APPLICATION OF DNA ANALYSIS APPROACH CONTRIBUTES TO THE IDENTIFICATION OF SEVERAL PLANT SPECIES IN TRUONG SA ARCHIPELAGO, KHANH HOA PROVINCE, VIETNAM

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SUMMARY

DNA barcoding has been widely used to assess species diversity in a variety of ecosystems, including temperate, subtropical, and tropical rain forests. However, due to the difficulties associated with field exploration, most of the species in Truong Sa archipelago have never been barcoded. The purpose of this study is to barcode five species of plants from the Truong Sa archipelago and to provide valuable evolutionary information that will aid in future understanding of the plant community assembly on those particular islands. Using DNA markers (*ITS-rDNA*), this study created a DNA barcode database for five plant species found on the Truong Sa archipelago. We used the sequence similarity and a phylogenetic based method to the identify 15 samples from five plant species collected in Truong Sa archipelago, Vietnam. Results showed that the PCR success rate for *ITS-rDNA* region was 100%. The success rate of bidirectional sequencing of PCR product was 100% for 650 bp long the *ITS-rDNA* region fragment. Phylogenetic analyses using maximum likelihood (ML) indicated that five plant species (PB, BT, BV, NH and TR) had a close relationship with *T. argentea*, *S. taccada, B. asiatica*, *M. citrifolia, M. citrifolia* and *C. uvifera*, respectively. The current study provided further evidence for *ITS-rDNA* region as a useful molecular marker for species identification found on other tropical coral islands.

Keywords: DNA barcodes, ITS-rDNA gene, phylogenetic tree, Truong Sa archipelago.

1. INTRODUCTION

DNA barcodes and environmental DNA (eDNA) are useful research tools for taxonomy, discovering new species, species identification, and samples derived from living or dead organisms, all based on sequence DNA (Kress and Erickson, 2008; Yang *et al.,* 2011; Thomsen and Willerslev, 2015; Gao *et al.,* 2017; Hosein *et al.,* 2017). DNA barcoding has been particularly valuable in the inventorying of biodiversity hotspots. Successful investigations have been carried out in Mount Kinabalu, Malaysia (Merckx *et al.,* 2015), and Ontario, Canada (Telfer *et al.,* 2015), providing a convenient and efficient way for recognition of nature in these regions. DNA barcoding can also be a powerful tool for addressing fundamental questions in ecology, evolution, and conservation biology (Kress *et al.,* 2015). A considerable number of cryptic and new species have been discovered based on evidence from DNA barcodes (García-Robledo *et al.,* 2013; Hamsher and Saunders, 2014; Hebert *et al.,* 2004; Silva *et al.,* 2014; Smith *et al.,* 2012; Winterbottom *et al.,* 2014). DNA barcoding data prodigiously contribute to understanding the evolutionary relations within a given community (Kress *et al.,* 2009). Many researchers are interested in using molecular biology to survey biodiversity on tropical coral islands (Hawlitschek *et al.,* 2013).

Tropical coral islands represent a unique ecosystem: They are far away from continental ecological systems with clear oceanic boundaries; Their species composition could be very different from those of the mainland; They often represent small geographical areas, where the species pool may come either from closely related species or from distantly related clades; They are of particular conservation and scientific interests in the global inventory of biodiversity (Monaghan *et al.,* 2006), and they

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badly need a comprehensive understanding on biodiversity and ecology due to the increasingly anthropogenic disturbance. Truong Sa archipelago, Khanh Hoa province (latitudes 6°30' to 12°00' and longitudes 111°20' to 117°20') are a group of tropical oceanic coral islands with 130 islands and shoals across an area of approximately 180,000 km^2, measuring approximately 800 km (East to West) and 600 km (North to South). There are only 23 small islets among 130 islands and shoals. In the exploration of ecology and biodiversity on Truong Sa archipelago, there are currently only a few studies about the plant being conducted on the Truong Sa archipelago (Nguyen Khac Khoi and Vu Xuan Phuong, 2001). However, there haven't been molecular biology approaches for study plant biodiversity in these islands.

In this study, we used the nuclear ribosomal internal transcribed spacer (*ITS-rDNA*) region to identify five plant species found on Truong Sa archipelago in Vietnam and provided additional information to emphasize the importance of species conservation, evolution, and systematics.

2. RESEARCH METHODOLOGY

2.1. Taxon sampling: Vu Dinh Duy and the research team in November 2020 collected 15 samples (young leaves) of five plant species during the field survey in the Truong Sa archipelago, Khanh Hoa province, Vietnam (Table 1 and Figure 1). In the field, young leaves were collected and placed in labeled plastic bags with silica gel and then transferred to the laboratory of Molecular Biology, Vietnam - Russia Tropical Centre and subsequently, stored at -30^oC until ready for use in DNA extraction. Herbarium specimens were also collected at studied localities and identified scientific names by Bui Van Thanh, the botanist of Institute of Ecology and Biological Resources, VAST.

Table 1. Locations of five plant species in the Truong Sa archipelago, Vietnam

Vietnamese names	Scientific names	Voucher No.	Symbol specimens	Samples collected	GenBank code
Phong ba	*Tournefortia argentea*	PB1.1-PB1.3	TSL47	03	MZ497173
Bão táp	*Scaevola taccada*	BT2.1-BT2.3	TSL01	03	MZ497174
Bàng vuông	*Barringtonia asiatica*	BV3.1-BV3.3	TSL49	03	MZ497175
Nhàu	*Morinda citrifolia*	NH4.1-NH4.3	TSL18	03	MZ497176
Tra	*Coccoloba uvifera*	TR5.1-TR5.3	TSL20	03	MZ497178

Figure 1. Flowers and fruits of five different plant species collected in Truong Sa archipelago
(Tournefortia foertherianum (A); *Scaevola taccada* (B); *Barringtonia asiatica* (C); *Morinda citrifolia* (D); *Coccoloba uvifera* (E) [Photo: Bui Van Thanh])

2.2. DNA isolation

Total genomic DNA was extracted using the plant/fungi DNA isolation Kit (Norgenbiotek, Canada). The total DNA purity and integrity were tested by NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, DE, USA) and then diluted to a concentration of 20ng/µl.

2.3. PCR amplification

The *ITS-rDNA* gene region was amplified through the following PCR cycling profile: an initial heating step at 94ºC for 3 min; followed by incubating for 40 cycles of 94ºC for 1 min, 55ºC for 1 min, respectively, and 72ºC for 1 min, and completed by incubating at 72ºC for 10 min. All PCR reactions were performed in 25 µl volumes using Gene Amp PCR Systems 9700. Pairs of primers: N10F: AGGAGAAGTCGTAACAAG (Wen and Zimmer, 1996) and C26AR: GTTTCTTTTCCTCCGCT (Suh *et al.,* 1996).

2.4. Sequencing of the ITS-rDNA region

Sequencing was performed on an Avant 3100 Automated DNA sequencer using the Dye Terminator Cycle sequencing kit (PE Applied Biosystems). Sequencing of the fifteen studied five species used the primers N10F and C26AR.

2.5. Phylogenetic analyses

Chromas Pro 2.1.6 software (Technelysium Pty Ltd., Tewantin, Australia) was used to edit the sequences. Sequence alignments were made with Bioedit v7.0.5.2 (Hall, 1999). We used MEGA 7.0 (Kumar *et al.,* 2016) to analyze our data. Nucleotide sequence divergences were calculated using the Kimura two-parameter (K2P). Phylogenetic trees were performed using maximum likelihood (ML) on MEGA 7.0 software with 1000 replicates.

3. RESULTS AND DISCUSSION

3.1. Total DNA extraction

DNA extraction is the first and most critical step in the general study of molecular biology. Total DNA extraction is used to extract DNA from the structure of the cell. The most critical aspect is to obtain DNA in an intact state free of decomposition and impurities in order to have a high-quality material for subsequent experiments. In this study, we extracted total DNA from 15 leaf samples of five plant species in the Truong Sa archipelago using the Plant DNA isolation Kit (Table 1). DNA test electrophoresis on 1% agarose gel results showed that each samples have a single band, sharp and bold bands indicate successful DNA extraction (Figure 2). To determine the quantity and purity of extracted DNA using the spectrophotometric method on the NanoDrop 2000, DNA samples were measured using absorption spectroscopy at wavelengths between 260 nm and 280 nm. In Table 2, OD260 nm/OD280 nm fluctuated within the allowable range from 1.8 to 2.0, which indicated that the extracted total DNA samples are suitable as a template for PCR reactions. DNA was diluted to 20 ng/µl H_2O for PCR reaction.

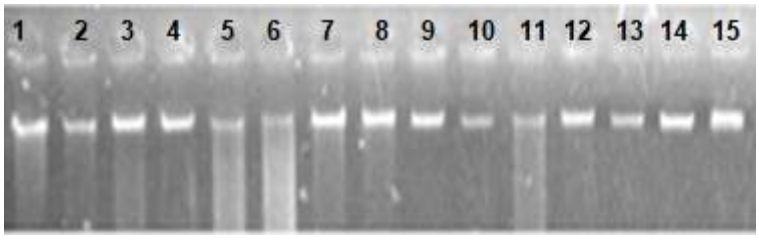

Figure 2. Electrophoresis of total DNA from 15 samples of 05 plant species in Truong Sa archipelago using 1% agarose gel with 1-3 (PB1.1-PB1.3); 4-6 (BT2.1-BT2.3); 7-9 (BV3.1-BV3.3); 10-12 (NH4.1-NH4.3); 13-15 (TR5.1-TR5.3).

Table 2. The quantity and purity of extracted DNA from 15 leaf samples of five plant species in Truong Sa archipelago

No.	No. samples	OD_{260nm}	OD_{280nm}	DNA purity ($OD_{260nm/280nm}$)	DNA quantity (ng/µl)
1	PB1.1	1.095	0.560	1.96	54.8
2	PB1.2	0.383	0.196	1.95	19.1
3	PB1.3	0.815	0.416	1.96	40.8
4	BT2.1	0.561	0.310	1.81	28.0
5	BT2.2	0.592	0.302	1.96	29.6
6	BT2.3	0.270	0.145	1.87	13.5
7	BV3.1	0.609	0.330	1.84	30.4
8	BV3.2	0.285	0.173	1.65	14.3
9	BV3.3	0.826	0.446	1.85	41.3
10	NH4.1	1.015	0.518	1.96	50.7
11	NH4.2	0.805	0.424	1.90	40.3
12	NH4.3	0.668	0.348	1.92	33.4
13	TR5.1	0.757	0.414	1.83	37.8
14	TR5.2	0.448	0.248	1.81	22.4
15	TR5.3	0.916	0.484	1.89	45.8

3.2. Polymerase chain reaction (PCR)

The primer pair N10F and C26AR was successfully cloned for 15 samples at a primer temperature of 55ºC (Figure 3). The PCR product were approximately length of 700 bp. Electrophoresis on a 1.5% agarose gel revealed that the PCR product was of high quality with only a single brightband qualifying for nucleotide sequencing.

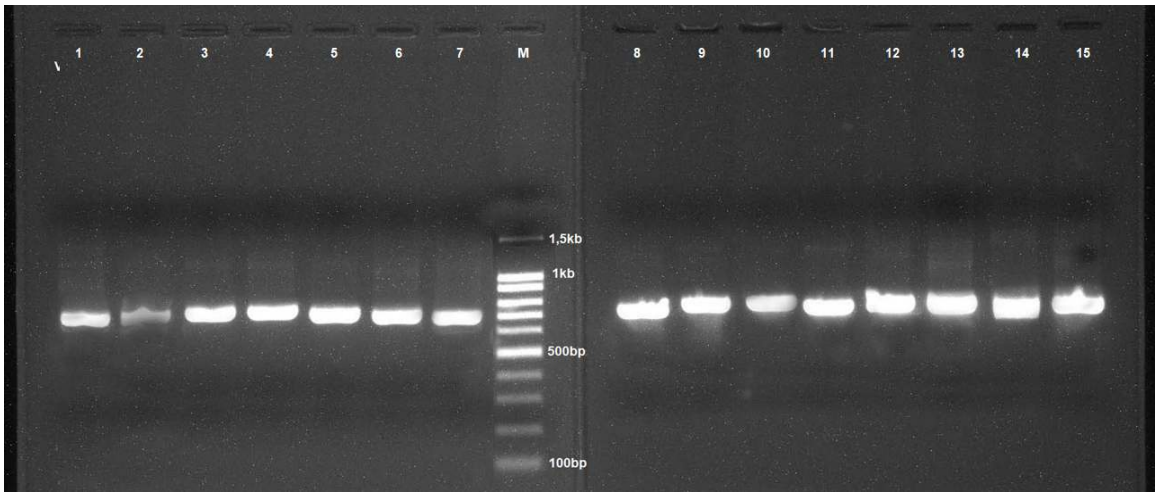

Figure 3. PCR products from five plant species were electrophoresed on 1.5% agarose gel (M: DNA ladder 100bp; 1-15: No. samples)

3.3. Nucleotide sequences (*ITS-rDNA*) and phylogenetic trees

Chromas Pro 2.1.6 software was used to display results and edit the sequences. After removing the two ends, we have identified the remaining sizes of the 15 samples of five plant species in Truong Sa archipelago: PB (600 bp), BT (650 bp), BV (612 bp), NH (570 bp), and TR (589 bp). Because the results indicated that there was no difference between three samples of one species in the study (100% similarity), we conducted a further analysis using the results of one sample. Nucleotide sequences of five plant species in Truong Sa archipelago have registered on GenBank (Table 1).

Using the NCBI's BLAST tool to compare five plant species in Truong Sa archipelago with similar sequences on GenBank. Results showed that species (PB) was 100% similar to *Tournefortia argentea* (MH768076); BT was 100% similar to *Scaevola taccada* (MH768165); BV 100% similar to *Barringtonia*

asiatica (AF208700); NH 100% similar to *Morinda citrifolia* (MK607923) and TR similar to *Coccoloba uvifera* - GQ206246 (100%).

In order to identify a species, sequences of *ITS–rDNA* of five species (PB, BT, BV, NH,and TR) were used to construct a phylogenetic tree together with one outgroup taxon (Figure 4, 5, 6, 7, 8). The maximum likelihood phylogenetic tree showed a clear separation of PB, BT, BV, NH, and TR inTruong Sa archipelago into one clade together with *T. argentea*, *S. taccada, B. asiatica*, *M. citrifolia, M. citrifolia* and *C. uvifera*, respectively with bootstrap value of 100% (Figure 4, 5, 6, 7, 8). PB/*T. argentea*, BT/*S. taccada,* BV/*B. asiatica*, NH/*M. citrifolia*, and TR/*C. uvifera* had highly identical *ITS* sequences.

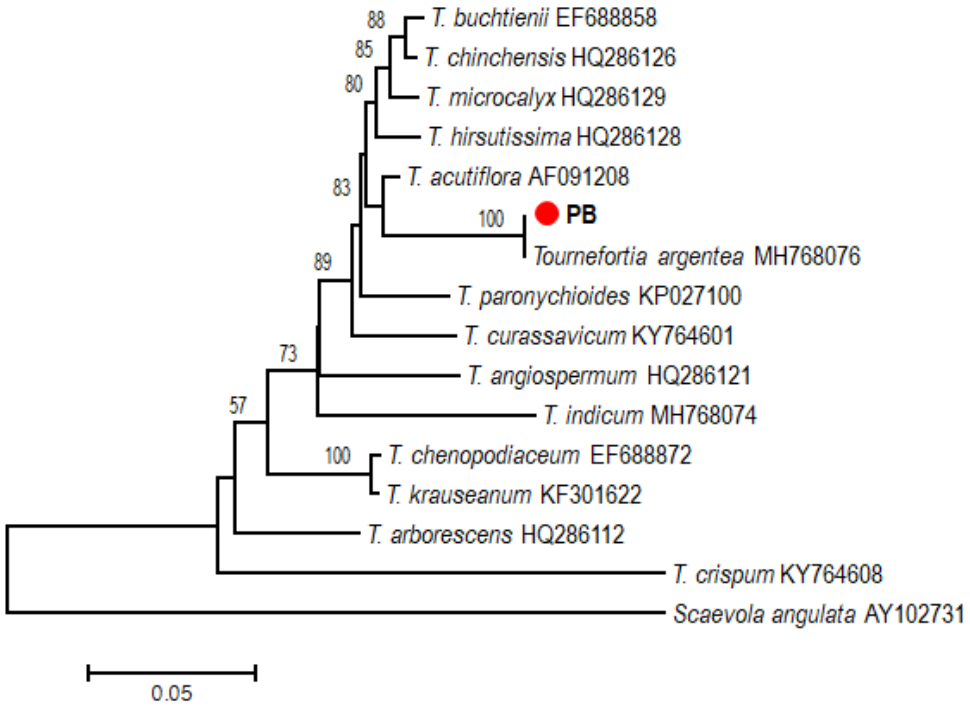

Figure 4. Molecular phylogenetic analysis of a species (PB) with other species using the Maximum Likelihood method (ML)

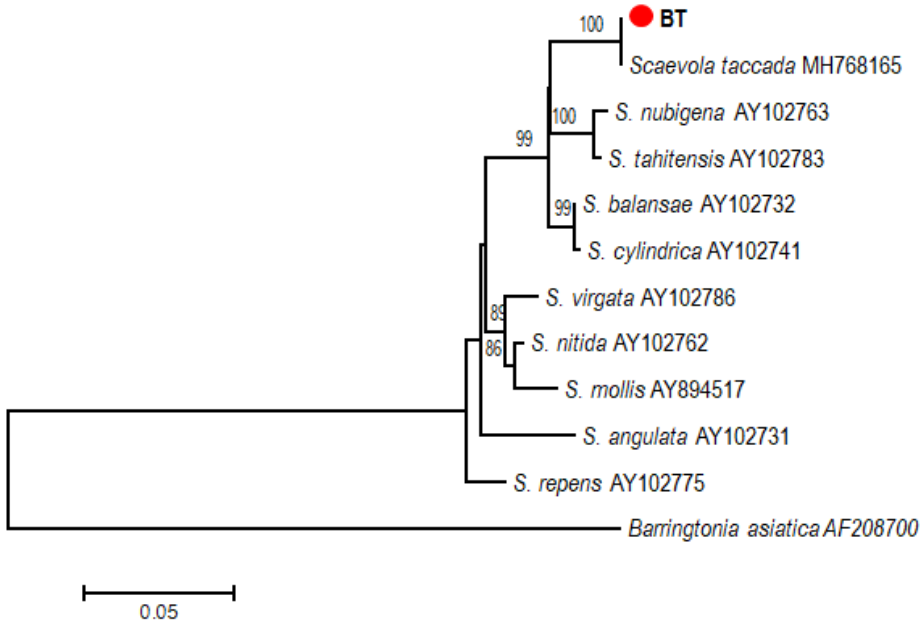

Figure 5. Molecular phylogenetic analysis of a species (BT) with other species using the Maximum Likelihood method (ML)

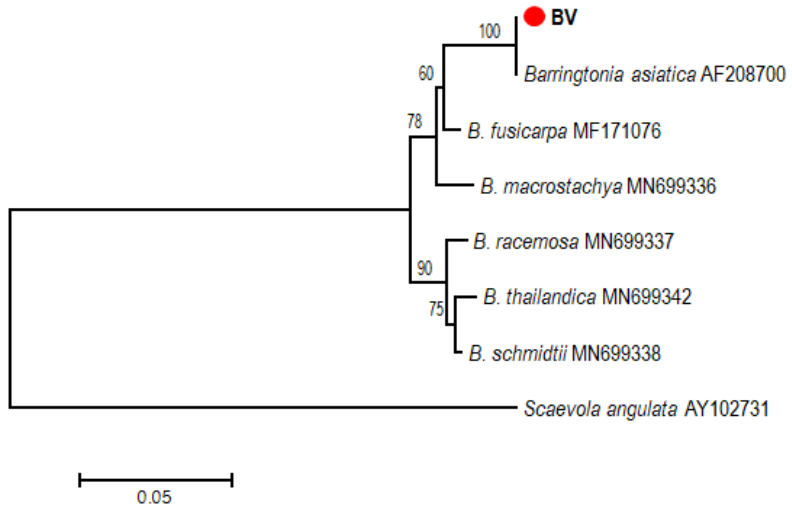

Figure 6. Molecular phylogenetic analysis of a species (BV) with other species using the Maximum Likelihood method (ML)

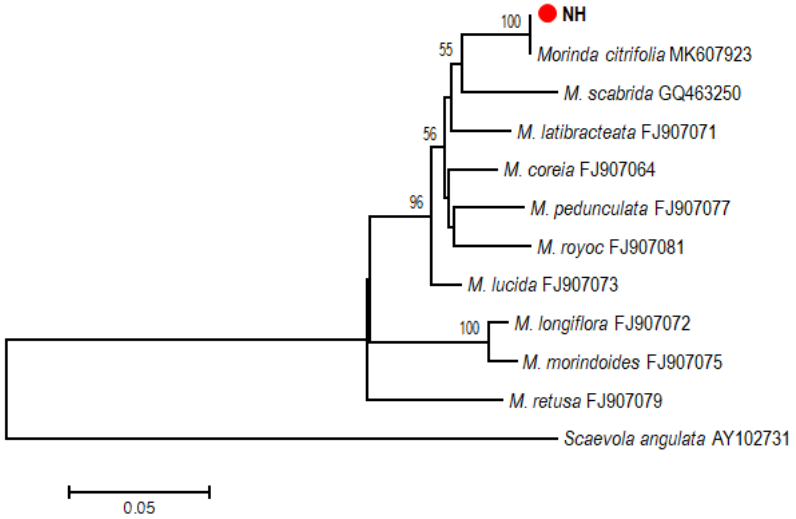

Figure 7. Molecular phylogenetic analysis of a species (NH) with other species using the Maximum Likelihood method (ML)

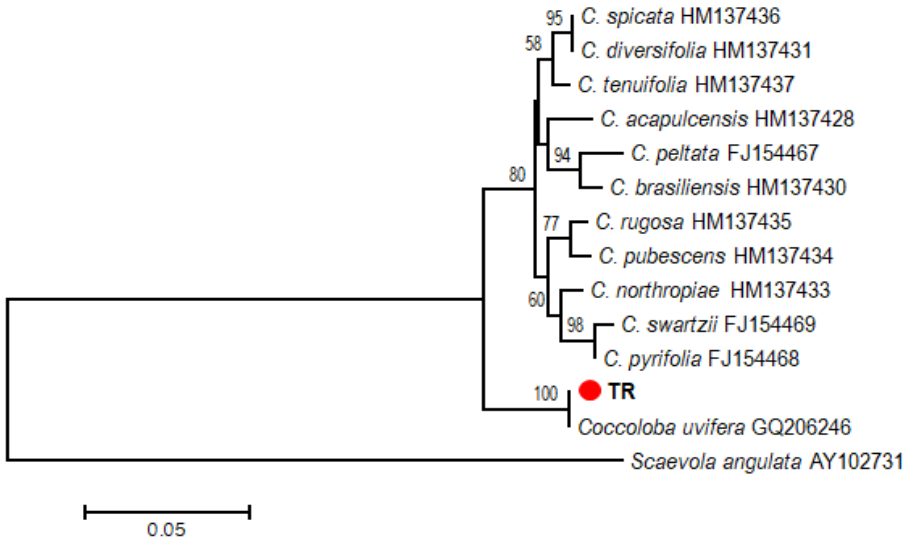

Figure 8. Molecular phylogenetic analysis of a species (TR) with other species using the Maximum Likelihood method (ML)

4. CONCLUSIONS

In the current study, we had used molecular biology to identify five species plant in Truong Sa archipelago, Khanh Hoa province, Vietnam belonging to *T. argentea*, *S. taccada, B. asiatica*, *M. citrifolia* and *C. uvifera*. Our results of the *ITS-rDNA* gene showed that the sequence of five specimens collected in Truong Sa archipelago have high similarity to *T. argentea*, *S. taccada, B. asiatica*, *M. citrifolia* and *C. uvifera* from GenBank. These sequences have been deposited in the GenBank (NCBI) under the accession number (MZ497173- MZ497176, MZ497178), thereby contributing to the development of the DNA barcode database for the study of species' evolutionary and biological systems.

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ỨNG DỤNG PHƯƠNG PHÁP PHÂN TÍCH ADN GÓP PHẦN NHẬN DẠNG MỘT SỐ LOÀI THỰC VẬT TẠI QUẦN ĐẢO TRƯỜNG SA, TỈNH KHÁNH HÒA, VIỆT NAM

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TÓM TẮT

Mã vạch DNA được sử dụng rộng rãi để đánh giá sự đa dạng của các loài trong nhiều hệ sinh thái: khu rừng mưa nhiệt đới, cận nhiệt đới và ôn đới. Tuy nhiên, do những khó khăn trong công tác thăm dò thực địa nên các loài thực vật ở quần đảo Trường Sa, tỉnh Khánh Hòa chưa được khám phá bằng kỹ thuật sinh học phân tử. Mục đích nghiên cứu này nhằm nhận dạng 5 loài thực vật thu được từ quần đảo Trường Sa bằng kỹ thuật sinh học phân tử và cung cấp thông tin tiến hóa có giá trị về quần thể thực vật trên một số đảo. Sử dụng chỉ thị DNA (gen *ITS-rDNA*) tạo ra cơ sở dữ liệu mã vạch DNA cho 5 loài thực vật được thu thập trên quần đảo Trường Sa. Chúng tôi đã sử dụng phương pháp so sánh sự tương đồng về trình tự DNA và xây dựng cây phát sinh loài để xác định 15 mẫu của 5 loài thực vật ở quần đảo Trường Sa. Kết quả cho thấy, tỷ lệ PCR thành công đối với vùng gen (*ITS-rDNA*) là 100%. Tỷ lệ thành công giải trình tự DNA hai chiều của sản phẩm PCR là 100% đối với vùng gen nghiên cứu có kích thước là 650 bp. Phân tích phát sinh loài sử dụng phương pháp ML chỉ ra rằng 5 loài thực vật (PB, BT, BV, NH và TR) có mối quan hệ chặt chẽ với *T. argentea*, *S. taccada*, *B. asiatica*, *M. citrifolia*, *M. citrifolia* và *C. uvifera*, tương ứng. Kết quả của nghiên cứu này đã cung cấp thêm bằng chứng cho vùng gen (*ITS-rDNA*) như một chỉ thị phân tử hữu ích có thể sử dụng để xác định các loài thực vật khác trên các đảo san hô nhiệt đới.

Từ khóa: cây phát sinh loài, DNA mã vạch, quần đảo Trường Sa, vùng gen ITS-rDNA.

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