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Universal primers for amplification and sequencing a noncoding spacer between the *atpB* and *rbc*L genes of chloroplast DNA

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Abstract. Universal primers for amplifying and sequencing a noncoding spacer between the *atpB* and *rbc*L genes of the chloroplast DNA were constructed from the published sequences of *Marchantia* (a liverwort), tobacco, and rice. Our results indicate an evolutionary trend of increasing spacer size from liverworts, through mosses, to vascular plants. This *atpB-rbc*L spacer is AT-rich, consistent with other chloroplast noncoding spacers. Due to weak functional constraints, the spacer is evolving rapidly. A sequence identity of 92.2% was observed between spacers of two closely related moss species, *Rhytidiadelphus loreus* and *R. triquestrus*. Insertion/deletion events are common in the evolution of this spacer. A 23 bp deletion occurrs in *R. loreus*. Variation is found between two populations of *Amorphophallus henryi* (Araceae) and between individuals in a populations of *Pasania formosana* (Fagaceae). We suggest that this spacer will be useful for molecular systematics at the subspecific, specific, and generic levels and, in some species, for population genetics studies.

Keywords: *Amorphophallus henryi*; *atpB-rbc*L chloroplast spacer; PCR; *Rhytidiadelphus* sp.; Universal primers.

Introduction

The chloroplast genome evolves slowly in general (Clegg et al., 1991), making it an ideal system to assess phylogenetic relationships among genera (e.g., Onagraceae, Conti et al., 1993; Apiaceae, Downie et al., 1996), families (e.g., Asteridae, Olmstead et al., 1992) or higher levels (e.g., seed plants, Chase et al., 1993; monocots, Davis, 1995; angiosperms, Nickrent and Soltis, 1995). Nevertheless, some recent studies have revealed genetic variation between populations based on RFLP's of the total chloroplast genome (see Schaal and Leverich, 1996) or on specific sequences, such as noncoding spacers between several tRNAs of chloroplast DNA (Demesure et al., 1995, 1996).

Several universal primers for amplifying noncoding spacers of the chloroplast genome have been reported (Taberlet et al., 1991; Demesure et al., 1995; Dumolin-Lapegue et al., 1997). Most of the primers were designed for amplifying spacers between tRNA genes, which have been proved variable among species or populations (Demesure et al., 1996). In this study we describe one pair of universal primers for another noncoding spacer region that lies between the large subunit of ribulose-1,5-bisphosphate-carboxyalse (*rbc*L) and the beta-subunit of the chloroplast ATP-synthase (*atpB*) genes. Compared to the primers designed for specific taxa (e.g., Rubiaceae, Manen et al., 1994), the primers proposed here work for plants of a wider phylogenetic array from bryophytes to flowering plants. The spacer is useful for both phylogenetic assessment (Golenberg et al., 1993; Manen et al., 1994; Natali et al., 1995; Savolainen et al., 1994) and species level or population studies.

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Materials and Methods

Material Collection

Species of mosses and vascular plants representing a phylogenetic array were collected for testing the primers designed in this study. Two mosses, *Rhytidiadelphus triquetrus* and *R. loreus* (Hylocomiaceae), one fern (*Angiopteris*), one gymnosperm [*Cunninghamia lanceolata* (Taxodiaceae)], three monocots [i.e., *Imperata cylindrica*, *Miscanthus transmorrisonensis* (Poaceae) and *Amorphophallus henryi* (Araceae)], and three dicots [i.e., *Begonia aptera* (Begoniaceae), *Glycine soja* (Fabaceae), and *Pasania formosana* (Fagaceae)] were collected in the field in Taiwan, China, Canada or USA (Table 1). Vouchers are deposited at HAST herbarium. Leaf tissue, fast dried in silica gel after collecting, was powdered in liquid nitrogen and kept in a -70°C freezer.

DNA Isolation

Genomic DNA was extracted by a CTAB method (Doyle and Doyle, 1987) from frozen tissue. Isolated DNA of mosses was purified on low melting agarose gels to remove secondary compounds and RNA (Chiang, 1994).

Table 1. Materials collected for amplification of *atpB-rbc*L spacer of cpDNA with size variation.

Taxon	Sources	Size (bp)
MOSSES		
Rhytidiadelphus loreus (Hylocomiaceae)	**Canada**: British Columbia	524
R. triquestrus (Hylocomiaceae)	**USA**: Smoky Mt.	549
FERN		
Angiopteris lygodiifolia (Marattiaceae)	**Taiwan**: Yangmingshan	629
GYMNOSPERM		
Cunninghamia lanceolata (Taxodiaceae)	**China**: Chekiang Prov.	1,000
ANGIOSPERMS		
Monocots:		
Miscanthus transmorrisonensis (Poaceae)	**Taiwan**: Hohuanshan	800
Imperata cylindrica (Poaceae)	**Taiwan**: Tainan Co.	800
Amorphophallus henryi (clone 1) (Araceae)	**Taiwan**: Tainan Co.	800
A. henryi (clone 2) (Araceae)	**Taiwan**: Kaohsiung City	800
Dicots:		
Glycine soja (Fabaceae)	**Taiwan**: Taipei Hsien	900
Begonia aptera (Begoniaceae)	**Taiwan**: Taipei Hsien	841
Pasania formosana (Fagaceae)	**Taiwan**: Pingtung Hsien	900

Primer Design and PCR

One pair of universal primers were designed by comparing the sequences of *Marchantia* (a liverwort, Umesono et al., 1988), *Angiopteris* (a fern, EMBL X58429), tobacco (Shinozawa et al., 1986), and rice (Nishizawa and Hirai, 1987). Primers, *atpB*-1: 5'-ACATCKARTACKGGACC AATAA-3' and *rbc*L-1: 5'-AACACCAGCTTTRAATCCAA-3', were used for PCR and sequencing. PCR amplification conditions were 30 cycles of 94°C denaturing for 45 s, 49°C annealing for 1 min 15 s, and 72°C extension for 1 min 15 s, followed by 72°C extension for 10 min and 4°C for storing. PCR products were electrophoresed in a 1% agarose gel.

Nucleotide Sequencing

PCR products were separated and eluted using agarose gel purification (BM) and ligated to a pT7 Blue T-vector (Novagen). Plasmid DNA was purified using Wizard Plus SV kit (Promega) and quantified for further sequencing. Cycle sequencing based on dideoxy-mediated chain-termination methodology (Sanger et al., 1977) with *Taq* polymerase was performed using the fmol™ Sequencing System (Promega). Double strands were sequenced from both ends, using primers for PCR amplification and additional primers in between as well, with overlapping of about 100 bp. Clones of mosses and *Amorphophallus henryi* were sequenced completely. DNA fragments from the other species were sequenced only with one of the primers, *atpB*-1, to confirm the nature of the amplified segments.

Results and Discussion

PCR products show size variation of the *atpB-rbc*L spacer among groups. Our results indicate an evolutionary trend of increasing *atpB-rbc*L spacer size, from liverworts (507 bp in *Marchantia*), through mosses (ca. 550 bp in *Rhytidiadelphus*), to vascular plants including ferns (629 bp in *Angiopteris*), conifers (ca. 1,000 bp in *Cunninghamia lanceolata*), monocots (ca. 800 bp) and dicots (ca. 850–950 bp) (Table 1).

This is also the first report of variation of cpDNA sequences between closely related species of bryophytes. 553 nucleotide bases of the *atpB-rbc*L noncoding spacer were aligned (Figure 1). Clones of *Rhytidiadelphus loreus* (EMBL no. L21722) and *R. triquestrus* (EMBL no. L23410) shared 92.2% identity. Substitutions occured with no bias in transitions (5 times) versus transversions (6 times). Insertion/deletion is a common phenomenon in this spacer. Several length mutations were observed between the two taxa. *Rhytidiadelphus loreus* lacked a three bases (TAG) and a 13 bases (positions 381–403) sequences in contrast to *R. triquestrus*. Additionally, *R. triquestrus* lacked a three bases sequence (TTC) in a different portion of the sequence. The variability of this spacer among species is close to that of the internal transcribed spacer (ITS region) of nuclear ribosomal DNA, in which 87.6–93.7% identity between *Raillardella* species (family Asteraceae, Baldwin, 1992) has been reported.

Sequences (Figure 2) of the spacer in *Amorphophallus henryi* showed variation between two populations. In 810 aligned bases 11 sites (1.4%) were variable. Two indels occured at positions 169 and 718. Nine substitutions were observed with more transitions (6 sites) than transversions (3 sites), which is consistent with reports for other genes (cf. Li, 1997). The *atpB-rbc*L spacer is AT rich. In the sequence of *A. henryi*, 29.9% of the bases (242 out of 810) were either C or G. Most noncoding spacers and pseudogenes are AT rich due to low functional constraints (Li, 1997). Lack of functional constraints also results in a faster rate of evolution for most spacer regions. Our preliminary data

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R-l 1    TACAACTCAA TTGATAACTC TTGCAAGGAT TAGGTCTGCT CGACAAATAA
R-t      .......... .......... .......... .......... ..........

R-l 51   GTTTTTTTAA TCTTATAAAA AAACATCTTT GGTATTTAAT TTTGTCTATT
R-t      .......... .......... .......... .......... ..........

R-l 101  TGTAGACAAA GTTATTTTAC TATAACAAAA CAGTATCATT GTATAGTATT
R-t      .T........ .......... .......... .......... ..........

R-l 151  TTTTACATTT GATGCAACTC AGATTATTTT TAGTAAATGA --T---TTTT
R-t      .......... .......... .......... .......... GC.TAG....

R-l 201  ATTTGTATAA GCATTGACCT AATAATCTTT TGAATGTTAA ACTAATTAAT
R-t      ...C...... .......... .......... .......... ..........

R-l 251  AAAAATAAGA TTTAATTAAA AACATAGTAA GAAATAAAAA AATAAACTAT
R-t      ........A. .......... .......... .......G.. .TA...-...

R-l 301  TACCTAATTC AATTTATTTT TTGAAAATAA AATAAAATTT TTTTATTTAA
R-T      .........T .......... .......... .......... ..........

R-l 351  ATATATTTAT TATTTACTAA TATTTTTAGA ---------- ----------
R-t      .......... .......... ......G... TTGTAAAAAA TATGTATGTC

R-l 401  ---CAGTTTT TAATTTTTTT TATTTTAGAT ACTTATATCT ATATATAAGT
R-t      TTA..C.... .......... .......T.. .......... ..........

R-l 451  AATTAATTGA AAGTAATTTT TTTTCTTCAA TAATTAAATG ATCGAGTTGA
R-t      .......... .......... .....---.. .......... ..........

R-l 501  TACTAAATAT TTTTT-CGAT ATAATCAGCA ACTAATTTAT TACTTCTAAA
R-t      .......... .....T.A.. .......... .......... ..........

R-l 551  TTT
R-t      ...
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Figure 1. Alignment of sequences of *atpB-rbc*L spacer of *Rhytidiadelphus loreus* (R-l) and *R. triquestrus* (R-t), with identical sequences (•) and deletions (–).

on *Pasania formosana* shows much higher diversity (data not shown) in the nucleotide sequence of the *atpB-rbc*L spacer than has been detected in any other plant groups reported previously, such as the taxa of Rubiacaeae (Manen et al., 1994; Natali et al., 1995). Here we find variation among individuals within populations of *Pasania*. Therefore, we suggest that this spacer will be useful for molecular systematics at the subspecific, specific, and generic levels and, in some cases, for population genetic studies.

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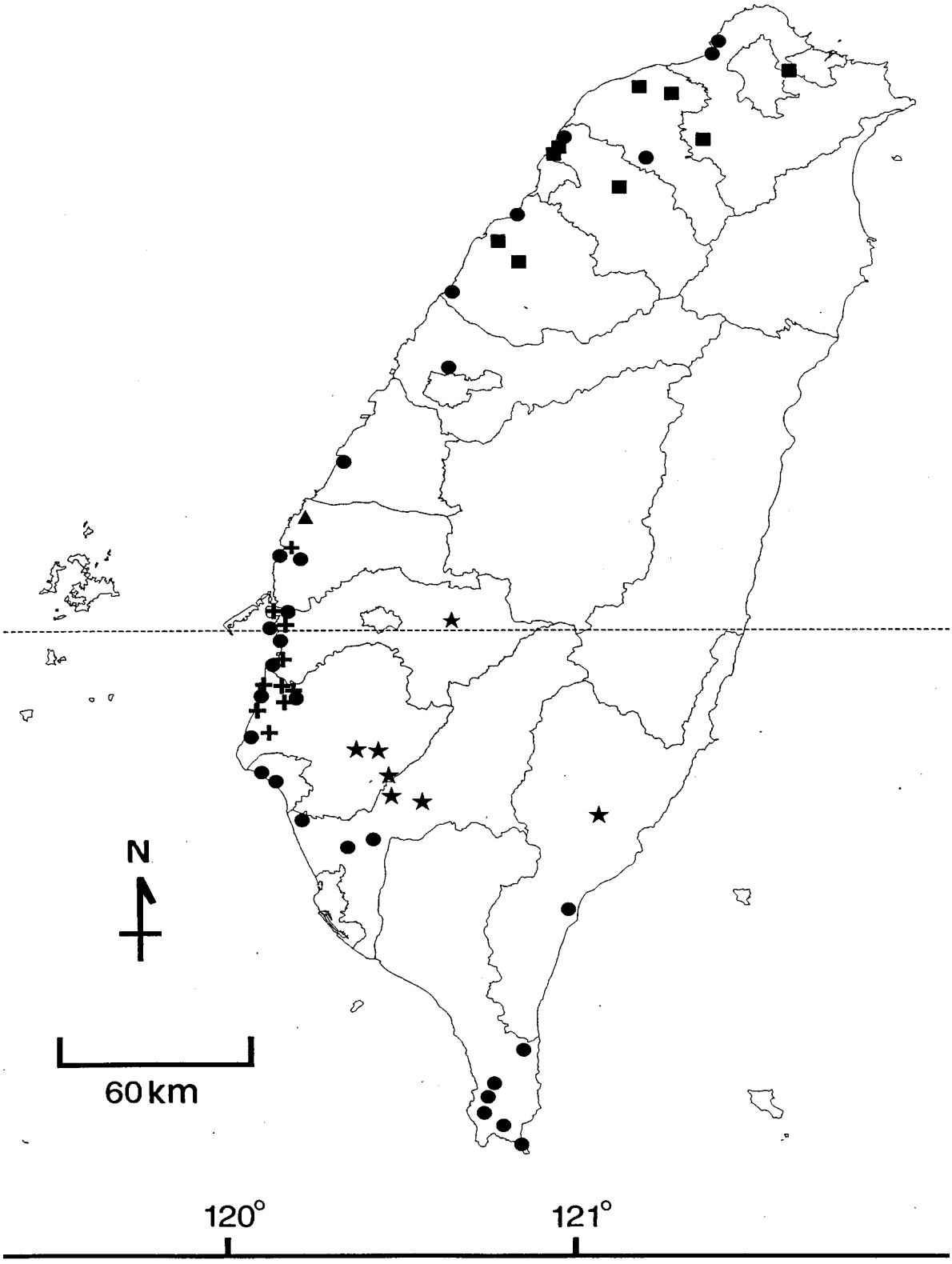

Figure 2. Nucleotide sequences of *atpB-rbc*L chloroplast DNA spacer of clones (Hen 1 and Hen 2) of *Amophorphalus henryi*, with identical sequences (•) and deletions (–).

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