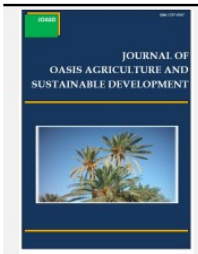

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Evaluating ITS barcode as a molecular tool for determining orchid protocorms genetic identity

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Abstract

Orchidaceae, one of the largest families of flowering plants, includes numerous endangered species. Identifying orchid species, especially those with fertile parts, poses significant challenges. In this study, a molecular analysis based on sequence analysis of the internal transcribed spacer (ITS) region of ribosomal DNA was used to determine the genetic identity of protocorms found in the main habitat of 4 co-occurring orchid species in Samsun/Türkiye. We collected 100 protocorms from the soil of the main habitat during the vegetative stage. Then, an aligned sequence of the complete ITS region was obtained using the universal primers ITS1/ITS4 to determine the genetic distances and the phylogenetic tree. As a result, the sequence of the protocorms aligned very closely with the sequence of *Orchis provincialis*. ITS has proven a useful DNA barcode for distinguishing orchid protocorms from different orchid species. Nevertheless, additional DNA regions are necessary to expedite research and conservation efforts for radiated orchid species.

1. INTRODUCTION

The Orchidaceae family holds significant ornamental, medicinal, scientific, and ecological importance (Gutiérrez 2010; Kim et al., 2014). Many species, such as Cymbidium, Phalaenopsis, Cypripedium, and Anacamptis, are valued for their attractive flowers and pleasant fragrances, making them popular ornamental plants (Watanabe et al., 2007; Gutiérrez 2010; Kim et al., 2014; Kabacaoğlu and Budak 2019; Harzlı and Kömpe 2024). Additionally, numerous Orchidaceae species contain bioactive compounds, including polysaccharides, alkaloids, phenanthrene, and dibenzyls, traditionally used in herbal medicine for disease treatment (Feng et al., 2015). These characteristics drive high economic value, contributing to the growing orchid demand. However, over the past decades, human activities, including overharvesting and habitat destruction, have placed many orchid species at serious risk of extinction (Jacquemyn et al., 2011; Kim et al., 2014).

Accurate identification of Orchidaceae plants is crucial for their safe use, biodiversity preservation, and the conservation of genetic resources (Asahina et al., 2010; Feng et al., 2015; De Boer et al., 2017). Traditional identification methods rely on morphological characteristics; however, many Orchidaceae species show minimal morphological differences before flowering, and these traits can be affected by environmental factors (Asahina et al., 2010; Techen et al., 2014). Additionally, there is a shortage of experts skilled in morphological identification, making the process increasingly time-consuming and labor-intensive (Devos et al., 2005; Kim et al., 2014; Techen et al., 2014; Zhang et al., 2017). Therefore, there is an urgent need for a rapid, accessible, and reliable identification method.

DNA barcoding is an innovative molecular identification technology that uses short, standardized DNA sequences to identify species (Devos et al., 2005; Asahina et al., 2010; Feng et al., 2015; Coissac et al., 2016; De Boer et al., 2017). Initially applied to microorganisms (Plant

2009), DNA barcoding now enables rapid and precise species identification, unaffected by factors such as developmental stage, internal morphological variation, environmental conditions, or the user's level of expertise (Techen et al., 2014; Coissac et al., 2016). Consequently, DNA barcoding has seen widespread application in species identification, biosystematics, biodiversity studies, ecological community analysis, species conservation, archaeological sample identification, and other fields (Cameron et al., 1999; Lahaye et al., 2008; Ma et al., 2014; Xu et al., 2015). Various genomic regions have been evaluated to establish a standardized DNA barcode for plants, identifying a few loci with sufficient discrimination power for specific plant groups (Lahaye et al., 2008; Ragupathy et al., 2009; Mahadani et al., 2013). The China Plant BOL Group suggested including ITS as a standard barcode for seed plants (China Plant et al., 2011). Across Türkiye, orchids represent a significant component of the biodiversity, with 191 taxa of which 39 are endemics (Kreutz and Çolak 2009). These species inhabit various environments and altitudes throughout the country. Although a few studies have explored orchid identification in Türkiye using ITS DNA barcoding (Şenel et al., 2018; Hürkan and Taşkın 2021), research in this area remains limited, especially concerning identifying orchid species from structures except fresh or dry leaves, young juveniles, and stems. In the present study, we aimed at (1) testing the potential of ITS barcode in the genetic identification of orchid species based on protocorms and (2) evaluating the potential of this barcode for identifying genetic identity among four co-occurring orchid species: Ancaamptis papillionacea, Neotinea tridentata, Orchis provincialis, and Serapias orientalis (Fig.1).

2. MATERIALS AND METHODS

2.1. Collection of orchid samples

This study was conducted in Samsun, the Northeast Province of Turkey (12.01°N/48.00°E). The four orchid species (Fig. 1) co-occur in a small area (400-450 m^2) on the Ondokuz Mayis University campus. When the four species were not dormant, 100 newly germinated protocorms in the soil were collected in March 2023. Following surface cleaning with sterile water, the protocorm samples (Fig. 2) were promptly placed into Eppendorf tubes and transported to the laboratory on the same day at 4 °C. Subsequently, the protocorms were subjected to surface sterilization by immersion in a 10% NaClO solution for 1 min, followed by thorough rinsing with distilled water. Then, the protocorms were frozen in liquid nitrogen and stored at −80 °C until further use.

2.2. DNA extraction and PCR amplification

Genomic DNA was extracted and purified from 100 mg of frozen protocorms using the NucleoSpin® Plant II isolation kit (Macherey–

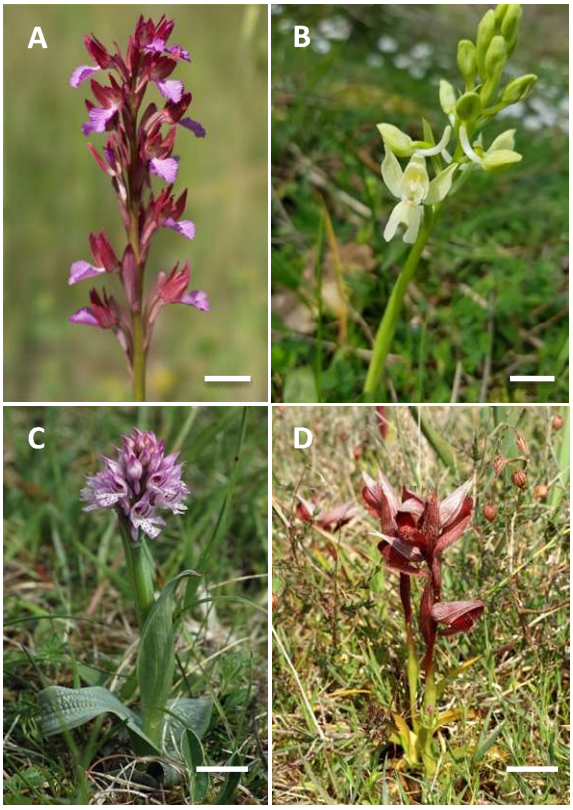

Fig. 1. Investigated orchid species: A) *Anacamptis papilionacea*, B) *Orchis provincialis*, C) *Neotinea tridentata*, D) *Serapias orientalis*, Scale Bar: 2cm.

Fig.2. Orchid protocorms collected from the study site. Scale Bar: 1mm.

Nagel™, Fisher Scientific) following the manufacturer's instructions. The quantity and quality of the isolated genomic DNA were assessed using a Biophotometer (Eppendorf Biophotometer® D30) and agarose gel electrophoresis. The PCR protocol employed for the amplification of the ITS gene consisted of an initial denaturation step at 96 °C for 3 minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 55 °C for 30 seconds, extension at 72 °C for 45 seconds, and a final extension step at 72 °C for 7 minutes. The PCR setup included a reaction mixture with the components listed in Table 1, and the primer sequences used for PCR amplification are detailed in Table 2.

Table 1. PCR components used to amplify the ITS region of protocorms

Components	Stock concentration	Final concentration	Volume to set 25 µl reaction
Milli-Q water			15.75 µl
dNTP mix	2 mM	0.2 mM	1 µl
Taq buffer	10X	1X[2]	5 µl
Forward primer	3 µM	0.5 µM	1 µl
Reverse primer	3 µM	0.5 µM	1 µl
DNA template	100 ng/µl	100 ng	1 µl
Taq DNA polymerase	5U/µl	1U	0.25 µl

Table 2. Primer pairs used to amplify the rDNA ITS region in protocorm samples in this study

Primer name	Sequence	Reference
ITS1	5'-GACGTCGCGAGAAGTCCA-3'	White et al. 1990
ITS4	5'-TCCTCCGCTTATTGATATGC-3'	White et al. 1990

The sequencing of PCR products was carried out by Macrogen Corporation (Macrogene INC., Seoul, Republic of Korea) using the ABI 3730 XL DNA sequence. The sequence of the PCR products was amalgamated using BioEdit 7.2.5 software. To examine the phylogenetic relationships of rDNA ITS regions, BLAST searches were conducted using sequences obtained from these sequences and comparable sequences available in the National Center for Biotechnology Information (NCBI) GenBank database (www.ncbi.nlm.nih.gov/genbank; accessed June 12, 2023) that showed high similarity (≥97.0%) to our sequences. *Malaxis ophrydis* (AY907114.1) sequence was used as an outgroup. Phylogenetic analysis was performed using MAFFT v7.310 with the L-INS-I strategy (Katoh et al., 2002). Phylogenetic and molecular evolutionary analyses were conducted in MEGA v. 11 (Tamura et al., 2021) using a maximum-likelihood (ML) approach, the Kimura two-parameter model, and a gamma distribution with invariant sites (G + I). Branch support was assessed using the bootstrap method using 1,000 replicates, and the rDNA ITS region sequence was submitted to GenBank.

3. RESULTS & DISCUSSION

In this study, sequencing of the ITS region showed high PCR amplification success (90%) with a product size of 600 bp, and the sequence was submitted to GenBank under accession number PP550860. These data were used to construct a phylogenetic tree employing the maximum likelihood method, as illustrated in Fig. 3. The ITS marker provided sufficient information to confirm that the collected protocorms belonged to *Orchis provincialis*, the orchid species under investigation. According to the phylogenetic tree, the protocorm sequence showed a 100% match to *Orchis provincialis* (Z9401.1) in the NCBI database, while it distinctly diverged from sequences of other orchid species, including *Anacamptis papilionacea* (KY512514.1), *Neotinea tridentata* (MH050855.1), and *Serapias orientalis* (KY512512.1) (Fig. 3).

DNA barcoding can be used for species identification through a DNA fragment shared across all species. This fragment must have sufficient variability for distinguishing species while also including conserved regions to design universal primers (Hebert and Gregory 2005; Chen et al., 2010; Kumar et al., 2016). The difference between intraspecific and interspecific values is referred to as the 'barcode gap' (Lahaye et al., 2008). Despite some

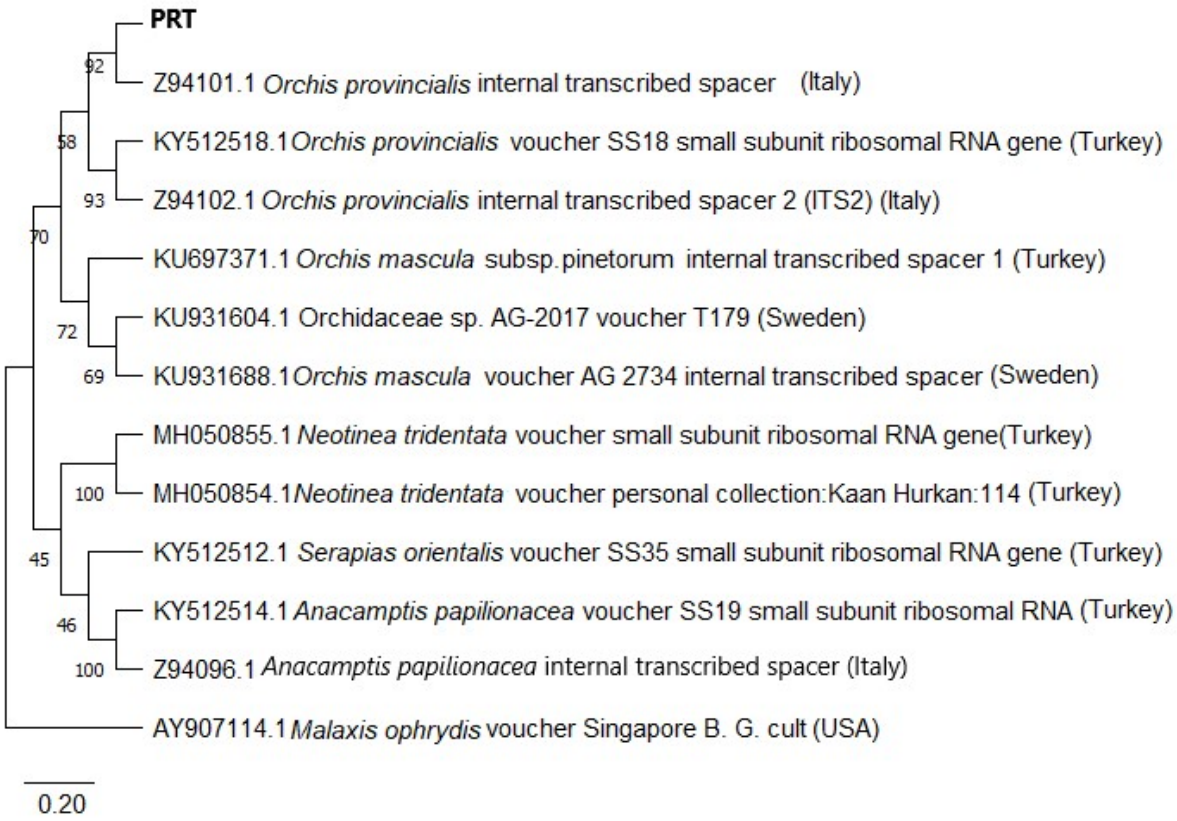

Fig. 3. Maximum likelihood tree of the rDNA ITS1-5.8-ITS2 regions of *O. provincialis* protocorms. *Malaxis ophrydis* (accession number: Y907114.1) was used to root the ML tree. The sequence of the *O. provincialis* protocorm is shown in bold.

limitations in using the ITS sequence as a universal barcode (Kress and Erickson 2007; Sass et al., 2007), we found that the ITS possessed a high success rate for amplification and sequencing (90%) in the samples examined. Previous studies have shown that the nuclear ribosomal DNA region (ITS) evolves rapidly, allowing it to establish genetic identities (Pang et al., 2010; Xiang et al., 2011; Kim et al., 2014; Xu et al., 2015; Li et al., 2016) and generate genetic distances capable of distinguishing closely related species within the same genus (Sass et al., 2007; Giudicelli et al., 2015; Zhang et al., 2017). As far as we know, it is the first time that genetic analysis in species identification of Orchidaceae with such a unique structure formed as a seed germinates, based on rDNA ITS regions in Türkiye. The sequence obtained from the protocorms was very similar to the sequences related to *Orchis provincialis* that co-occur with 3 orchid species with 92% similarity (Fig.3).

A limitation of this study is its reliance solely on the ITS region for genetic identification of orchid protocorms, which, while widely used, may not provide a comprehensive view of genetic diversity across all orchid species. Although the ITS region is effective for distinguishing many orchid taxa, previous studies suggest that single-locus barcoding can sometimes lack resolution, especially for closely related species, due to insufficient sequence variation within this marker alone (Chase et al., 2007; Kress and Erickson 2007; Chattopadhyay et al., 2017). To enhance the accuracy and universality of orchid identification based on protocorm material, it is recommended that future studies incorporate additional loci, such as the matK or rbcL regions, which are often used in plant barcoding and may complement ITS by providing greater phylogenetic resolution (Group et al., 2009). Furthermore, employing a combination of different barcode markers could improve the reliability of identification, ensuring more robust and universal application in orchid species differentiation from protocorms. Such multi-locus approaches could address potential limitations and help confirm the effectiveness of DNA barcoding protocols for diverse orchid taxa.

4. CONCLUSION

The ITS sequences determine the genetic identity of protocorms. Our results indicate that ITS sequence data could facilitate accurate species identification for orchid plants, enabling similarity-based identification accessible to non-experts. Additionally, our study, using distance, BLAST, and tree-building methods, suggests that ITS is the most effective region to be used as a single-locus barcode for identifying orchid species in Türkiye. However, inaccurate sequence information in reference databases may mislead non-expert users. Therefore, a user-friendly DNA barcode database specific to orchids would be valuable to assist non-experts in reliable identification.

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