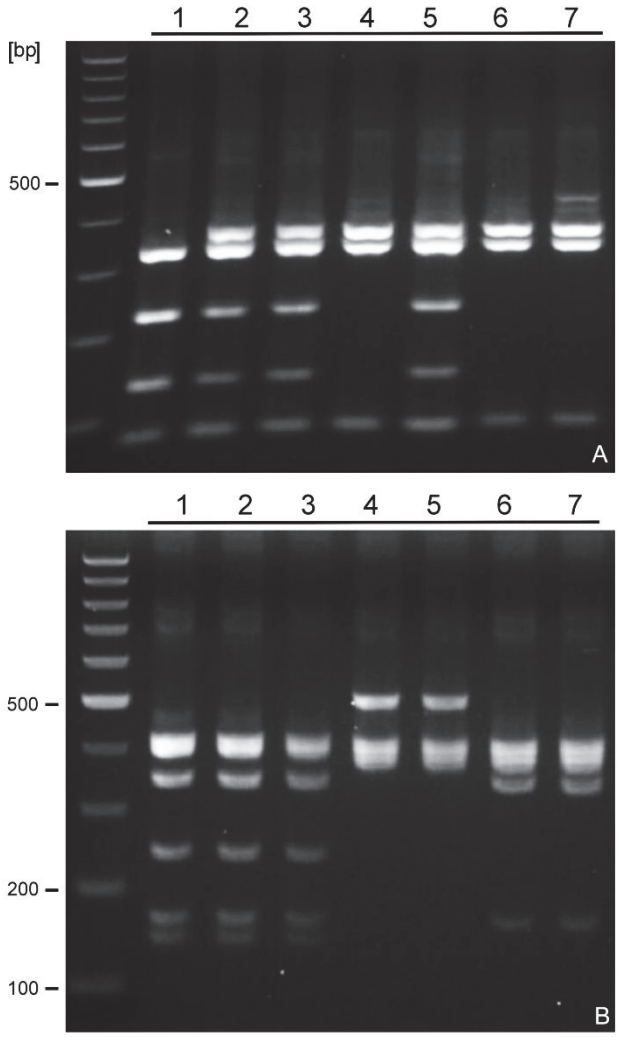

Fig. 3. CAPS analysis of *Lud. discolor*, *A. formosanum*, and intergeneric hybrids based on ITS regions (*A*) and *matK* gene (*B*). *Lane* 1 - *Lus. discolor*, lane 2 - F_1 hybrids of *Lus. discolor* × *A. formosanus* (LA), *lane 3* - *Lus. Jin-Chai*, *lane* 4 - *A. formosanus*, *lane* 5 - F_1 hybrid of *A. formosanus* × *Lus. discolor* (AL), lane 6 - *A. koshunensis*, *lane* 7 - *A. roxburghii*.

Because the CAPS markers of ITS regions and *matK* sequence are identical in *A. koshunensis* and *A. roxburghii*, and no *matK* sequence of *Anoectochilus* species has been reported, the ITS regions amplified by ITS and ITS-U primers were sequenced and the fragments were 760 and 765 bp in length, respectively. Sequence alignments reveal that ITS and ITS-U flanked the same rDNA regions and slip at the 5' and 3' ends. The ITS regions belonged to *A. formosanus*, *A. koshunensis* (EU700340), and *A. roxburghii* (EU817408) and two to three single nuclear polymorphism (SNP) were found among each other. Previous studies have revealed that the highly conservative nature of ITS sequences within *Anoectochilus* makes it difficult to detect polymorphism under this genus through the ITS sequence in most cases (Gao *et al.* 2009). The *matK* genes and partial tRNA-*Lys* genes belong to the *Anoectochilus* species and vary in length from 1784 to 1786 bp due to separate base substitution and single nucleotide insertion/deletion events. There were 11 SNP between *A. formosanus* (EU797513) and *A. koshunensis* (EU797512), 17 SNP between *A. koshunensis* and *A. roxburghii* (EU817409), and 23 SNP between *A. formosanus* and *A. roxburghii* (Fig. 4). These results are consistent with the CAPS analysis and indicate that *A. roxburghii* and *A. koshunensis* contained more variation than do *A. formosanus* and *A. koshunensis*. Furthermore, examination of the translated *matK* genes revealed the premature stop codons, and only small potential open reading frames were present (Fig. 4) leading to non-functionality, such as *Lus. discolor* and *Valeriana matK* pseudogene (Hidalgo *et al*. 2004). Hence, the results support assertion that the plastid *matK* sequence is a pseudogene in *Anoectochilus*. Non-functional *matK* pseudogenes have been found in genera *Corallorhiza*, *Aplectrum*, and *Oreorchis*, revealed by the 5' deletion and resulting in a truncated *matK* pseudogene, which might be a common phenomenon in *Orchidaceae* (Freudenstein and Senyo 2008).

Chloroplast genes are characterized by their maternal inheritance properties and have been used to analyze a wide range of plant species (Hidalgo *et al.* 2004, Hidayat *et al.* 2005, Lee *et al.* 2011). Stegemann *et al.* (2003) developed a transgenic chloroplast system for selecting and analyzing DNA transfer events from the chloroplast genome to the nuclear genome. They demonstrated a high frequency and an ongoing process of gene transfer out of the chloroplast into the nucleus and thus providing a mechanism not only causing an intraspecific but also intraorganismic genetic variation in multicellular eukaryotes. In addition, genome fragments of chloroplast DNA may have no clear functional roles but may provide a raw material for converting organellar genes into functional nuclear genes. In another words, chloroplast *matK* genes pseudogenization might undergo transferring from the chloroplast genome to the nucleus (Yoshida *et al.* 2014). The inheritance of *matK* pseudogenes is not anymore exclusively maternal. In the present study, intergeneric hybrid *Lud. Jin-Chai* revealed a heterozygous pattern in the CAPS markers of ITS regions as well as of reciprocal crosses of F_1 progenies LA and AL (Fig. 3). The *matK* CAPS markers belonging to *Lud. Jin-Chai* and LA were identical with their maternal source – *Lus. discolor*, whereas the *matK* CAPS marker of AL was identical with *A. formosanus*. Despite *Anoectochilus matK* gene being a pseudogene, the exclusively maternal inheritance of this *matK* sequence was observed. Indeed, the chloproplast profiles obtained for the hybrids LA and AL were different but identical to their respective mothers.

In conclusion, the *Anoectochilus* genus is closely related to *Lus. discolor* based on the similarity between the ITS regions and *matK* pseudogene in the present study. However, all the data do not seem to explain whether the origin of *Anoectochilus matK* pseudogene derived from species possessing with common polymorphic ancestor or just a mutational event occasionally. The *matK* pseudosequence accumulated many variations, but it is highly conserved in the flanking region and thus the same primer sets were used to amplify the *Anoectochilus* and *Ludisia* genus *matK* pseudogenes. Moreover, the molecular marker described in this study is also useful in a positive identification of the pedigree of the new intergeneric hybrid BC_1F_1 cultivar *Lud. Jin-Chai*. The ITS and *matK* markers developed in this study are valuable tools for discrimination of *Anoectochilus* species and other *Orchidaceae* plants.

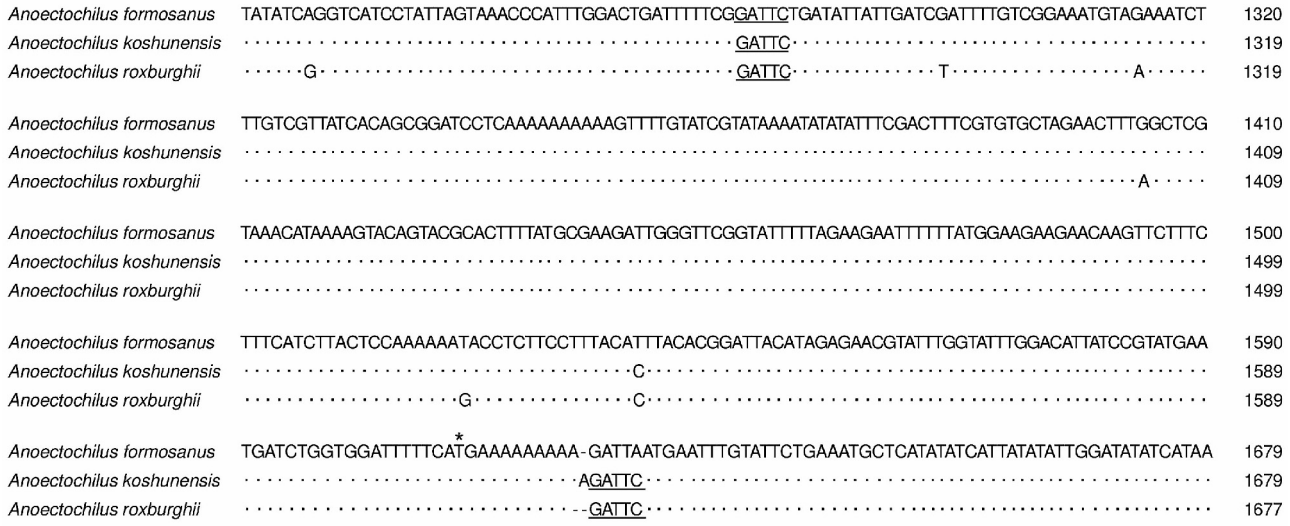

Fig. 4. Phylogenetic informative sites in multiple sequence alignments of plastid *matK* genes and partial tRNA-*Lys* genes for *Anoectochilus formosanus* (EU797513), *A. koshunensis* (EU797512), and *A. roxburghii* (EU817409). *Underlines* indicate recognition sites of restriction endo-nuclease *Hinf*I, and an *asterisk* indicates the stop codon.

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