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GENETIC RELATIONSHIPS OF *PHRAGMIPEDIUM* SPECIES (ORCHIDACEAE) USING AMPLIFIED FRAGMENT LENGTH POLYMORPHISM (AFLP) ANALYSIS

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ABSTRACT. The objective of this study is to evaluate the genetic relationships of eight Peruvian species of the genus *Phragmipedium* using amplified fragment length polymorphisms and a phenetic analysis (UPGMA). The analysis showed that the species are phenetically grouped in four large clusters. The first cluster includes species from the *Himantopetalum* (*P. pearcei* and *P. richteri*) and *Lorifolia* (*P. boissierianum*) sections. The second cluster comprised the species of the *Micropetalum* section (*P. besseae* and *P. schlimii*). The third one included *P. kovachii* in the *Schluckebieria* section. The fourth cluster contains species of the *Phragmipedium* (*P. caudatum* and *P. wallisii*) section. The results show that AFLP analysis is a powerful genetic tool for the analysis of the taxonomy of orchids from the diverse and complex *Phragmipedium* genus.

RESUMEN**.** El presente estudio tiene como objetivo evaluar la diversidad y distancia genética de 8 especies del género *Phragmipedium* usando la técnica de amplified fragment length polymorphisms (AFLP) y el método de análisis fenético (UPGMA). Las especies son agrupadas feneticamente en cuatro clusters mayores. El primer cluster comprende especies de las secciones *Himantopetalum* (*P. pearcei* y *P. richteri*) y *Lorifolia* (*P. boissierianum*). El segundo cluster incluye las especies de la sección *Micropetalum* (*P. besseae* y *P. schlimii*). El tercer cluster contiene a la especie de la sección *Schluckebieria* (*P. kovachii*). El cuarto cluster contiene a las especies de la sección *Phragmipedium* (*P. caudatum y P. wallisii)*. Los resultados muestran que AFLP es una herramienta genética poderosa para el análisis taxonómico del diverso y complejo género *Phragmipedium*..

KEY WORDS: Orchidaceae, DNA markers, *Phragmipedium*, *Phragmipedium kovachii,* AFLP, Fingerprinting.

Introduction

The orchid genus *Phragmipedium* Rolfe comprises species distributed in six sections including; *Phragmipedium, Himantopetalum, Platypetalum, Lorifolia, Micropetalum,* and *Schluckebieria.*(Gruss 2003; Braem 2004).

Concerns about the taxonomic placement of some species in this genus have frequently been raised in this genus, since some accessions show morphological characteristics that are intermediate between different species. Improvements in the understanding of taxonomic relationships are likely to occur with the extensive analysis of molecular markers that unravel the genetic relationships and allow us to produce a phylogeny of the new species, thus aiding the identification of varieties.

Analysis of the genetic diversity of endangered plant species is one of the key elements in the estimation of the viability of its populations, and in the establishment of efficient *in situ* as well as *ex situ* preservation.

The AFLP (amplified fragment length polymorphism) method (Vos *et al*. 1995) is a valuable DNA-based technique that informs about multiple polymorphic anonymous loci, generally in the nuclear genome of a species and can be used to determine genetic diversity in many plant species, particularly when there are few characterized molecular genetic markers (Mackil *et al*.1996; Segovia-Lerma *et al.* 2003; Nguyen *et al*. 2004)

The objective of this study was to evaluate genetic distance among 8 species of genus *Phragmipedium* by using AFLP analysis

Materials and Methods

PLANT MATERIAL. Plants were maintained under greenhouse conditions in the orchid collection at Peruanino Nursery (Vivero Peruanino) located in San Ramon, Region Junín. The plants were labeled by assigning an individual code in order to keep them identified. For this work, permission was granted to collect simples from registered plants by the Peruvian authority of Natural Resources (INRENA). We collected young leaves from the plants and kept them at -70°C in the lab until use.

Our study comprises eight species corresponding to five of the six sections of *Phragmipedium* genus: *P. pearcei, P. besseae, P. boissierianum, P. caudatum, P. wallisii, P. richteri, P. schlimii* and *P. kovachii.*

DNA ISOLATION**.** DNA was isolated from 4-5 grams of plant material. DNA extraction was performed as described by Doyle and Doyle (1990) and Ghislain *et al*. (1997).

AFLP ANALYSIS**.** AFLP analysis and the bands on gel were visualized by silver staining method. (Vos *et al*. 1995; Ghislain *et al.* 1997). AFLP analysis was conducted as follow: 0.5 mg of genomic DNA was digested simultaneously by two restriction enzymes *Eco*RI and *Mse*I(for 3 h and 15 min at 37 ^{O}C). Following heat inactivation of the restriction enzymes (70 ^{O}C), the DNA digested was ligated with *Eco*RI and *Mse*I adapters for 3 h at 20 ^{O}C to generate template DNA for amplification. 20 ng of the template DNA generated were first pre-amplified by PCR: 1 cycle (72 ^{O}C for 1 min), 26 cycles (94 ^{O}C for 30 sec, 56 ^{O}C for 1 min, 72 ^{O}C for 1 min), 1 cycle (72 ^{O}C for 5 min) and 4 ^{O}C using *Eco*RI and *Mse*I primers with one selective nucleotide. The selective amplification AFLP reactions were performed using two primers *Mse*I and *Eco*RI with three selective nucleotides by PCR: 1 cycle (94 ^{O}C for 2 min, 65 ^{O}C for 20 sec, and 72 ^{O}C for 2 min), 12 cycle (94 ^{O}C for 30 sec, 65 ^{O}C for 30 sec, and 72 ^{O}C for 2 min), 20 cycle (94 ^{O}C for 30 sec, 56 ^{O}C for 30 sec, and 72 ^{O}C for 1 min) and 4 ^{O}C, the selective amplification reactions were performed with 18 primer combinations acquired from Invitrogen™. (*Eco*RI 36/*Mse*I 48, *Eco*RI 36/*Mse*I 55, *Eco*RI 38/*Mse*I 60, *Eco*RI 36/*Mse*I 60, *Eco*RI 38/*Mse*I 50, EcoRI 36/*Mse*I 50, *Eco*RI 35/*Mse*I 55, *Eco*RI 35/*Mse*I 60, *Eco*RI 38/*Mse*I 55, *Eco*RI 35/*Mse*I 50, *Eco*RI 38/*Mse*I 48, *Eco*RI 42/*Mse*I 50, *Eco*RI 35/*Mse*I 48, *Eco*RI 42/*Mse*I 55, *Eco*RI 40/*Mse*I 50, *Eco*RI 31/*Mse*I 60, *Eco*RI 45/*Mse*I 60, *Eco*RI 45/*Mse*I 45) (Fig. 1).

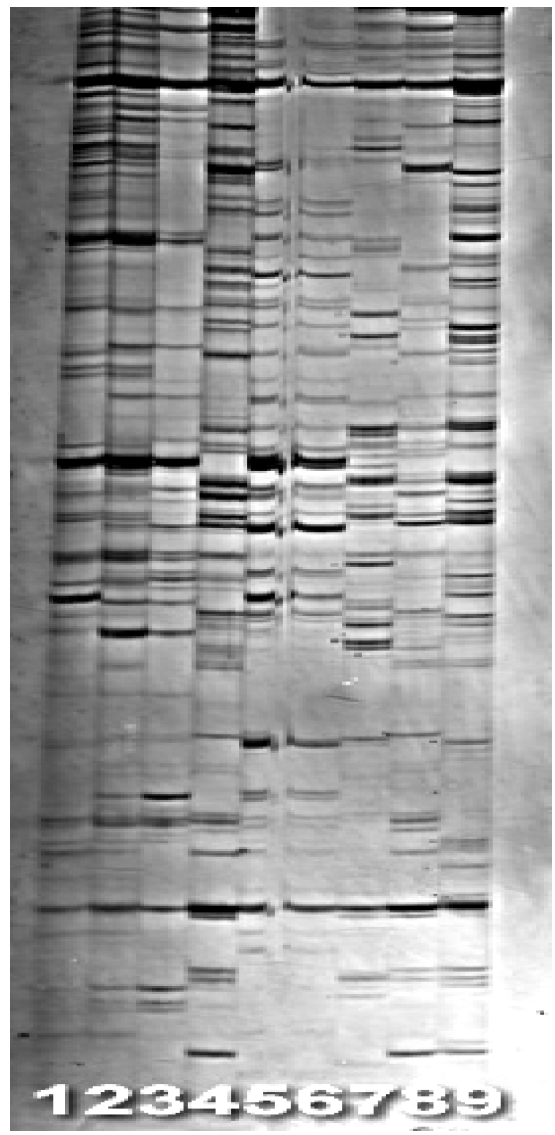

FIGURE 1. The AFLP fragments in denaturing polyacrylamide gel of orchids species from genus *Phragmipedium*: 1. *P. pearcei;* 2. *P. richteri;* 3. *P. boissierianum;* 4,8. *P. besseae;* 5. *P. wallisii;* 6. *P. caudatum;* 7. *P. kovachii;* 9. *P. schlimii.*

DATA ANALYSIS. AFLP products were scored as the presence (1) or absence (0) of bands in each species to form a binary matrix. Only AFLP fragments that

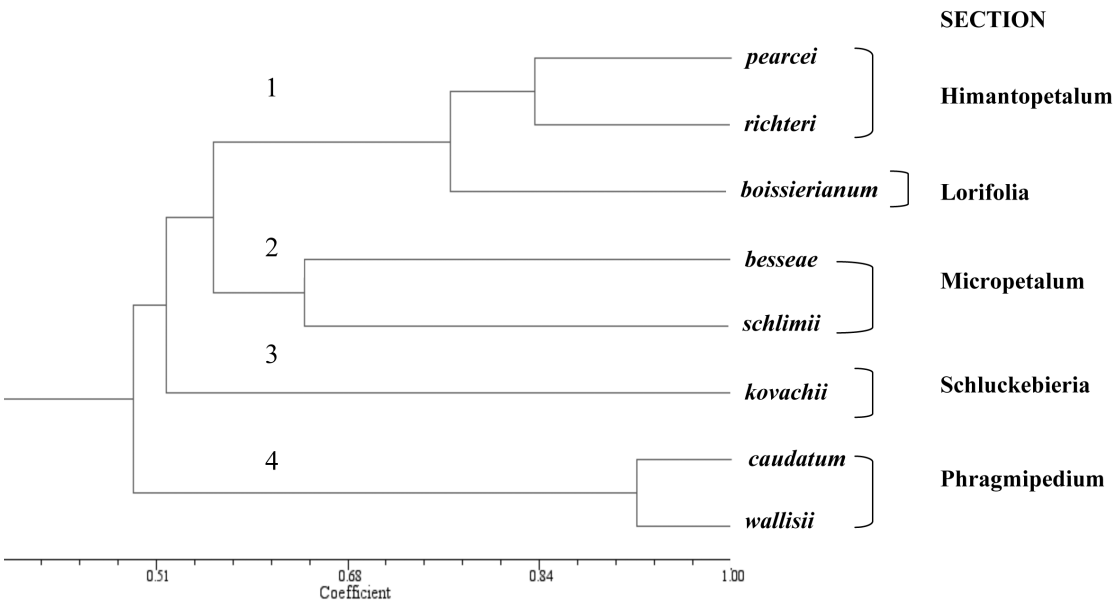

FIGURE 2. UPGMA dendogram of genetic relationships among 8 species in 5 sections of *Phragmipedium* based on genetic distance coefficients using AFLP markers.

could be scored unambiguously were included in the analysis. The genetic distance matrix was obtained using the genetic distance described by Jaccard (Sokal 1963), and used to construct a UPGMA tree. The analyses were performed using the NTSYS program software.

Results

The AFLP analysis of the orchid species were performed by using 18 primer combinations that amplified a total of 732 bands (220 bands for *P. pearcei,* 259 *P. besseae,* 169 *P. boissierianum,* 303 *P. caudatum,* 283 *P. wallisii,* 229 *P. richteri,* 250 *P. schlimii* and 311 *P. kovachii*). The genetic distance among the species determined by UPGMA analysis is shown in Figure 2. The species included in 5 sections were grouped into four major clusters. The first cluster comprised species of the sections *Himantopetalum* (*P. pearcei* and *P. richteri*) and *Lorifolia* (*P. boissierianum*), the second one comprised the species analyzed of the sections *Micropetalum* (*P. besseae* and *P. schlimii*), the third group contained *P. kovachii* of the section *Schluckebieria*, and the fourth group comprised *P. caudatum* and *P. wallisii* both of the section *Phragmipedium*.

Discussion

The results suggest that AFLP is a very useful technique for a rapid analysis of the genetic distance of the *Phragmipedium* species, in which there are no well characterized molecular genetic markers, such as microsatellites and SNPs. The sections *Micropetalum* and *Schluckebieria* were separated on the basis of the differences (among others) of the size of the flowers between *P. kovachii* and other of the species from section *Micropetalum* (Braem 2004), but this taxonomical classification was not supported by ITS sequence analysis (Damian *et al*. 2005). Despite using a single accession from each species, the grouping based on multiple anonymous genetic markers produced by AFLP supported the tentative inclusion of *P. kovachii* within section *Schluckebieria* which is in agreement with the previous taxonomical analysis based on phenotype (Braem 2004). However, this classification still requires further analysis with more accessions for each species before well-supported inferences may be made and with that in mind we are currently developing a semi-automated AFLP analysis of orchids using a capillary DNA sequencer.

Phragmipedium kovachii is a recently discovered species endemic to Peru, where it is apparentlyt

restricted to the Region of San Martín (Atwood *et al*. 2002; Christenson *et al*. 2002). This species is in danger of extinction, and classified as vulnerable by CITES, due to the indiscriminate extraction. AFLP analysis is a potential tool to aid in studies aimed at conservation of vulnerable orchid species such as *P. kovachii,* since DNA markers are valuable in providing information on genetic diversity of populations and to implement traceability of collected species.

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