PRIMERS FOR PHYLOGENY RECONSTRUCTION IN BIGNONIEAE
(BIGNONIACEAE) USING HERBARIUM SAMPLES

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• Premise of the study: New primers were developed for Bignonieae to enable phylogenetic studies within this clade using herbarium samples.
• Methods and Results: Internal primers were designed based on available sequences of the plastid ndhF gene and the rpl32-trnL intergenic spacer region, and the nuclear gene PepC. The resulting primers were used to amplify DNA extracted from herbarium materials. High-quality data were obtained from herbarium samples up to 53 yr old.
• Conclusions: The standardized methodology allows the inclusion of herbarium materials as alternative sources of DNA for phylogenetic studies in Bignonieae.

Key words: Bignonieae; herbarium specimens; ndhF; PepC; phylogeny; rpl32-trnL spacer.

The tribe Bignonieae includes 393 neotropical species (Lohmann and Taylor, in press), representing almost half of the species in the Bignoniaceae. The tribe is mainly composed of lianas and shrubs with widespread or highly endemic distribution patterns (Lohmann and Taylor, in press).

The first molecular phylogenetic study for the tribe (Lohmann, 2006) was mainly based on recently collected samples that did not present any problems for the amplification of large DNA fragments. However, approximately 10% of the currently recognized species of Bignonieae (37 of the 393) are highly endemic and were not encountered in the field. For those species, only five or fewer herbarium samples are available (Lohmann, unpublished data), making those specimens the only source of DNA material for phylogenetic studies.

Here, we propose new primers and protocols that allow the amplification of medium-sized DNA fragments (~500 bp) from herbarium samples. The novel protocols here proposed are critical for the inclusion of rare and poorly known species of Bignonieae into a comprehensive phylogeny of the whole tribe.

METHODS AND RESULTS

DNA extraction—Total DNA of six herbarium samples (dating up to 53 yr old) was extracted with Invisorb Plant Mini Kit (Invitek, Berlin, Germany). The manufacturer’s protocol was followed, except for the final step, in which 50 μL of elution buffer was used instead of the suggested 200 μL.

Primer development—Selected sequences of the plastid ndhF and nuclear PepC genes for Bignonieae from Lohmann (2006) were combined with newly generated sequences for the plastid rpl32-trnL intergenic spacer region following Shaw et al. (2007). Vouchers and GenBank accessions of the sequences used and/or generated in this paper are presented in Appendix 1. The data sets corresponding to the individual data partitions were aligned in Geneious 5.4 (Drummond et al., 2010) using the algorithm MUSCLE (Edgar, 2004). A thorough search for primer pairs was also conducted in Geneious, using the software Primer3 (Rozen and Skaletsky, 2000). The objective of this search was to design primers placed in highly conserved regions that would only amplify medium-sized fragments (~500 bp) and would overlap adjacent amplicons (~70 bp). Given that the nuclear marker PepC is present in multiple copies, with two sizes (Lohmann, 2006), we focused on the amplification of the larger fragment, which covers all of intron 4 and holds 85% of the informative sites (Lohmann, 2006). In total, 17 primers were initially developed (Table 1).

DNA amplification, cloning, and sequencing—PCR conditions were optimized using a common 25 μL master mix containing the following ingredients: 5 μL of 5X buffer, 2.5 μL of MgCl2 (25 mM), 1 μL of dNTP (10 mM), 0.5 μL of bovine serum albumin (BSA; New England Biolabs, Ipswich, Massachusetts, USA), 0.5 μL of each primer (10 μM), 1 unit of GoTaq Hot Start Polymerase (Promega Corporation, Madison, Wisconsin, USA), and 1 μL of genomic DNA. For the PepC mix, 0.25 μL of dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Missouri, USA) was also added. A standard PCR program was implemented as follows: one initial step at 95°C for 5 min; 40 cycles at 95°C for 30 s, 48–56°C for 30 s, and 72°C for 30 s to 2 min; and a final step at 72°C for 10 min. The specific annealing temperature and elongation time for each primer pair is presented in Table 2.

The optimized PCR conditions were applied using the common mix. For ndhF and rpl32-trnL, products were purified by adding 1.5 μL of Illustra ExoStar (GE Healthcare Life Sciences, Buckinghamshire, United Kingdom) and submitting the samples to the thermal treatment as indicated by the manufacturer, with an additional step of 62°C for 15 min to renature the DNA. For PepC, PCR products were purified with the Illustra GFX purification kit (GE Healthcare Life Sciences), due to the presence of primer dimer, and then used in a ligation protocol with pGEM Easy Vector System (Promega Corporation). JM109 Competent E. coli cells (Promega Corporation) were used for the heat-shock transformation protocol. After incubation, transformant colonies were resuspended in 10 μL of 0.5X TE buffer and boiled for 10 min in a thermocycler. Up
TABLE 1. Primer sequences used and/or developed to amplify and sequence selected loci for Bignonieae.

| Region | Primer | Primer sequences (5′–3′) | Reference |
|--------|--------|--------------------------|-----------|
| ndhF   | 5F     | ATGGAAACAGACATATCATTAGTGG | Olmstead and Sweere, 1995 |
|        | 1318R  | CGGAATTATATAATAAGCGTAATCC | Olmstead and Sweere, 1994 |
|        | 972F   | GCTCAATGTTGTATAGATTG     | Olmstead and Sweere, 1994 |
|        | 3R     | CCGYATTTTCTGATCTTCGCC   | Olmstead and Sweere, 1995 |
|        | 370F   | TCTATGGTGGGAGATGTTACTAGC | This paper |
|        | 478R   | AGGTCTGTGTGAAACAAAAAC   | This paper |
|        | 741F   | AGGGACACGCYCTATTGCGCT   | This paper |
|        | 808F   | AGTCGCGCCCTCCTCCTTTTTT | This paper |
|        | 849R   | GGGTATACAAAGAGAAATCTT   | This paper |
|        | 1290F  | CAACAGATTTACCGCATTATT   | This paper |
|        | 1336R  | CTGTTAAATGCGGCTTCAAAA  | This paper |
|        | 1393R  | AGGGGTATTGGTGGCTTCACCTT | This paper |
|        | 1680F  | TGGATGTCCTAGGAAATTTTTT | This paper |
|        | 1835R  | CCGTTAAATATTCGGAAATAAGC | This paper |
|        | 2117R  | GAACACTTATAATACACACACCTTTT | This paper |
| rpl32-trnL | trnL(UAG)| CTGCCTCCTAAATAGCACGCT | Shaw et al., 2007 |
|        | rpl32-F | CAGTCTCCAAAAATGCTTCTC  | Shaw et al., 2007 |
|        | trnL_479R | TAGAAGCGCGATAGAAAACCT  | This paper |
|        | trnL_365F | TGCCTGATTGATGGYAGGAGA | This paper |
|        | trnL_407F | AGAGACTTCCAGAAGAAAAATCT | This paper |
|        | rpl32_146R | TCCGTGAAATACAGAAGAAA  | This paper |
|        | rpl32_241F | ATCATTTCCAACCCGGAGA  | This paper |
|        | rpl32_619R | TGGTTCATGTTGGGATTAGTTACTAGC | This paper |
|        | rpl32_682F | CGGACGCTAGCCTTTTACAGAGT | This paper |
| PepC   | 4F     | AATCAGCAGAAGTGGAGT     | Ayres et al., 2009 |
|        | 5R     | GCGGCTATCCATTCCTG    | Ayres et al., 2009 |
|        | IV_119F | ACGCAGTGYTGAGACCTGTYTG | This paper |
|        | IV_197F | RCTCTGAGAGTGGDGDSGGGATGCGG | This paper |
|        | V_25R  | ACTCGAGGGRCTACCTAGGATTGC | This paper |

TABLE 2. Optimized PCR conditions used in this study.

| Region     | Forward primer | Reverse primer | T<sub>a</sub> (°C) | Elongation duration |
|------------|----------------|----------------|---------------------|---------------------|
| ndhF       | 5F             | 1318R          | 48                  | 2 min               |
|            | 972F           | 3R             | 48                  | 2 min               |
|            | 5F             | 478R           | 52                  | 45 s                |
|            | 370F           | 849R           | 56                  | 45 s                |
|            | 741F           | 1393R          | 52                  | 45 s                |
|            | 808F           | 1336R          | 55                  | 45 s                |
|            | 1290F          | 1835R          | 51                  | 45 s                |
|            | 1680F          | 2117R          | 50                  | 45 s                |
| rpl32-trnL | trnL(UAG)      | rpl32-F        | 48                  | 2 min               |
|            | rpl32-F        | 479R           | 48                  | 1 min               |
|            | 407F/365F      | rpl32-F        | 48                  | 1 min               |
| PepC       | IV_119F        | V_25R          | 48                  | 1 min               |
|            | IV_197F        | V_25R          | 48                  | 1 min               |

Note: T<sub>a</sub> = annealing temperature.

CONCLUSIONS

The 21 new primers here proposed, combined with the eight previously available primers (Fig. 1) and optimized protocols, led to high-quality sequences for the three selected molecular markers (ndhF, PepC, and rpl32-trnL). Those results demonstrate that herbarium materials can provide an excellent source of information for molecular phylogenetic studies in the plant family Bignoniaceae. These primers are now being used to obtain a comprehensive phylogeny for the whole tribe (Lohmann et al., in prep.). Given that the primers designed here were positioned in conserved regions, we believe that those primers will also yield high-quality sequences in other clades of the Bignoniaceae and other closely related families.
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http://www.bioone.org/loi/apps
APPENDIX 1. Vouchers and GenBank accessions used and/or generated in this study. Information presented: species; voucher (herbarium), ndhF, rpl32-trnL, PepC clones. Asterisks indicate sequences generated in this work.

**Amphilophium bauhinioides** (Bureau ex Baill.) L. G. Lohmann (ined.; Lohmann and Taylor, in press); Lohmann 655 (CVRD, MO), DQ222586, KC914599*, DQ222734. **Anemopaegma robustum** Bureau & K. Schum.; Assunção 126(INPA, MO), DQ222538, KC914598*, DQ222663. **Bignonia bracteomana** (K. Schum. ex Sprague) L. G. Lohmann (ined.; Lohmann and Taylor, in press); Wojtkowski 5637 (MO), KC914588*, KC914594*, KC914610*, KC914611*, KC914612*. **Bignonia convolvuloides** (Bureau & K. Schum.) L. G. Lohmann (ined.; Lohmann and Taylor, in press); Carvalho 2 (SPF), KC914586*, KC914592*, KC914605*, KC914606*, KC914607*; Gomes 278 (SPF), KC914591*, KC914597*, KC914617*, KC914618*, KC914619*. **Bignonia hyacinthina** (Standl.) L. G. Lohmann; Lohmann 642 (MO, MOL), DQ222614, KC914602*, DQ222775. **Bignonia potosina** (K. Schum. & Loes.) L. G. Lohmann (ined.; Lohmann and Taylor, in press); Álvarez 5353 (MO), KC914587*, KC914593*, KC914608*, KC914609*; Carnevali 6840 (MO), KC914590*, KC914596*, KC914614*, KC914615*, KC914616*. **Bignonia uleana** (Kraenzl.) L. G. Lohmann (ined.; Lohmann and Taylor, in press); Lohmann 617 (MO, MOL), DQ222572, KC914601*, DQ222709; Nee 39466 (MO, NY, TEX), KC914589*, KC914595*, KC914613*. **Dolichandra unguis-cati** (L.) L. G. Lohmann; Lombardi 2432 (BHC, MO), DQ222595, KC914603*, DQ222749. **Fridericia speciosa** Mart.; Lombardi 2521 (BHC, MO), DQ222584, KC914604*. **Mansoa diffi cilis** (Cham.) Bureau & K. Schum.; Lohmann 662 (CVRD, MO), DQ222598, KC914600*, DQ222752.