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Source: Applications in Plant Sciences, 1(9)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300018

Applications in Plant Sciences 2013 1(9): 1300018

Primer Note

Primers for phylogeny reconstruction in Bignonieae (Bignoniaceae) using herbarium samples[1]

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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 For those species, only five or fewer herbarium samples are available (Lohmann, unpublished data), making those specimens the only source of DNA material for phylogenetic studies.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 5× buffer, 2.5 μL of $MgCl_2$ (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 0.5× TE buffer and boiled for 10 min in a thermocycler. Up

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[1] Manuscript received 5 March 2013; revision accepted 23 April 2013.

The authors thank the Fundação de Amparo à Pesquisa do Estado da São Paulo (FAPESP) for scholarships to A.R.Z. (2011/09160-5) and L.H.M.F. (2009/12508-3), a regular research grant to L.G.L. (2011/50859-2), and a collaborative research grant between NSF-FAPESP (2012/50260-6). We also thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for a Pq-2 grant to L.G.L. We are extremely grateful to the Missouri Botanical Garden for authorizing DNA extraction from selected specimens.

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doi:10.3732/apps.1300018

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	ATGGAACAGACATATCAATATGSGTGG	Olmstead and Sweere, 1995
	1318R	CGAAACATATAAAATGCRGTTAATCC	Olmstead and Sweere, 1994
	972F	GTCTCAATTGGGTTATATGATG	Olmstead and Sweere, 1994
	3R	CCCYASATATTTGATACCTTCKCCG	Olmstead and Sweere, 1995
	370F	TTCCATGTTGGGATTAGTTACTAGC	This paper
	478R	AGGTCGTGTGAACCAAAACC	This paper
	741F	AGGGACCCACYCCTATTTCGGCT	This paper
	808F	AGCTCGCCTTCTTCCTCTTT	This paper
	849R	GGCCTATCAAAGAGATAAAATTCA	This paper
	1290F	CAGCAGGATTAACCGCATTT	This paper
	1336R	CGTTTAAATGCCCCTCAAAA	This paper
	1393R	AGGGGTATTTTGGCTGCCACTGT	This paper
	1680F	TTGGATCCCTAGGAATTYCTTTT	This paper
	1835R	CGCTAAAAATATTCCGAAATAAGC	This paper
	2117R	GAAACTCATAATACCAACCCCATT	This paper
rpl32-trnL	trnL$^{\text{(UAG)}}$	CTGCTTCCTAAGAGCAGCGT	Shaw et al., 2007
	rpl32-F	CAGTTCCAAAAAAACGTACTTC	Shaw et al., 2007
	trnL_479R	TAGAAGGGCGGATAGAAAATCT	This paper
	trnL_365F	TGCCTGGATTGATGGYGAGAGA	This paper
	trnL_407F	AGAAGACTTCAGAGAAAATTCAAAA	This paper
	rpl32_146R	TCGGTAAGGTAAACAGAAGAA	This paper
	rpl32_241F	ATCATTTCCAAGCCGAGGA	This paper
	rpl32_619R	TTCTTTTAATGAACTGTTTTTGA	This paper
	rpl32_682F	CGGACGATCGAGTTTTACAAGAGT	This paper
PepC	4F	ACTCCACAGGATGAGATGAG	Ayres et al., 2009
	5R	GCAGCCATCATTCTAGCCAA	Ayres et al., 2009
	IV_119F	ACGRCGTGTYGACACTGCTYTGA	This paper
	IV_197F	RTCCTGGATGGGDGGGGATCGKG	This paper
	V_25R	ACTTCAGGRGTTACCCTAGGATTGC	This paper

to four colonies were amplified using M13 primers and the common mix adjusted to a final volume of 10 μL. These amplifications used an initial step of 95°C for 5 min; 30 cycles of 95°C for 45 s, 53°C for 1 min, and 72°C for 90 s; and a final step of 72°C for 10 min. PCR products were purified with 0.7 μL of Illustra ExoStar (GE Healthcare Life Sciences).

All samples were sequenced at Macrogen (Seoul, Korea), assembled in Geneious 5.4, and deposited in GenBank (Appendix 1). Annotations for *ndhF* and *PepC* follow Lohmann (2006), and those for newly generated sequences of *rpl32-trnL* were established using the complete plastid genomes of *Nicotiana sylvestris* Speg. & S. Comes (NC_007500) and *Olea europaea* L. (NC_013707). All cloned sequences were screened for vector contamination by comparison with the UniVec Database (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/VecScreen/UniVec.html) prior to submission to GenBank. Species names follow Lohmann and Taylor (in press).

The three selected loci (*ndhF*, *PepC*, and *rpl32-trnL*) were successfully amplified from herbarium materials using the newly developed primer sets and proposed protocols. High-quality DNA sequences were obtained for most samples (55 of 62 sequenced fragments). In the rare cases in which low-quality sequences were generated, additional PCR optimizations were conducted, none of which led to higher-quality products. In those cases, a second PCR, using 0.5 μL of the unpurified product from the first PCR as template and the same PCR program, was adopted, leading to higher-quality products. With these optimizations, all fragments were successfully amplified and also led to high-quality sequences for *ndhF* and *PepC*. For the *rpl32-trnL* marker, the presence of two homopolymeric regions (polyA or polyT) was responsible for DNA polymerase slippage. As a result, low-quality sequences were seldom obtained immediately after this region (three of 13 sequences). To produce fully double-covered sequences, four primers (146R, 241F, 619R, and 682F; Table 1) were designed flanking the homopolymers. After these adjustments, high-quality sequences were produced for all samples. This protocol is already being used to reconstruct generic-level phylogenies in Bignonieae and has proved to be highly efficient in all of the genera it has been tested on (Zuntini and Lohmann, in prep.; Fonseca and Lohmann, in prep.; Medeiros and Lohmann, in prep.; Calió, Winkworth, and Lohmann, in prep.).

Table 2. Optimized PCR conditions used in this study.

Region	Forward primer	Reverse primer	T_a (°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$^{\text{(UAG)}}$	rpl32-F	48	2 min
	trnL$^{\text{(UAG)}}$	479R	48	1 min
	407F / 365F	rpl32-F	48	1 min
PepC	4F	5R	48	1 min
	IV_119F	V_25R	48	1 min
	IV_197F	V_25R	48	1 min

Note: T_a = 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.

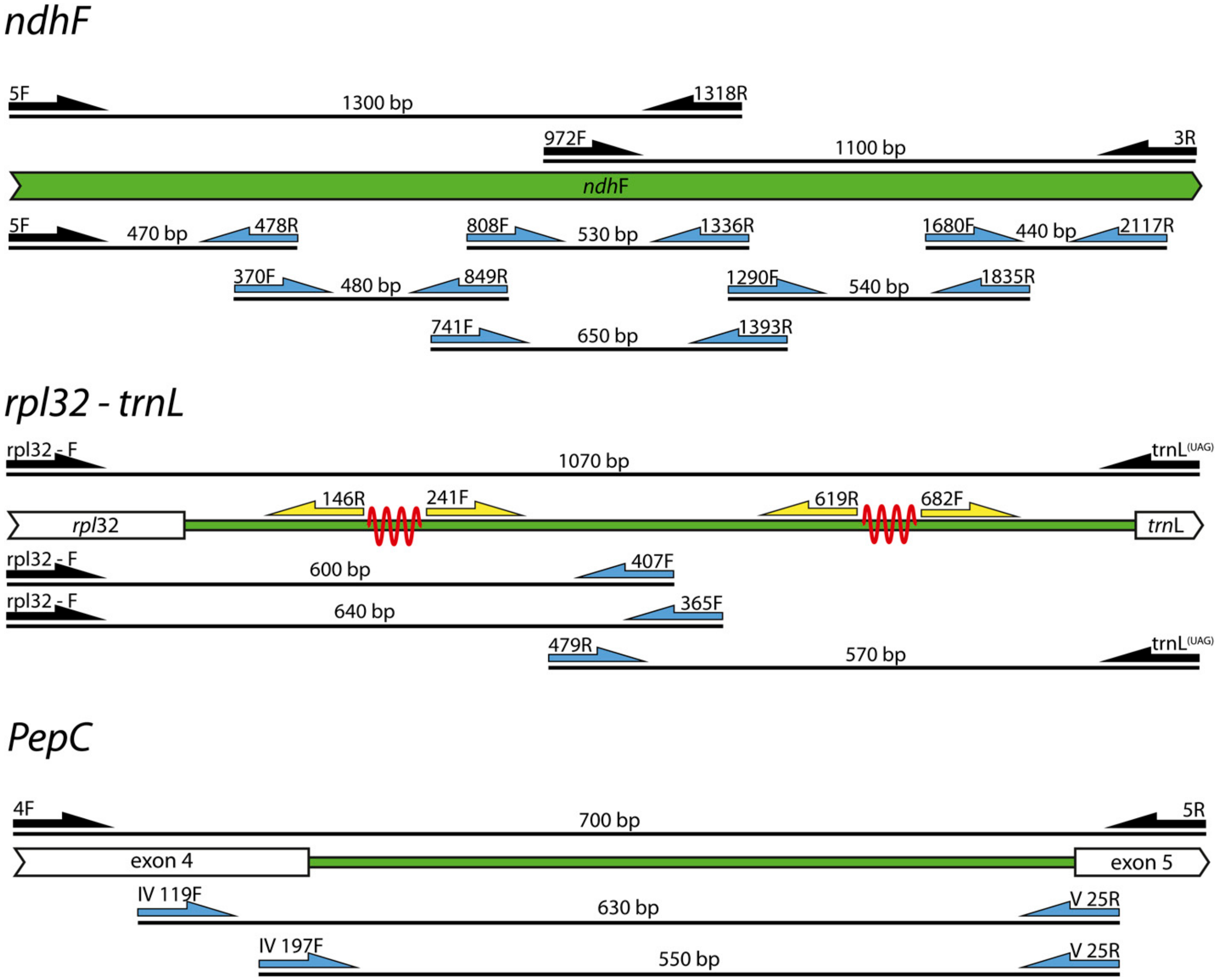

Fig. 1. Primer map for *ndhF*, *rpl32-trnL*, and *PepC* with regions of interest marked in green. Average fragment sizes are indicated between each primer pair, represented by arrows: black (previously published primers), blue (newly developed primers for amplification and sequencing), and yellow (additional sequencing primers developed). The red zigzag patterns represent the position of the homopolymeric regions occasionally found in *rpl32-trnL*.

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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); *Woytkowski 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* (BHCB, MO), DQ222595, KC914603*, DQ222749. ***Fridericia speciosa*** Mart.; *Lombardi 2521* (BHCB, MO), DQ222584, KC914604*, DQ222730. ***Mansoa difficilis*** (Cham.) Bureau & K. Schum.; *Lohmann 662* (CVRD, MO), DQ222598, KC914600*, DQ222752.