Exon-Primed Intron-Crossing (EPIC) Markers for Evolutionary Studies of Ficus and Other Taxa in the Fig Family (Moraceae)

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Source: Applications in Plant Sciences, 1(10)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1300037

Applications in Plant Sciences 2013 1(10): 1300037

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 tropical forests throughout the world. Fig trees are keystone species in many tropical forests, and their relationship with host-specific wasp pollinators has received much attention, although many questions remain unresolved regarding the levels of host specificity, cospeciation, and the role of hybridization in fig and wasp speciation. We developed exon-primed intron-crossing (EPIC) markers to obtain phylogenetic resolution needed to address these questions.
- *Methods and Results:* Expressed sequence tags (ESTs) from *F. elastica* were compared to *Arabidopsis* and *Populus* genomes to locate introns and to design primers in flanking exons. Primer pairs for 80 EPIC markers were tested in samples from divergent clades within *Ficus* and the outgroup *Poulsenia* (Moraceae).
- *Conclusions:* Thirty-one EPIC markers were successfully sequenced across *Ficus*, and 29 of the markers also amplified in *Poulsenia*, indicating broad transferability within Moraceae. All of the EPIC markers were polymorphic and showed levels of polymorphism similar to that of the widely used internal transcribed spacer (ITS).

Key words: exons; *Ficus*; Moraceae; nuclear DNA markers; phylogeny; transcriptome.

Ficus L. (Moraceae) is a pantropical genus comprised of ca. 750 species of trees, epiphytes, shrubs, vines, and stranglers found primarily 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., Rønsted 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*)

[1]Manuscript received 12 May 2013; revision accepted 17 June 2013.

Financial support was received from the National Science Foundation (DEB 0640379 to C.W.D.). The authors thank Allen Herre, Adalberto Gomez, and Katrin Heer for help in locating Panamanian *Ficus*, and the Smithsonian Tropical Research Institute and Autoridad Nacional del Ambiente (ANAM) for facilitating research and providing research permits for work in Panama.

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doi:10.3732/apps.1300037

TABLE 1. Characterization of 31 EPIC markers designed to amplify broadly across the genus *Ficus*.[a]

Locus[b]	Primer sequences (5′–3′)	Total/intron length (bp) (+range)	No. of polymorphic sites	Nucleotide diversity	GenBank accession no.	Reference locus[c]	Gene abbreviation[d]
FA08190	F: CCAAATTGTTGCGAGGATCT	484/435 (+2)	24	0.05053	JQ341915	AT1G08190	ATVAM2
	R: TTTAGAGCATCGGTCATGGA				JQ341916		
FA02580	F: CTAGATCTTGCACAGCAGCAG	487/381 (+2)	22	0.04593	JQ341917	AT4G02580	T10P11_14
	R: GCATGGTGTAGTGCCACAAA				JQ341918		
FA03310	F: GCGGGTATAAGAAGGGAACC	740/581 (+2)	43	0.05866	JQ341919	AT3G03310	LCAT3
	R: GGTGCATTGACCACCTTGAT				JQ341920		
FA07360a	F: GCTGATAAAATGGTTGCTGCTG	540/287	29	0.05410	JQ341921	AT2G07360	T13E11.13
	R: CCCCTTGATCTTCCCCATTACT				JQ341922		
FA08510	F: TGCTGGACTTCTTGGTGATG	893/741	51	0.05724	JQ341923	AT1G08510	FATB
	R: CAATCACGACGCATACCATTC				JQ341924		
FA11980	F: AGTTGGGCCATGCATCAGA	851/734 (+4)	35	0.04142	JQ341925	AT5G11980	F14F18_150
	R: ACCCAACGATGTGAATCCAA				JQ341926		
FA14000	F: TCCAGTGCTGATCATTTGAAAG	443/278 (+7)	23	0.05349	JQ341927	AT1G14000	F7A19_9
	R: GGCTGCCTCATAAGGCTCA				JQ341928		
FA16180b	F: CGGACTTATGGAACCAGAGTAATTC	417/281 (+3)	21	0.05147	JQ341929	AT4G16180	DL4130C
	R: GATGCTCCAGTACAATGACAACAT				JQ341930		
FA16690b	F: TCACAATTCTCCAGTGGTCATAAT	964/674 (+3)	40	0.04171	JQ341931	AT5G16690	ATORC3
	R: TTCTCAGAAAAACTGCAACCTT				JQ341932		
FA19690*	F: ACTTGGCCTTCTTACTTCATGG	386/258 (+2)	12	0.03158	JQ341933	AT5G19690	STT3A
	R: AGCAATCCCAGACATGATGC				JQ341934		
FA23640*	F: ATTCCTTTTGGTCCTCCACATC	1032/821 (+1)	55	0.05478	JQ341935	AT3G23640	HGL1
	R: ACCCCCAATCCAGGGAACTA				JQ341936		
FA24620a	F: CCTTACAAGGACAGCCTTTTG	513/323	20	0.04219	JQ341937	AT4G24620	PGI1
	R: CTCAAGCTCCCAATCATGG				JQ341938		
FA24620b	F: TGGCTAGATTTCCCATGTTTG	980/827 (+4)	50	0.05149	JQ341939	AT4G24620	PGI1
	R: AGCTGCTGCAGATACCGACT				JQ341940		
FA26990	F: GGAAGCGTACAGGGTGATGT	476/246	13	0.02760	JQ341941	AT2G26990	FUS12
	R: CATCAGAGCCATCTTCCTTTTG				JQ341942		
FA32180	F: TGCTCGAACTAAGGGAAGAATG	741/628 (+13)	38	0.05163	JQ341943	AT4G32180	ATPANK2
	R: GCTGCAAGAACACCTTCAATAA				JQ341944		
FA32910	F: GGTTGGAATTCTTGGAGAAAATAC	455/284 (+2)	12	0.02655	JQ341945	AT4G32910	F26P21_30
	R: GTGAAGCCAAAACTTGAGCATA				JQ341946		
FA36880b	F: GCTGTTGGGACATTGTTGAC	1044/896 (+6)	41	0.03958	JQ341947	AT5G36880	F5H8_15
	R: ATAACCGCTACACTCCCCTTC				JQ341948		
FA45300	F: GGAGGACTTGGTCTTGGTTACTT	890/684	41	0.04622	JQ341949	AT3G45300	ATIVD
	R: CCATTAGTGCACCACATCTTGT				JQ341950		
FA48520*	F: TCATCCATATTTGGTCGGAGAT	1059/890 (+4)	71	0.07305	JQ341951	AT5G48520	ATAUG3
	R: CCACCCATTGTCTTTCACTTG				JQ341952		
FA73180	F: CGGGACTTATCTTCAGACTTTTCA	470/235	18	0.03863	JQ341953	AT1G73180	T18K17_15
	R: GTGCCTTAGAAAGCTCAACTGC				JQ341954		
FP04090b	F: GGAATGCAAGCAATTGATGA	438/275 (+10)	15	0.03529	JQ341955	POPTR_0006s00800	CYP97B3
	R: AGGTCCAGCAACCTCAGCTA				JQ341956		
FP08470	F: GCGATGTGCTGCGTGTATTT	550/404 (+7)	25	0.04630	JQ341957	POPTR_0017s08470	BGAL9
	R: GGTCCATAAAGACTTGGAGAGG				JQ341958		
FP08550	F: CCGCTATCCTTTGGCTGTTA	741/451 (+5)	36	0.05233	JQ341959	POPTR_0006s08550	F6E21_100
	R: CACATGCTTCTGCACGTTCT				JQ341960		
FP09670	F: GCAGCAACGTGGTGATAAGA	642/509	32	0.05016	JQ341961	POPTR_0001s09670	XPB1
	R: ATCACATTAGCCTCGGGAATATC				JQ341962		
FP10430	F: GTGGGATGTCAGTTTGGATTT	1021/658 (+161)	44	0.05176	JQ341963	POPTR_0009s10430	FUT11
	R: CAGCCCAGGAAAAGTATCCA				JQ341964		
FP10550	F: GGTGAAGGTGCAGTTGATCAGT	473/325 (+1)	24	0.05172	JQ341965	POPTR_0008s10550	ALDH22a1
	R: GCTTGACAGCCTCTTCATCAGT				JQ341966		

TABLE 1. Continued.

Locus[b]	Primer sequences (5′–3′)	Total/intron length (bp) (+range)	No. of polymorphic sites	Nucleotide diversity	GenBank accession no.	Reference locus[c]	Gene abbreviation[d]
FP11540b	F: GATTACAACAACCTCTGCCAGT	661/496 (+4)	28	0.04328	JQ341967	POPTR_0017s11540	MZN14.21
	R: AGCATGTGCTTGTACTCATCAACAAC				JQ341968		
FP12610a	F: GGATGCACTGGTTATGGTCA	362/238	14	0.03889	JQ341969	POPTR_0011s12610	uncharacterized
	R: TCGTAAGGAGCACCAGCAAC				JQ341970		
FP13070	F: GGCACATTTGCTTCCATTCT	844/748 (+2)	38	0.04612	JQ341971	POPTR_0013s13070	uncharacterized
	R: TAATGCATGATTCCTGTTCCAA				JQ341972		
FP17290	F: CTCACATGCCTCACTCATGC	781/642 (+2)	33	0.04465	JQ341973	POPTR_0001s17290	F18B13_28
	R: GTCTCCACACGGTCCTTTCT				JQ341974		
FP35460	F: TCTCTGGTTGTTGCTGATTTTGG	735/634 (+8)	41	0.05840	JQ341975	POPTR_0001s35460	unknown
	R: TGGGGTCTGCTCCTCCAGT				JQ341976		

[a]The locus descriptions (total and intron length, polymorphism) represent comparisons between *Ficus obtusifolia* (sect. *Americana*) and *F. maxima* (sect. *Pharmacocysea*).

[b]The first two letters of the marker name indicate the genomic comparisons (e.g., FA = *Ficus*/*Arabidopsis*; FP = *Ficus*/*Populus*) followed by the numerical locus identifier of the reference genome.

[c]Full reference genome locus name.

[d]Abbreviation for the putative gene function.

* Denotes markers that were not transferable to the *Poulsenia armata* (Moraceae) outgroup.

and the strangler figs (subg. *Urostigma* (Gasp.) Miq. sect. *Americana* Miq.). Sect. *Pharmacocysea* is sister to all the other fig subgenera, and therefore our sect. *Americana* and sect. *Pharmacocysea* 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 (Rønsted et al., 2005). All primers were tested on *F. obtusifolia* Kunth (sect. *Pharmacocysea*) 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 armata* (Miq.) Standl., which is a monotypic genus in the fig family Moraceae (Datwyler and Weiblen, 2004). Botanical vouchers (*Dick and Gomez 234*, *F. obtusifolia*; *Dick and Gomez 240*, *F. maxima*; and *Dick and Gomez 180*, *P. armata*) 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* (Brassicaeae) and *P. trichocarpa* (Salicaceae) using the informatics 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 *P. 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 interface program (Rozen and Skaletsky, 2000).

***Primer assays*—**PCR was performed in a final volume of 20 μL containing 10 mM Tris–HCl (pH 8.4), 50 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.1 μM each primer, 2 ng 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 pMD 18-T plasmid vector (Promega Corporation, Madison, Wisconsin, USA) and transformed into *E. coli* strain (DH5α, Promega Corporation). Insert-positive plasmids were isolated using the E.Z.N.A. Plasmid Mini Kit I (Omega Bio-Tek, Norcross, Georgia, USA) and amplified using M13 primers. Forward and reverse strands of each amplicon were sequenced on an ABI 3730xL DNA sequencer (Applied Biosystems, Carlsbad, California, USA) at the University of Michigan Sequencing Core Facility. All *Ficus* insert sequences have been deposited in GenBank (accession numbers JQ341915–JQ341980; also see Table 1). For comparisons with ITS, we also obtained ITS sequences for *F. obtusifolia*, *F. maxima*, and *P. armata* (GenBank accessions JX137113–JX137114) using standard methods.

***Data analyses*—**DNA chromatograms were edited using the SEQUENCHER 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 MEGA 5 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 further tested in *P. armata*, 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.

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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