PRIMER NOTE

EXON-PRIMED INTRON-CROSSING (EPIC) MARKERS FOR EVOLUTIONARY STUDIES OF Ficus AND OTHER TAXA IN THE FIG FAMILY (MORACEAE)¹

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• Premise of the study: The genus Ficus (fig trees) comprises ca. 750 species of trees, vines, and stranglers found in humid tropical forests. As a year-round source of calcium-rich fig fruits, Ficus trees are often described as keystone species. However, Ficus may be best known for their pollination mutualism with small (1–2 mm), short-lived (1–2 d) “fig wasps” in the family Agaonidae (Weiblen, 2002; Herre et al., 2008). Female fig wasps pollinate flowers and oviposit within the enclosed inflorescence (syconium or “fig”), in which the larvae develop before emerging to pollinate and oviposit in the syconia of asynchronously flowering conspecific trees. For sustained reproduction of the figs and the wasps, the wasps must exhibit a high degree of host-specificity, and the host population must provide access to flowers (i.e., figs) throughout the year.

Although the fig-wasp pollination mutualism is one of the tightest known in terms of host-pollinator specificity, there are many exceptions to the one pollinator species/one host species rule. In some cases, two or more wasp species pollinate the same host species in different parts of its geographic range, and multiple wasp species have been found in a single host tree (Herre et al., 2008). Furthermore, in Central America and in South Africa some wasp species have been shown to use more than one fig species in the local fig community (reviewed in Herre et al., 2008). The nonspecificity of some pollinators, in addition to some genetic studies (e.g., Machado et al., 2005), suggests that hybridization is possible.

Most phylogenetic studies of Ficus have used chloroplast DNA and/or one or two commonly used nuclear DNA markers (e.g., internal transcribed spacer [ITS]) (e.g., Ronsted et al., 2005). These markers are insufficient in number for studies of introgression, and they do not resolve phylogenies of closely related species or phylogeographic structure in widespread species (C. Dick, unpublished). To address the deficiency in nuclear genomic markers for Ficus, we have developed a set of exon-primed intron-crossing (EPIC) markers by comparing an expressed sequence tag (EST)–library for F. elastica Roxb. ex Hornem. with the annotated genomes of Populus trichocarpa Torr. & A. Gray (Salicaceae) and Arabidopsis thaliana (L.) Heynh. (Brassicaceae) using a bioinformatics pipeline developed by Li et al. (2010).

METHODS AND RESULTS

Selection of taxa—Neotropical Ficus contains two distinct and phylogenetically distant subgenera, which represent two important neotropical life forms: the free-standing fig trees (subg. Pharmacosycea (Miq.) Miq. sect. Pharmacosycea) and other taxa in the fig family (Moraceae).
| Locus | Primer sequences (5′→3′) | Total/intron length (bp) (+range) | No. of polymorphic sites | Nucleotide diversity | GenBank accession no. | Reference locus | Gene abbreviation |
|-------|--------------------------|----------------------------------|--------------------------|---------------------|----------------------|------------------|------------------|
| FA08190 | F: CCAAATTGTCTCGAGGAGACCTC T: TTATAGAATGTGTCGTCGTAAGA | 484/435 (+2) | 24 | 0.0503 | JQ341915 | AT1G08190 | ATVAM2 |
| FA02580 | F: CTAGATCTTCCACAGACAGACAG T: CTTGCGTGAGCCGACACAG | 487/381 (+2) | 22 | 0.0493 | JQ341917 | AT4G02580 | T10P11_14 |
| FA03310 | F: GCCTGTCAATTGAGAGAACACAG T: GGTCATTGACCGACTCGTTA | 740/581 (+2) | 43 | 0.0586 | JQ341919 | AT3G03310 | CAT3 |
| FA07360a | F: GCTGATAAAATGGTTGCTGCTG T: CTTGAGGAGGACAGACAGACAG | 540/287 | 29 | 0.0541 | JQ341921 | AT2G07360 | T13E11.13 |
| FA08510 | F: TCGTCGACTCTGGTTGAGAT | 893/741 | 51 | 0.0572 | JQ341923 | AT1G08510 | FATB |
| FA11980 | F: ATGGGAGGTCGTCGTCGTAAGA T: ACCCAAGTCTTGAGACACAG | 851/734 (+4) | 35 | 0.0414 | JQ341925 | AT5G11980 | F14F18_150 |
| FA14000 | F: TCCAGTCGACTCTGGTTGAGAT | 443/378 (+7) | 23 | 0.0142 | JQ341927 | AT3G14000 | F7A19_9 |
| FA16180b | F: CGACTATGGAACAGACAGAACAGACAG T: CATCAGTCAATGGAACAGAG | 417/281 (+3) | 21 | 0.0514 | JQ341928 | AT4G16180 | DL4130C |
| FA16990b | F: TCAACTTTCCTGGGTGAGAT | 964/674 (+3) | 40 | 0.0417 | JQ341930 | AT5G16990 | ATORC3 |
| FA19690a | F: ACTTGGCCTTCTTACTTCATGG T: AGCAATCCCAGACATGATGC | 386/258 (+2) | 12 | 0.0315 | JQ341933 | AT5G19690 | STT3A |
| FA23640* | F: ATTCCTTTTGGTCCTCCACATC T: ACCCACTTCCAGGAGAAGA | 1032/821 (+1) | 55 | 0.0547 | JQ341935 | AT3G23640 | HGL1 |
| FA24620a | F: CCTTACAAGGACAGCCTTTTG T: CTCAAGTTCCTGGAAGAAGA | 513/323 | 20 | 0.0421 | JQ341937 | AT4G24620 | PG1 |
| FA24620b | F: TGGCTAGATTTCCCATGTTTG T: CATCAGTCAATGGAACAGAG | 980/827 (+4) | 50 | 0.0514 | JQ341939 | AT4G24620 | PG1 |
| FA26990 | F: GGANGCTAGCTGCTGAGAT | 476/246 | 13 | 0.0276 | JQ341941 | AT2G26990 | FUS12 |
| FA32180 | F: GGTGCAAGAAGAAGAAGAAGAAGA | 741/628 (+13) | 38 | 0.0516 | JQ341943 | AT4G32180 | ATPK2 |
| FA32910 | F: GTGTGATAATGGTTGAGAT | 455/284 (+2) | 12 | 0.0265 | JQ341947 | AT4G32910 | F26P21_30 |
| FA36880b | F: GCTGTTGGGACATTGTTGAC | 1044/896 (+6) | 41 | 0.0514 | JQ341948 | AT5G36880 | F5H8_15 |
| FA45300 | F: GGAGGACTTGGTCTTGGTTACTT | 890/684 | 41 | 0.0462 | JQ341949 | AT3G45300 | ATV1D |
| FA48520* | F: TCATCCATATTTGGTCGGAGAT | 1059/890 (+4) | 71 | 0.0730 | JQ341951 | AT5G48520 | ATUAG3 |
| FA73180 | F: GACCACTTACTGGACTCGTTA T: ATGCAATCCCAGACACTCTCTC | 470/235 | 18 | 0.0383 | JQ341953 | AT1G73180 | T18K17_15 |
| FP04090b | F: GAATGGCTAGCGACAGATGATA | 438/275 (+10) | 15 | 0.0529 | JQ341955 | POPTR_0006s00800 | CYP97B3 |
| FP08470 | F: GCACTAGCTGCTGCTGAGAT | 550/404 (+7) | 25 | 0.0463 | JQ341957 | POPTR_0017s08470 | BGA19 |
| FP08550 | F: CGCTGTACCTGCTGCTGAGAT | 741/561 (+5) | 36 | 0.0523 | JQ341959 | POPTR_0006s08550 | F6E21_100 |
| FP09670 | F: GCACTAGCTGCTGCTGAGAT | 642/509 | 32 | 0.0516 | JQ341960 | POPTR_0006s08550 | F6E21_100 |
| FP10430 | F: GTGGAGTCTGAGTCTGCTGAGAT | 1021/658 (+161) | 44 | 0.0517 | JQ341961 | POPTR_0009s10430 | FUT11 |
| FP10550 | F: GTGGAGTCTGAGTCTGCTGAGAT | 473/325 (+1) | 24 | 0.0517 | JQ341963 | POPTR_0008s10550 | ALDH22a1 |
and the stranger fgs (subg. Urostigma (Gasp.) Miq. sect. Americana Miq.). Sect. Pharmacocysa is sister to all the other fgs subgenera, and therefore our sect. Americana and sect. Pharmacocysa samples share a most recent common ancestor that is the base of the entire Ficus crown clade, which, based on fossil records, dates back to at least 60 million years before present (Ronsted et al., 2005). All primers were tested on F. obtusifolia Kunth (sect. Pharmacocysa) and F. maxima Mill. (sect. Americana), which were collected from the Barro Colorado National Monument (BCNM) in central Panama. The subset of primers that amplified in both Ficus species were also tested on Poulsenia arnata (Miq.) Standl., which is a monotypic genus in the fig family Moraceae (Datwyler and Weiblen, 2004). Botanical vouchers (Dick and Gomes; 234, F. obtusifolia; Dick and Gomes; 240, F. maxima; and Dick and Gomes; 180, P. arnata) were deposited at the herbaria of the University of Panama (PMA) and University of Michigan, Ann Arbor (MICH). Genomic DNA was extracted with the cetyltrimethylammonium bromide (CTAB) method of Doyle and Doyle (1987).

**Bioinformatics pipeline**—Researchers from the United States Department of Agriculture (USDA) previously developed an EST library of F. elastica to characterize the genetic basis of rubber biosynthesis (McMahan and Whalen, personal communication). We compared 9289 unique F. elastica ESTs from the National Center for Biotechnology Information (NCBI) database with the annotated genomes of A. thaliana (Brassicaceae) and F. trichocarpa (Salicaceae) using the bioinformatics pipeline developed by Li et al. (2010). Briefly, we (1) retrieved coding sequences (CDS) that were longer than 100 bp from the annotated genomes of A. thaliana and F. trichocarpa. (2) We compared those CDS with the genome of the same species to identify “single-copy” CDS. (3) The candidate single-copy CDS thus identified were subsequently compared to the EST library of F. elastica to find markers that were conserved (identity >80%) among all three species. (4) After locating the single-copy conserved CDS, we screened for CDS flanking small introns, which were smaller than 1000 bp in the compared genomes, to facilitate the subsequent PCR and sequencing steps. Primers based on the F. elastica exons were initially designed by eye and subsequently checked with the Primer3 web program (Rozen and Skaltsky, 2000).

**Primer assays**—PCR was performed in a final volume of 20 μL containing 10 mM Tris–HCl (pH 8.4), 50 mM (NH₄)₂SO₄, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.1 μM each primer, 2 μg of genomic DNA, and 0.5 units of Taq polymerase (BioTherm, Gaithersburg, Maryland, USA). The amplification profiles included an initial denaturing at 94°C for 5 min; followed by 35 cycles of 50 s at 94°C, 50 s at 54°C, and 1 min at 72°C; and a final extension step of 10 min at 72°C. PCR products were ligated into the pmD18-T plasmid vector (Promega Corporation). Insert-positive plasmids were isolated using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, Norcross, Georgia, USA) and screened for CDS flanking small introns, which were smaller than 1000 bp in the compared genomes, to facilitate the subsequent PCR and sequencing steps. Primers based on the F. elastica exons were initially designed by eye and subsequently checked with the Primer3 web program (Rozen and Skaltsky, 2000).

**Data analyses**—DNA chromatograms were edited using the SEQUENCER program (Gene Codes Corporation, Ann Arbor, Michigan, USA). DNA sequences were initially aligned using ClustalX version 1.81 (Thompson et al., 1997) with default settings, and subsequently aligned manually using Se-Al (Rambaut, 1996). We determined number of polymorphic sites, nucleotide diversity (π), and GC content using MEGA5 software (Kumar et al., 2008).

**Results**—We identified 200 ESTs that satisfied our criterion of 80% exon identity with the published genomes. Based on intron length, we selected a subset of 80 ESTs for further marker development, of which 31 amplified successfully in Ficus species from both subgenera, 16 amplified in one species only, and 33 did not amplify in either species. The 31 cross-amplifying primer pairs were those that amplified in P. arnata of which 29 amplified successfully (Table 1). The number of polymorphic sites in F. obtusifolia and F. maxima comparisons ranged from 12 to 71 (mean = 32), whereas nucleotide diversity ranged from 0.02655 to 0.07305 (mean = 0.0470) (Table 1). In comparison, there were 45 variable sites in ITS between F. obtusifolia and F. maxima, falling within the range of the EPIC marker variation.

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**Table 1.** Primer information for EPIC markers for Ficus

| Locus | Primer sequences (5′–3′) | No. of polymorphic sites | GenBank accession no. | Reference genome | Nucleotide diversity | Gene abbreviation |
|-------|--------------------------|--------------------------|-----------------------|-----------------|---------------------|-------------------|
| FP11540b | F: GATTACAACAACCTCTGCCAGT; R: TCGTAAGGAGCACCAGCAAC | 28 | JQ341967 | POPTR_0017s11540 | 0.04328 | MZN14.21 |
| | | 107/80 (+4) | | | |
| FP11540b | F: GGCACATTTGCTTCCATTCT; R: TGGGGTCTGCTCCTCCAGT | 38 | JQ341971 | POPTR_0013s13070 | 0.04612 | uncharacterized |
| | | 844/748 (+2) | | | |
| FP17290 | F: GGCACATTTGCTTCCATTCT; R: TGGGGTCTGCTCCTCCAGT | 33 | JQ341973 | POPTR_0001s17290 | 0.04465 | F18B13_28 |
| | | 781/642 (+2) | | | |
| FP35460 | F: TCTCTGGTTGTTGCTGATTTTGG; R: TGGGGTCTGCTCCTCCAGT | 41 | JQ341975 | POPTR_0001s35460 | 0.05840 | unknown |
| | | 735/634 (+8) | | | |
CONCLUSIONS

The 31 EPIC markers that amplified between the two Ficus subgenera indicate that these markers might be useful across the full phylogenetic breadth of the >60 Ma genus and its >750 species. The markers that transfer to Poulsenia indicate an even broader phylogenetic utility within the Moraceae (ca. 40 genera and 1000 species), which probably originated in the Cretaceous. These markers should therefore be extremely useful for phylogenetic analysis at the family level and potentially beyond. The markers show a level of intron divergence that is of a similar magnitude as ITS, which is one of the most informative and broadly used markers in plant molecular systematics. These EPIC loci should be useful for analyzing recent divergences in which incomplete lineage sorting and/or introgression may be factors, including recent speciation, hybridization, and comparative phylogeography. In combination with EPIC markers developed for chalcid wasps (Lohse et al., 2011), it should now be possible to jointly analyze wasp and host plant phylogenies to study coevolution at both population and phylogenetic scales.

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