Molecular Identification of *Podocarpus* spp. from Bukidnon and Batanes, Philippines using rbcL sequences

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***Abstract*—*Podocarpus* (Podocarpaceae) consists of approximately 100 species spreading in the tropical and subtropical regions worldwide and also in temperate regions in the Southern Hemisphere. It is often difficult to accurately identify *Podocarpus*. DNA barcoding technique is helpful to provide rapid and accurate taxonomic identification using a specific DNA region. In this study, unknown *Podocarpus* samples are identified using rbcL sequences. Leaf samples were collected from Bukidnon and Batanes, Philippines. DNA extraction, PCR amplification and DNA sequencing were consequently conducted. The rbcL sequence of *Podocarpus* is about 700 bp in length. Molecular identification using Basic Local Alignment Search Tool (BLAST) for nucleotides reveals that the sample from Bukidnon is *Podocarpus macrophyllus* while that from Batanes is *Podocarpus costalis*. This identification may be helpful as an addition to the reference library of Podocarpaceae. This may allow researchers unfamiliar with the family's morphology and anatomy make accurate identification.**

***Keywords*— Batanes, Bukidnon, conifers, *Podocarpus*, Philippines, rbcL sequence**

I. Introduction

Podocarpus is a genus of conifers, the most numerous and widely distributed of the podocarp family, Podocarpaceae. *Podocarpus* are evergreen shrubs or trees usually from 1 to 25 meters tall, known to reach 40 meters at times. The leaves are 0.5 to 15 cm long, lanceolate to oblong or falcate (sickle-shaped) in some species, with a distinct midrib. They are arranged spirally, though in some species twisted to appear in two horizontal ranks. The cones have two to five fused scales, of which only one, rarely two, are fertile, each fertile scale has one apical seed. At maturity, the scales become berry-like, swollen, brightly coloured red to purple and fleshy, and are eaten by birds which then disperse the seeds in their droppings. The male (pollen) cones are 5 to 20 mm long, often clustered several together. Many species, though not all, are dioecious. There are approximately 104 to 107 species in the genus [1].

Species of Podocarpaceae are of conservation interest because of their small population sizes and limited habitat. In the International Union for the Conservation of Nature [2], there are twenty-seven species of Podocarpaceae that are included in the red list. Ten species are included under the vulnerable category, 14 species are endangered, and three species are critically endangered. Two species are included in the appendices of the Convention on International Trade in Endangered Species [3]: *Podocarpus parlatorei* is listed in Appendix I (trade is not allowed) and *Podocarpus neriifolius* is listed in Appendix III (trade with, some limitations, is allowed).

Podocarpaceae have a minor role in commerce. *Nageia nagi*, when labeled as Asian bayberry, can legally be sold in the United States of America as an herbal dietary supplement [4]. The seeds are processed into an edible oil that is also used in manufacturing [5]. The young leaves are also edible, but not typically consumed [6]. The conspicuous fleshy reproductive structures (receptacles or epimatium) of *Afrocarpus falcatus*, *Dacrycarpus dacrydioides*, *Dacrydium cupressinum*, *Podocarpus elatus*, *Podocarpus macrophyllus*, *Podocarpus totara*, and *Prumnopitys taxifolia* are eaten either raw or cooked [6].

Podocarpaceae are also known to have medicinal properties that benefit humans and animals [7, 8]. The receptacles and leaves contain a variety of bio–active compounds such as antioxidants, nordi–terpenes, podocarpic acid, and tatarol (7, 9, 10]. Some of these compounds have antimicrobial, fungistatic, or bacteriostatic properties (7, 11, 12]. Other compounds have cytotoxic properties that may be useful in destroying cancer (13, 14, 15, 16, 17].

Accurate identification of Podocarpaceae is often very difficult. The most easily accessed material is usually sterile. If fertile material is present, it is frequently either inaccessible or detached from the tree making it difficult to convincingly associate the fertile and sterile portions. Although sterile material of Podocarpaceae can usually be identified to genus using phyllotaxis and leaf form [18, 19], accurate species identification often requires careful microscopic examination of internal, and external characteristics. Proper use of the existing identification tools requires training in botanical terminology, skill in microtechnique, and familiarity with Podocarpaceae [20].

Thus, DNA barcoding is a technique which is helpful to provide rapid and accurate taxonomic identification using a specific DNA region [21]. It has become a useful tool for species identification [22, 23], and discovering new or cryptic species [24, 25]. With DNA barcoding, universality of primers

Manuscript received March 29, 2016.

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for polymerase chain reaction (PCR) and sequencing is one of the most important criteria [26, 22, 27, 28]. In the core barcode, the primers for rbcL show a high level of universality in land plants [29, 30].

In this study, two species of *Podocarpus* from Bukidnon and Batanes, Philippines were identified using rbcL sequences.

II. MATERIALS AND METHODS

A. Sample collection

The leaf samples used in this study were from Bukidnon and Batanes, Philippines. Leaf samples from Bukidnon were cleaned with sterile water and then placed individually in small plastic bags with silica gel. These were transported to DLSU – Manila and stored frozen until needed for DNA extraction. Samples from Batanes were fresh potted plant material.

B. DNA extraction

Leaf samples were frozen with liquid nitrogen and poured into powder form. These were temporarily stored in the refrigerator at 4°C. DNA from individual samples was extracted using the CTAB method as described by Doyle and Doyle [31]. The genomic DNA was dissolved in TE buffer (10 mmol/L tris-HCL, pH 8.0, 1 mmol/L EDTA) to a final concentration of 40-50 µg/µL to avoid any variation in PCR success due to DNA concentration differences. The extracts were consequently subjected to agarose gel electrophoresis (0.8%).

C. PCR amplification and DNA sequencing

The Polymerase Chain Reaction (PCR) was used to amplify rbcL in a 40 µL volume containing: 1X PCR buffer, 2.5 mM $MgCl_2$, 0.3 mM dNTPs, 0.3 µM of rbcLa_Forward (5' –ATGTCACCAACAAACAGAGACTAAAGC - 3'), 0.3 µM of rbcLa_Reverse (5' - GTYAAATCAAGTCCACCYCG - 3'), 0.05 units Taq, and 1 µL genomic DNA. The reaction mixture was incubated for 5 mins at 94°C, followed by 34 cycles of denaturation at 94°C for 40 seconds, primer annealing at 55°C for 90 seconds, and extension at 72°C for 90 seconds. PCR products were sent to 1st Base Inc. for sequencing.

D. Sequence analysis

The chromatogram of the DNA sequences of the individual samples were viewed, corrected and analysed using Chromas LITE version 2.0 (Technelysium Pty. Ltd., Gold Coast, Australia). The sequences were then compared with available sequences in the Genbank using the Basic Local Alignment Search Tool (BLAST) to determine sequence homology of the species and to confirm the identity of the sequenced sample.

III. RESULTS AND DISCUSSION

Podocarpus and the Podocarpaceae were endemic to the ancient supercontinent of Gondwana, which broke up into Africa, South America, India, Australia-New Guinea, New Zealand, and New Caledonia between 105 and 45 million years ago. *Podocarpus* is a characteristic tree of the Antarctic flora, which originated in the cool, moist climate of southern Gondwana, and elements of the flora survive in the humid temperate regions of the former supercontinent. As the continents drifted north and became drier and hotter, *Podocarpus* and other members of the Antarctic flora generally retreated to humid regions, especially in Australia, where sclerophyll genera like Acacia and Eucalyptus became predominant, and the old Antarctic flora retreated to pockets that presently cover only 2% of the continent. As Australia drifted north toward Asia, the collision pushed up the Indonesian archipelago and the mountains of New Guinea, which allowed podocarp species to hop across the narrow straits into humid Asia, with *P. macrophyllus* reaching north to southern China and Japan. The flora of Malesia, which includes the Malay peninsula, Indonesia, the Philippines, and New Guinea, is generally derived from Asia but includes many elements of the old Gondwana flora, including several other genera in the Podocarpaceae (Dacrycarpus, Dacrydium, Falcatifolium, Nageia, Phyllocladus, and the Malesian endemic Sundacarpus), and also Agathis in the Araucariaceae [32].

Two unknown species of *Podocarpus* from Bukidnon and Batanes, Philippines were identified using DNA barcoding techniques. The sample from Bukidnon was coded R1 while the sample from Batanes was coded R3. R1 and R3 are about 700 bp in length (Fig. 1). The sequences of R1 and R3 are shown in Figs. 2 and 3, respectively. Molecular identification using Basic Local Alignment Search Tool (BLAST) for nucleotides gave the following identities: R1 is *Podocarpus macrophyllus*; R3 is *Podocarpus costalis* (Table I).

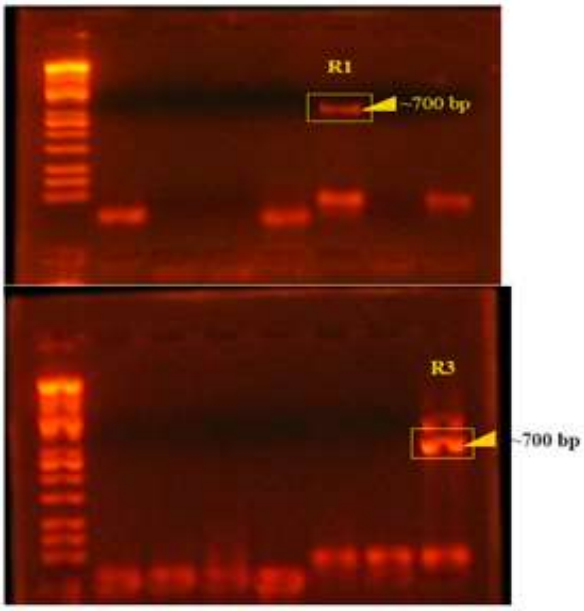

Fig. 1. Gel electrophoresis results of the PCR amplification for the rbcL gene from *Podocarpus* samples from Bukidnon [R1, (left)] and from Batanes [R3, (right)].

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>Contig1
ATGTCACCCCAAACAGAGACTAAAGCAAGTGCTGGGTTCAAAGCTGGTGTTAAAGATTAC
AGATTAACTTATTATACTCCGGAATATCCGACCAAAGATACTGATATCTTGGCGGCATTC
CGAGTCACTCCTCAACCCGGAGTGCCCCCCGAGGAAGCAGGAGCGGCAGTAGCTGCCGAA
TCTTCCACTGGTACATGGACTACTGTTTGGACCGATGGACTTACCAGCCTCGATCGTTAC
AAGGGGCGATGCTATGGCATCGAACCTATTCCTGGAGAAGAAAGTCAATTTATTGCCTAT
GTAGCTTATCCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACCTGTTCACCTCCATT
GTGGGTAATGTTTTTGGATTCAAAGCCCTACGGGCTATACGTCTGGAAGATCTGCGAATT
CCTCCTTCTTATTCCAAAACTTTCCAAGGTCCCCCACATGGTATCCAGGTAGAAAGGGAT
AAATTAAATAAATATGGCCGCCCCTTATTGGGATGTACCATCAAACCAAAATTGGGTCTG
TCTGCAAAGAATTATGGTAGAGCAGTTTACGAATGTCTTC
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Fig. 2. Nucleotide sequence of the *Podocarpu*s sample from Bukidnon.

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>Contig1
CAAACAGAGACTAAAGCAAGTGCTGGGTTCAAAGCTGGTGTTAAAGATTACAGATTAACT
TATTATACTCCGGAATATCCGACCAAAGATACTGATATCTTGGCGGCATTCCGAGTCACT
CCTCAACCCGGAGTGCCCCCCGAGGAAGCAGGAGCGGCAGTAGCTGCCGAATCTTCCACT
GGTACATGGACTACTGTTTGGACCGATGGACTTACCAGCCTCGATCGTTACAAGGGGCGA
TGCTATGGCATCGAACCTATTCCTGGAGAAGAAAGTCAATTTATTGCCTATGTAGCTTAT
CCCTTAGACCTTTTTGAAGAAGGTTCTGTTACTAACCTGTTCACCTCCATTGTGGGTAAT
GTTTTTGGATTCAAAGCCCTACGGGCTATACGTCTGGAAGATCTGCGAATTCCTCCTTCT
TATTCCAAAACTTTCCAAGGTCCCCCACATGGTATCCAGGTAGAAAGGGATAAATTAAAT
AAATATGGCCGCCCCTTATTGGGATGTACCATCAAACCAAAATTGGGTCTGTCTGCAAAG
AATTATGGTAGAGCAGTTTACGAATGTCTTCGAGGTGGA
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Fig. 3. Nucleotide sequence of the *Podocarpu*s sample from Batanes.

TABLE I: IDENTITY OF PODOCARPUS SAMPLES COLLECTED FROM BUKIDNON AND BATANES, PHILIPPINES.

Isolate no.	Collection area	Identification	E-value	Identity	Reference
R1	Bukidnon	Podocarpus macrophyllus	0.00	99%	JQ512600.1
R3	Batanes	Podocarpus costalis	0.00	99%	HM593635.1

The unknown *Podocarpus* spp. from Bukidnon was identified as *Podocarpus macrophyllus*. In the latest revision of the genus *Podocarpus* following Buchholz and Gray [33], Laubenfels [34] proposed an infrageneric classification of the genus that recognized two subgenera with 18 sections. *Podocarpus macrophyllus*, is classified in section Polystachyus (type: P. polystachyus R. Br.) in subgenus Foliolatus. According to Laubenfels [34], section Polystachyus consists of nine species ranging from Japan and S China through Malaya to W New Guinea and NE Australia. Laubenfels distinguished *Podocarpus macrophyllus* from *Podocarpus chinensis* (Roxb.) Wall. ex J. Forbes by differences in the shape and size of the leaves; *Podocarpus macrophyllus* has leaves "with revolute margins, narrowing gradually at the base", and those of *Podocarpus chinensis* are "narrowing gradually at the base, less than 7 mm wide" and "at least 4 cm long, and at least 10 times as long as wide." Fu and Mill [35] reduced *Podocarpus chinensis* to synonymy under *Podocarpus macrophyllus* and treated it as var. maki [sensu Siebold & Zucc.]. They distinguished the varieties by the length and width of leaves: leaves of var. macrophyllus are 7–12 cm long, (5–) 7– 10 mm wide, those of var. maki are 1.7–7 cm long, 5–7 mm wide [36].

The *Podocarpus* spp. sample from Batanes was identified as *Podocarpus costalis*. *Podocarpus costalis*, locally known as Arius, is a species of conifer in the Podocarpaceae family, found and endemic in the Philippines and Taiwan. It is sometimes misidentified as *Podocarpus polystachyus* R. Brown ex Endlicher (from Indonesia, Malaysia, and the Philippines) by several authors dealing with the Chinese flora. In the Philippines, it is endemic to Palawan and Luzon. *P. costalis* is distributed in Batanes and in the Babuyan Islands in Northern Luzon. It is home to coastal bluffs near sea-level to at least 300 meters [37].

IV. CONCLUSION

Identification of *Podocarpus* and Podocarpacea is often difficult. DNA barcoding technique using rbcL sequences in this study provides rapid and accurate identification of *Podocarpus* samples from Bukidnon and Batanes, Philippines. The sample from Bukidnon was *Podocarpus macrophyllus*. The sample from Batanes was identified as *Podocarpus costalis*. This identification may be helpful as an addition to the reference library of Podocarpaceae DNA barcodes which will allow researchers unfamiliar with the family's morphology and anatomy to make accurate identifications.

ACKNOWLEDGMENT

We thank Lessie Mae dela Cruz-Mata for the assistance in making this study.

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