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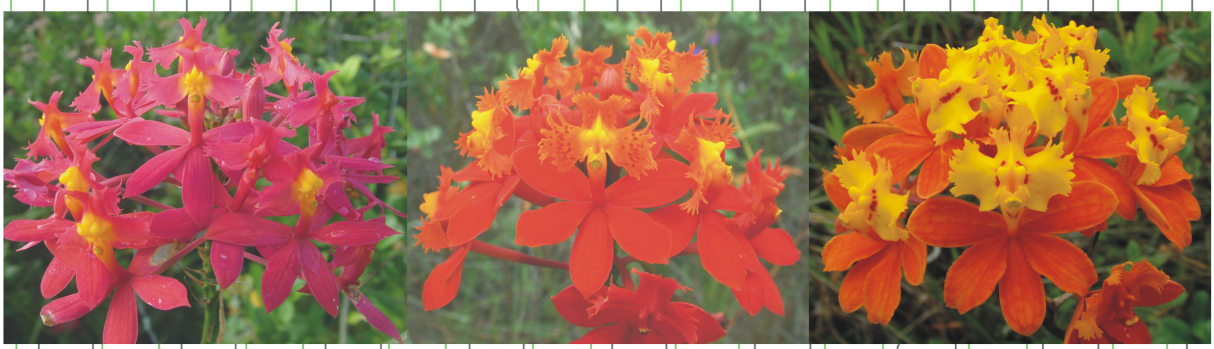

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À minha família,
com todo meu amor,
respeito e admiração

“How many generations are necessary for one species or race to absorb another by repeated crosses has often been discussed; and the requisite number has probably been much exaggerated … with plants, one species could be made to absorb another in from three to five generations…”

“With respect to the influence of the conditions of life on any two breeds which are allowed to cross freely…they will, in all probability, be unequally affected by the conditions, and this will modify the result.”

“There can be no doubt that crossing, with the aid of rigorous selection during several generations, has been a potent means in modifying old races, and in forming new ones.”

“…nature opposes no barrier to successful admixture; in the course of time, by the aid of selection and careful weeding, it is practicable to establish a new breed.”

“The crossing of distinct forms, which have already become variable, increases in the offspring the tendency to further variability, by the unequal commingling of the characters of the two parents, by the reappearance of long-lost characters, and by the appearance of absolutely new characters.”

“If we assume that each particular variation was from the beginning of all time preordained, then that plasticity of organisation, which leads to many injurious deviations of structure, as well as the redundant power of reproduction which inevitably leads to a struggle for existence, and, as a consequence, to the natural selection or survival of the fittest, must appear to us superfluous laws of nature. On the other hand, an omnipotent and omniscient Creator ordains everything and foresees everything. Thus we are brought face to face with a difficulty as insoluble as is that of free will and predestination.”

C.R. Darwin
The variation of animals and plants under domestication.
Volume 2, segunda edição.
John Murray, London. 1875.

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Índice

Introdução Geral

A importância de marcadores moleculares na caracterização dos processos evolutivos em populações naturais de orquídeas

O emprego de marcadores moleculares em estudos de evolução na família Orchidaceae

Com cerca de 24.000 espécies, Orchidaceae é a maior família dentre as Angiospermas (WORLD Checklist of the Monocotyledons, 2006). A diversidade da família é proporcional ao número de estratégias evolutivas que podem ser encontradas em diferentes espécies de orquídeas. Uma extensa variação morfológica, diferentes níveis de especialização ao hábitat e síndromes de polinização variadas mostram como a evolução de orquídeas pode ser investigada num contexto multidisciplinar.

O potencial que a família Orchidaceae oferece para a pesquisa e compreensão dos processos evolutivos (diversificação de linhagens, co-evolução, convergência evolutiva, especiação) foi primeiramente reconhecido por Darwin, que dedicou um livro inteiro ao assunto (DARWIN, 1877). Considerando a importância do livro de Darwin, orquídeas deveriam ter sido o sistema biológico preferido dos cientistas para a pesquisa em evolução (PEAKALL, 2007). Porém, orquídeas não receberam a atenção que se esperava da comunidade científica, e muitas lacunas de conhecimento ainda existem (DRESSLER, 1981), especialmente nas regiões tropicais. Para FAY & CHASE (2009), a falta de interesse da comunidade científica sobre orquídeas se deve ao extenso tamanho da família, que dificulta o estabelecimento de relações de parentesco em diversos níveis taxonômicos (subfamílias, subtribos, subgêneros). Este fato inibe o teste de hipóteses evolutivas, uma vez que existe controvérsia na escolha e delimitação dos táxons que serão utilizados no estudo. Porém, famílias botânicas que possuem uma diversidade comparável a Orchidaceae, como Asteraceae e Poaceae, foram intensamente abordadas em estudos de evolução ao longo da história, e possuem um número comparativamente maior de estudos sobre especiação, evolução e mecanismos de isolamento reprodutivo em plantas (PEAKALL, 2007).

Segundo PEAKALL (2007), orquídeas não possuem espécies de interesse agrícola, que sejam intensamente estudadas sob diversos aspectos biológicos, e que possam servir de modelo, para estudos similares com espécies selvagens. Este fato

pode ser constatado pela deficiência de marcadores moleculares específicos desenvolvidos para espécies de orquídeas ao longo dos anos (PEAKALL, 2007), se comparado ao número de marcadores desenvolvidos para famílias que possuem representantes com interesse agrícola. Este fato dificulta o progresso do conhecimento sobre processos evolutivos que ocorrem numa escala populacional, uma vez que para este tipo de trabalho, são necessários marcadores moleculares com um nível de especificidade maior do que aqueles empregados em estudos de filogenias, em níveis taxonômicos superiores.

Em espécies modelo, que possuem interesse comercial, há grande abundância de marcadores moleculares para estudos populacionais, fato que não acontece com Orchidaceae (PEAKALL, 2007). A carência de marcadores moleculares específicos para orquídeas é ainda mais notável na região Neotropical, que contraditoriamente, concentra a maior diversidade de espécies de orquídeas. Se se considerar, por exemplo, o número de trabalhos que isolaram e caracterizaram *loci* de microssatélites nucleares para Orchidaceae, apenas três, dos 15 publicados até o momento, foram realizados com espécies neotropicais (PINHEIRO, 2009). Claramente, um esforço maior deve ser empregado no sentido de tentar diminuir esta diferença, uma vez que as técnicas que permitem o desenvolvimento de marcadores moleculares específicos para espécies de interesse têm se tornado mais acessíveis nos últimos anos. Trabalhos que medem o potencial de amplificação cruzada destes marcadores também são importantes porque ampliam a utilização de marcadores previamente caracterizados (BÁRBARA *et al.*, 2007; PINHEIRO *et al.*, 2009).

Mesmo estando em desvantagem, se comparada a outras famílias, marcadores moleculares foram fundamentais para a expansão dos estudos evolutivos em Orchidaceae, principalmente quando integrados a outras áreas do conhecimento como ecologia, morfologia, fisiologia e sistemática (AVISE, 2004; PEAKALL, 2007). A existência de hipóteses filogenéticas baseadas em polimorfismos de DNA foi fundamental para que grupos de táxons fossem reconhecidos num contexto evolutivo (ACETO *et al.*, 1999). Tais filogenias foram utilizadas em estudos subseqüentes para testar diversos tipos de hipóteses de diversificação de linhagens e tendências evolutivas, principalmente em gêneros de Orchidaceae provenientes de regiões temperadas. O reconhecimento de processos demográficos históricos de expansão e retração populacional foi fundamental, por exemplo, para o entendimento dos padrões de diversificação dos gêneros *Dactylorhiza* (PILLON *et al.*, 2007) e *Anacamptis*

(COZZOLINO *et al.*, 2003). Eventos de hibridação também começaram a ser investigados e descritos para diversos grupos de orquídeas, revelando que ser, este, um processo bastante comum e difundido em diversos grupos, principalmente em orquídeas polinizadas por engodo (COZZOLINO & WIDMER, 2005).

Trabalhos que formularam hipóteses filogenéticas para grupos de orquídeas neotropicais foram fundamentais para que hipóteses evolutivas de diversificação de linhagens e especiação fossem testadas (KOEHLER *et al.*, 2008; CHASE *et al.*, 2009; VAN DEN BERG *et al.*, 2009; PINHEIRO *et al.*, no prelo). Porém, enquanto o número de filogenias para orquídeas neotropicais aumenta a cada ano, poucos são os trabalhos que utilizam filogenias pré-existentes como fontes de hipóteses a serem testadas. Deste modo, são raros os trabalhos que empregam marcadores moleculares num contexto multidisciplinar, com o objetivo de esclarecer processos evolutivos que ocorrem numa escala populacional. Como exemplo, pode-se destacar a série de estudos realizados com o gênero *Acianthera*, no qual marcadores moleculares (isoenzimas) foram integrados a dados ecológicos e morfológicos, num contexto multidisciplinar. Os resultados indicaram que a manutenção das barreiras reprodutivas se baseia na existência de polinizadores distintos e específicos para cada espécie de *Acianthera* (BORBA & SEMIR, 2001). A manutenção de elevados níveis de diversidade genética nas populações analisadas (BORBA *et al.*, 2001b) parecem estar correlacionados com a existência de auto-incompatibilidade e depressão por endocruzamento detectada em diversas populações (BORBA *et al.*, 2001a). Além disso, as semelhanças morfológicas entre as espécies não são devidas a similaridades genéticas, mas sim à existência de convergência floral entre espécies que possuem os mesmos polinizadores (BORBA *et al.*, 2002).

A popularização e a expansão do uso de marcadores nucleares e plastidiais em orquídeas, numa escala populacional, é um passo importante rumo à melhor compreensão dos processos que promovem e mantém o grande número de espécies em Orchidaceae, principalmente em regiões com elevada biodiversidade, como a região Neotropical.

Hibridação em populações naturais de plantas

O estudo de barreiras reprodutivas em grupos de espécies é fundamental para o entendimento dos processos que deram origem às espécies atuais, uma vez que a intensidade de fluxo gênico pode determinar o grau em que populações, linhagens

e/ou espécies se mantêm como unidades coesivas (COYNE & ORR, 2004). Neste contexto, a investigação de zonas de hibridação permite a avaliação da intensidade das barreiras reprodutivas entre espécies, revelando o quanto indivíduos híbridos facilitam, ou não, o fluxo gênico entre os táxons parentais (ARNOLD, 2006). Zonas de hibridação permitem que o sucesso reprodutivo do genótipo híbrido adulto seja avaliado sob condições naturais, o que é muito difícil de conseguir para espécies perenes com geração longa (RIESEBERG & BUERKLE, 2002; LEXER *et al*., 2003, 2005), como ocorre com muitas orquídeas por exemplo. O processo de hibridação, além de promover fluxo gênico entre espécies previamente isoladas, pode dar origem a novas linhagens e espécies (RIESEBERG, 1997), e pode ser responsável pela radiação adaptativa de diferentes grupos de organismos (SEEHAUSEN, 2004).

Para diversos grupos de plantas, o processo de hibridação possui uma grande conexão com hábitats perturbados ou compostos por mosaicos de vegetação que oferecem diferentes condições ecológicas (ARNOLD, 2006). O fato de indivíduos híbridos combinarem elementos genômicos de espécies distintas pode aumentar a amplitude ecológica e a diversidade de hábitats que estes indivíduos são capazes de aproveitar (JOHNSTON *et al.*, 2001; SEEHAUSEN, 2004; LEXER *et al.*, 2005). Além disso, eventos de hibridação podem originar indivíduos híbridos capazes de ocupar hábitats distintos daqueles ocupados pelas espécies parentais, dando origem a novas espécies (RIESEBERG, 1997; RIESEBERG *et al.*, 2003; ARNOLD, 2006).

As causas e as conseqüências da hibridação natural interespecífica, sob uma perspectiva conservacionista, sugerem que a perda de heterogeneidade de ambientes, devida à ação antrópica, poderia causar a perda da biodiversidade através do aumento do contato entre espécies previamente isoladas, promovendo sua hibridação e introgressão. Este fenômeno foi chamado de “especiação reversa”, e se baseia no princípio da perda de biodiversidade causada pela introgressão entre espécies previamente isoladas, as quais ocorriam em hábitats que se encontram em processo de fragmentação e deterioração (SEEHAUSEN *et al.*, 2008). A probabilidade do contato secundário entre populações ou espécies previamente isoladas tem sido alterada constantemente ao longo dos milhares de anos de evolução das espécies, mas recentemente tem aumentado de forma drástica devido ao crescente impacto do homem sobre a natureza.

A hibridação natural na família Orchidaceae é intensamente estudada em grupos de espécies européias, principalmente na região do Mediterrâneo, onde as

zonas híbridas descritas apresentam grande variação na arquitetura genética (COZZOLINO & WIDMER, 2005; COZZOLINO *et al.*, 2006; MOCCIA *et al.*, 2007). No gênero *Ophrys*, no qual se pensava que as barreiras pré-zigóticas eram bastante eficientes devido à alta fidelidade entre o polinizador e a espécie de orquídea polinizada, foram revelados diversos casos de hibridação e introgressão (SOLIVA & WIDMER, 2003; CORTIS *et al.*, 2009). Além disso, existem evidências de que eventos de hibridação em *Ophrys* podem gerar indivíduos que possuem flores capazes de produzir aromas distintos daqueles observados nas espécies parentais (CORTIS *et al.*, 2009), caracterizando um possível evento de especiação. Em outros gêneros, como *Orchis* e *Anacamptis*, a existência de hibridação também é comum, mas é restrita à formação de gerações F1, e a ausência de introgressão é decorrente de intensas barreiras pós-zigóticas (SCOPECE *et al.*, 2007), relacionadas principalmente a diferenças cariotípicas entre as espécies parentais (COZZOLINO *et al.*, 2004).

Na região Neotropical, eventos de hibridação em orquídeas são raramente estudados. Como exceções podem ser citados os trabalhos realizados com *Bulbophyllum* (BORBA & SEMIR, 1998; AZEVEDO *et al.*, 2006). Considerando que existe uma grande quantidade de híbridos artificiais produzidos para fins comerciais, utilizando espécies neotropicais, o potencial de descoberta e pesquisa de zonas de hibridação em populações naturais é bastante elevado. A disponibilidade de marcadores moleculares capazes de produzir polimorfismos suficientes para detectar e caracterizar a estrutura genética de zonas de hibridação é importante para propiciar maior número de estudos com hibridação em populações naturais. A integração de marcadores moleculares com experimentos de biologia reprodutiva permite avaliar o sucesso reprodutivo das espécies parentais e híbridos, e indica os mecanismos envolvidos na manutenção das barreiras reprodutivas e no surgimento de novas linhagens e/ou espécies (MOCCIA *et al.*, 2007; CORTIS *et al.*, 2009). A interface com outras áreas do conhecimento como morfologia (COZZOLINO *et al.*, 2006), citogenética (COZZOLINO *et al.*, 2004), e ecologia (CRUZAN & ARNOLD, 1993; JOHNSTON *et al.*, 2001) também são fundamentais, uma vez que o processo de hibridação é um fenômeno complexo, e sua pesquisa deve se inserir num contexto multidisciplinar (RIESEBERG & CARNEY, 1998).

Caracterização do sistema biológico de interesse – o gênero *Epidendrum*

Epidendrum L. é o maior gênero da família Orchidaceae na região Neotropical com cerca de 1.500 espécies, e sua distribuição geográfica vai desde o Sul da Flórida até o Norte da Argentina (HÁGSATER & SOTO-ARENAS, 2005). Como características principais do gênero podem ser ressaltadas a ampla variação morfológica e cromossômica, que dificulta a circunscrição do gênero e de diversos grupos de espécies, mais conhecidos como "complexos" (HÁGSATER & SOTO-ARENAS, 2005; PINHEIRO *et al*., no prelo). Hipóteses filogenéticas publicadas por VAN DEN BERG *et al*. (2000) e HÁGSATER & SOTO-ARENAS (2005) indicam que o gênero é monofilético e que os agrupamentos internos correspondem, em grande parte, a grupos previamente reconhecidos com base em caracteres morfológicos descritos em tratamentos taxonômicos históricos (LINDLEY, 1852-1859; BRIEGER, 1976-1977).

O subgênero *Amphiglottium*, descrito primeiramente por LINDLEY (1852-1859), reúne espécies que exibem extensa variação morfológica e cromossômica, e que possuem barreiras reprodutivas fracas, com diversos casos de hibridação descritos na literatura (DUNSTERVILLE, 1979; DRESSLER, 1989; HÁGSATER & SOTO-ARENAS, 2005; PANSARIN & AMARAL, 2008; PINHEIRO *et al*., no prelo). As espécies do grupo também compartilham características ecológicas, como a habilidade de ocupar hábitats em que elementos abióticos apresentam situação extrema, como dunas arenosas, afloramentos rochosos e terrenos alagados (HÁGSATER & SOTO-ARENAS, 2005). Ambientes originados por ação antrópica, como margens de rodovias e encostas desmatadas, também são freqüentemente ocupados por espécies do subgênero *Amphyglottium*, originando populações com características morfológicas distintas daquelas que ocorrem em áreas preservadas (PINHEIRO & BARROS, 2008) e zonas de hibridação entre espécies previamente isoladas (DUNSTERVILLE, 1979; HÁGSATER & SOTO-ARENAS, 2005).

Recentemente PINHEIRO *et al*. (no prelo) formularam uma hipótese filogenética para o subgênero *Amphyglottium* empregando marcadores moleculares nucleares (AFLP) e de cloroplasto (espaçador intergênico *trn*L-*trn*F). Com base nos resultados obtidos, grupos de espécies reconhecidos na filogenia puderam ser associados à presença de características comuns aos táxons que compõem os respectivos grupos, como caracteres morfológicos (subseção *Tuberculata*, incluindo *E. secundum*, *E. xanthinum* e *E. cochlidium*) e padrões biogeográficos, com espécies distribuídas ao longo da Cordilheira dos Andes e Planalto das Guianas (clado andino,

incluindo *E. calanthum*, *E. ibaguense* e *E. incisum*) e espécies distribuídas ao longo do litoral brasileiro, com ocorrência de populações disjuntas em áreas sob o domínio de Cerrado e Caatinga (clado Atlântico - *E. cinnabarinum*, *E. denticulatum*, *E. fulgens* e *E. puniceoluteum*).

As espécies que compõem o clado Atlântico possuem grande variação morfológica, que torna muitas vezes incerta a delimitação das espécies (PINHEIRO & BARROS, 2006). Além disso, populações podem ser encontradas ao longo de toda a distribuição costeira da Mata Atlântica, desde o Norte do Rio Grande do Sul até o Norte do Rio Grande do Norte, bem como em áreas inseridas em outros biomas, com populações disjuntas em Cerrado e inselbergues de Caatinga. Nas áreas de ocorrência simpátrica já foram relatados casos de hibridação (PINHEIRO & BARROS, 2006; PANSARIM & AMARAL, 2008; PINHEIRO, 2009), mas como este processo afeta a arquitetura genética das espécies ainda é uma questão a ser investigada.

De especial interesse são as populações simpátricas de *E. fulgens* e *E. puniceoluteum*, que se estendem desde o litoral sul de São Paulo até o sul de Santa Catarina. Nestas populações é possível observar diversos indivíduos com características morfológicas intermediárias entre *E. fulgens* e *E. puniceoluteum*, fato que dificulta a identificação de ambas as espécies e abre oportunidades para que a ocorrência de hibridação entre os táxons seja investigada (PINHEIRO & BARROS, 2006).

Os principais objetivos deste trabalho foram:

a) Isolar e caracterizar marcadores nucleares e plastidiais para *Epidendrum fulgens* e *E. puniceoluteum*;
b) Testar a taxa de transferência e utilidade destes marcadores em outras espécies de *Epidendrum* e em outros gêneros de Laeliinae;
c) Utilizar marcadores nucleares e plastidiais para investigar a ocorrência de hibridação entre *E. fulgens* e *E. puniceoluteum*.

Neste contexto, a composição genética das zonas híbridas, o papel de barreiras reprodutivas pré e pós-zigóticas no isolamento reprodutivo das espécies parentais, o potencial de indivíduos híbridos promoverem fluxo gênico entre as espécies parentais, e a existência de indivíduos híbridos que caracterizem novas linhagens e/ou espécies foram discutidos. Os resultados obtidos com marcadores moleculares também foram discutidos em um contexto ecológico, considerando o papel dos diferentes hábitats que as espécies parentais ocupam na manutenção do isolamento reprodutivo.

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Capítulo 1

Isolation and characterization of microsatellite loci in the Brazilian orchid *Epidendrum fulgens*

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TECHNICAL NOTE

Isolation and characterization of microsatellite loci in the Brazilian orchid *Epidendrum fulgens*

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Abstract *Epidendrum fulgens* has a patchy distribution along the Atlantic Rainforest in the Brazilian coast, due to the destruction of its native habitat. Here, we report on both the development of nine new microsatellite markers isolated from this species and the characterization of their allele variability in two distant and unrelated populations. The number of alleles observed for each locus ranged from 2 to 17 with an average of 6.4 alleles per locus. These microsatellites should be valuable tools for studying the effect of habitat fragmentation on the genetic structure of *E. fulgens* populations.

Keywords *Epidendrum* · Orchidaceae · Microsatellites · Atlantic Rainforest · Cross-amplification

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Epidendrum fulgens is an endemic orchid of coastal Brazilian Atlantic Rainforest. In the past, this orchid had a wide geographic distribution between the Brazilian States of Rio de Janeiro and Rio Grande do Sul but, nowadays, its populations are small and fragmented due to habitat loss. The knowledge of patterns of genetic diversity and gene flow is essential to guide conservation management decisions and for understanding the genetic consequences of habitat loss in fragmented populations. Therefore, the aim of this study was to develop a set of polymorphic microsatellite (simple sequence repeat—SSR) markers for *E. fulgens* for describing the population genetic structure of this threatened species.

Total DNA was extracted from silica gel exsiccated leaves of *E. fulgens* following the protocol of Doyle and Doyle (1990). Marker isolation involved the construction of a genomic library partially enriched for $(CT)_n$ and $(GT)_n$ repeats by using biotinylated oligonucleotide sequences bound to Streptavidin-coated magnetic particles as described by Kijas et al. (1994) with modifications by Billote et al. (1999). Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy vector (Promega) as described by supplier and used to transform XL1Blue competent *E. coli* cells (Stratagene). A total of 96 recombinant colonies were obtained and sequenced using the BigDye v3.1 terminator kit on the ABI PRISM 3130 Sequence Analyser (Applied Biosystems). For 25 clones, containing SSR motifs, forward and reverse sequences were aligned in SeqMan (DNASTAR package), and primers were designed for 16 loci using the PRIMER3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

For each SSR, the forward primers were synthesized with a 19 bp long M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the amplification method of Schuelke (2000), which involved three primers: a forward

Table 1 Characteristics of microsatellite loci from *Epidendrum fulgens*, including locus name, primer sequences, repeat type (interrupted microsatellites are indicated by a (...) between repeats), no. of alleles (A), allele size range, observed (H_O) and expected (H_E) heterozygosity for each population, and test for departure from Hardy–Weinberg equilibrium (HWE)

Locus	Primer sequences[a]	Repeat	A	Size range (bp)	Guaratuba		Imbituba	
					H_O	H_E	H_O	H_E
EFF06	F: TCAAGCCTATCATAAGTGCTCCA	$(CA)_8$	4	364–370	0.500	0.567	0.500	0.633
	R: CCTTGTTGCAACTGGGTGTT							
EFF26	F: TGTCCTAAGTCAAGTGGGGTTT	$(GT)_{15}$	4	199–205	0.400	0.343	0.550	0.680
	R: TCCGAGTCTGTCGGTCTTTT							
EFF29	F: TCCGCTGATTTGAGTTTGCT	$(TC)_{33}$	17	185–229	0.900	0.910	0.700	0.915*
	R: CTGGTCCCGTAAGATCAATCAC							
EFF43	F: TGCCCCACAGACAATTAAGC	$(GA)_9$	6	148–160	0.100	0.097	0.700	0.584
	R: CCTCGATGGAACCCCATAAT							
EFF45	F: TTGGGTTTCGTCTCACATCA	$(CT)_{11}...(CT)_4$	4	288–294	0.450	0.514	0.550	0.670
	R: CCCTCAGTATCCGCCACTT							
EFF51	F: CTTGTCTACGTGAGGGCACTG	$(GT)_8$	5	369–377	0.700	0.744*	0.600	0.573
	R: TCAACAACGTGAAAAGCCATC							
EFF58	F: TGAATGCTTATACTCTCCCATCA	$(CA)_7$	2	210–212	0.200	0.184	0.200	0.430*
	R: AAGTGGCAAAGCACCATGTA							
EFF61	F: TGTCCCCTATATTCTGATGGTG	$(CA)_9$	2	264–266	0.050	0.050	0.350	0.357
	R: AGGGTTTTAGGTCAAAGTGCTC							
EFF70	F: CGCGAGATTGTTCCAAACC	$(AG)_{30}$	14	321–349	0.750	0.866*	0.700	0.896*
	R: GCTCCACGCAAAACCTTTTTA							

Genbank Accession nos. EU363791–EU363799

[a] All forward primers were M13-tailed at the 5′ end. Significant departures from HWE: $*P < 0.001$

Table 2 Cross-species and genera amplification of nine microsatellite primers from *Epidendrum fulgens* within the subtribe Laeliinae

Species	EFF6	EFF26	EFF29	EFF43	EFF45	EFF51	EFF58	EFF61	EFF70
Epidendrum xanthinum	−	+	W	+	+	−	+	W	+
Epidendrum secundum	−	+	W	+	+	+	+	+	++
Pseudolaelia cipoensis	−	+	W	+	−	−	W	W	−
Cattleya eldorado	−	−	W	+	+	−	W	+	−
Prosthechea vespa	−	+	W	+	−	−	W	+	−

Successful amplifications with a single band visualized (+), successful amplifications with more than one band visualized (++), weak amplifications (W) and failed amplifications (−) are indicated

SSR-specific primer with the M13 tail at its 5′ end, a reverse locus-specific primer, and a universal M13 primer labelled with a fluorescent dyes, 6-FAM or JOE (Applied Biosystems) respectively. All PCR amplifications were performed in a Applied Biosystems 2700 thermocycler in 10 µl reactions containing: 10 ng DNA template, 1× PCR buffer, 2 mM $MgCl_2$, 100 µM dNTPs, 1 pmol forward primer, 4 pmol reverse primer, 0.4 pmol universal M13 primer and 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech). A 'touchdown' cycling program was used: 95°C for 3 min, then 10 cycles of 94°C for 30 s, 58°C decreasing to 48°C at 1°C per cycle for 30 s, 72°C for 30 s followed by 40 cycles of 94°C for 30 s, 48°C for 30 s, 72°C for 30 s, followed by a final extension of 20 min at 72°C. Microsatellite alleles were resolved on a 3130 DNA Sequence Analyser and were sized with LIZ (500) standard by using GENEMAPPER v3.7 software (Applied Biosystems).

A total of 40 individuals from two Brazilian populations of *E. fulgens* (Imbituba and Guaratuba) were analyzed to evaluate SSR polymorphism. ARLEQUIN 3.11 (Excoffier et al. 2005) software was used to calculate observed (H_O) and expected (H_E) heterozygosity, to test for departure from Hardy–Weinberg equilibrium (HWE) and for linkage disequilibrium between all pairs of loci. MICRO-CHECKER (Van Oosterhout et al. 2004) software was used to quantify genotyping errors. Nine SSRs were polymorphic, with number of alleles per locus ranging from 2 to 17 with an average of 6.4 alleles per locus. The observed and expected heterozygosities (H_O and H_E) ranged from 0.10 to 0.90 and 0.05 to 0.91, with averages of 0.494 and 0.556, respectively (Table 1). Four loci showed a significant departure from Hardy–Weinberg equilibrium ($P < 0.001$), three due to heterozygote deficiency (EFF29, EFF58 and EFF70) in Imbituba population, and one due to heterozygote excess (EFF51) in the Guaratuba population. The small size of the Imbituba population and fragmentation history of the region may be the factors promoting the local observed low levels of heterozygosity. No linkage disequilibrium was detected among any pair of loci, and no genotyping errors due to presence of null alleles, short allele dominance or scoring of stutter peaks was detected.

Cross-species and cross-genera amplification were performed on *Epidendrum* species and allied genera, on single individuals by using the same amplification conditions used for *E. fulgens*. Several positive amplifications occurred across all tested species (Table 2).

The primers proved to be useful in revealing levels of diversity in *E. fulgens* and thus can be used to explore the genetic structure of scattered populations across its actual geographical range.

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Capítulo 2

Isolation and characterization of microsatellite loci in *Epidendrum puniceoluteum*, an endemic orchid from the Atlantic Rainforest

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PERMANENT GENETIC RESOURCES

Isolation and characterization of microsatellite loci in *Epidendrum puniceoluteum*, an endemic orchid from the Atlantic Rainforest

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Abstract

***Epidendrum puniceoluteum* is an endemic orchid of Atlantic Rainforest, restricted to few populations only due to the destruction and fragmentation of its native habitat. Here, we report on the development of 10 microsatellite markers isolated from this orchid species. Genetic variability was characterized in two distant populations from Brazil coast. The number of alleles observed for each locus ranged from two to 12 and with an average of 6.4 alleles per locus. These microsatellites should be valuable tools for studying both fine-scale genetic structure of scattered *E. puniceoluteum* population and patterns will be useful genetic markers for other closely related taxa.**

Keywords: Atlantic Rainforest, cross-amplification, *Epidendrum*, microsatellites, Orchidaceae

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The fragmentation of the Atlantic Rainforest, a worldwide biodiversity hotspot, represents a dramatic process with a strong impact on genetic diversity of local plant community (Cardoso *et al*. 2005). Knowledge about demographic events that shape the genetic patterns in fragmented populations can help conservation efforts to preserve threatened species. Among the several Neotropic orchids of the Atlantic Rainforest, *Epidendrum* is the largest genus showing great morphological diversity that has recently generated many taxonomic doubts about its generic classification and species delimitation (Hágsater 1984). *Epidendrum puniceoluteum* is a recently described orchid species (Pinheiro & Barros 2006) that had a wide distribution in the past, from São Paulo and Rio Grande do Sul States, Brazil, but is now restricted to few populations only due to the destruction of its native habitat. Here, we report on the development of a set of polymorphic microsatellite [simple sequence repeat (SSR)] markers for *E. puniceoluteum* that will be useful in addressing questions on the genetic structure of this endangered species.

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Total genomic DNA was extracted from silica gel-exsiccated leaves of *E. puniceoluteum* following the protocol of Doyle & Doyle (1990). Markers isolation involved the construction of a genomic library partially enriched for $(CT)_n$ and $(GT)_n$ repeats by using biotinylated oligonucleotide sequences bound to streptavidin-coated magnetic particles as described by Kijas *et al*. (1994) with modifications by Billote *et al*. (1999). Microsatellite-enriched DNA fragments were ligated into pGEM-T Easy vector (Promega) as described by the supplier and used to transform XL1-Blue competent *Escherichia coli* cells (Stratagene). A total of 96 recombinant colonies were obtained and sequenced using the BigDye version 3.1 terminator kit on the ABI PRISM 3130 Sequence Analyser (Applied Biosystems). For 21 clones, containing SSR motifs, forward and reverse sequences were aligned in SEQMAN (DNASTAR package), and primers were designed for 14 loci using the PRIMER 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

Table 1 Characteristics of microsatellite loci from *Epidendrum puniceoluteum*, including locus name, primer sequences, repeat type (interrupted microsatellites are indicated by a (...) between repeats), no. of alleles (A), allele size range, observed (H_O) and expected (H_E) heterozygosity for each population, and the significance of the test for departure from Hardy–Weinberg equilibrium (HWE). Locus EPP49 was monomorphic for Imbituba population. GenBank Accession nos EU326290–EU326299

Locus	Primer sequences†	Repeat	A	Size range (bp)	Pontal		Imbituba	
					H_O	H_E	H_O	H_E
EPP08	F: TGTTCAAGAACAACATCGGACT R: TCTTGCTGGTTGGCATTATCT	$(GA)_9$	3	219–223	0.100	0.184	0	0.405*
EPP10	F: GGAGGCCAATGTGATGAAAC R: TCGAATAAGCTCCTGCATCC	$(GT)_5 \ldots (AG)_9 \ldots (AG)_{25}$	6	234–250	0.350	0.344	0.400	0.478
EPP12	F: GTCGGTGAGGGTCCAGAAA R: CACCATCTTCTCTCCCCTGAG	$(GA)_{21}$	9	177–197	0.600	0.715	0.400	0.457
EPP17	F: AGCACATCCGGGCCTAACTA R: TGCCTGGCATCCATAATGAC	$(TC)_{13}T(TC)_9$	10	203–223	0.700	0.750	0.250	0.708*
EPP18	F: TGCATACGTAACAACTGGAGGT R: GGAAGGTCATTCTAACCAGGAA	$(AG)_{24}$	12	288–324	0.350	0.321	0.400	0.503
EPP49	F: GCAAAGGGAGACGATTTGAG R: AGCATTTTTCGCCCTTAACA	$(GA)_{17}$	2	182–186	0.150	0.142	mono	mono
EPP56	F: ACGCTCTTTGGCTGGAACT R: CTCACATGCCTTTAGCCTCAC	$(TC)_{16}$	2	136–144	0.150	0.142	0.300	0.492
EPP86	F: CAGCCTTTAGGCATTCTTGG R: GCTCATTGGCCTTAGTGACC	$(GA)_{14}$	11	215–239	0.550	0.650	0.950	0.846
EPP89	F: TTCTTGTTGTCGCCTTCGAT R: TCAGAGAGCTCGTCCGACA	$(GA)_3AA(GA)_3 \ldots (GA)_{10}$ GG$(GA)_5$	4	284–290	0.300	0.328	0.350	0.314
EPP96	F: TCTAACATGCGAAGGCAAAA R: TTTGGTTGTTAAGCCCCATT	$(AG)_{12}$	5	291–299	0.650	0.544	0.600	0.635

†All forward primers were M13-tailed at the 5′ end. Significant departures from HWE: *$P < 0.001$.

For each SSR, the forward primers were synthesized with a 19-bp long 5′ M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following the amplification method of Schuelke (2000). All polymerase chain reaction (PCR) amplifications were performed in an Applied Biosystems 2700 thermocycler in 10-μL reactions containing: 10 ng template, 1× PCR buffer (Amersham Pharmacia Biotech), 2 mM $MgCl_2$, 100 μM dNTPs, 1 pmol forward primer, 4 pmol reverse primer, 0.4 pmol label (6-FAM or JOE: Applied Biosystems) M13 primer and 0.5 U *Taq* polymerase (Amersham Pharmacia Biotech). A 'touchdown' cycling programme was used: 95 °C for 3 min, then 10 cycles of 94 °C for 30 s, 58 °C decreasing to 48 °C at 1 °C per cycle for 30 s, 72 °C for 30 s followed by 40 cycles of 94 °C for 30 s, 48 °C for 30 s, 72 °C for 30 s, followed by a final extension of 20 min at 72 °C. PCR products were resolved on a 3130 DNA Sequence Analyser and were sized with Genescan 500 LIZ size standard using GENEMAPPER version 3.7 software (Applied Biosystems).

A total of 40 individuals from two Brazilian populations of *E. puniceoluteum* (Imbituba and Pontal do Paraná) were analysed to evaluate SSR polymorphism. ARLEQUIN 3.11 (Excoffier *et al*. 2005) software was used to calculate observed and expected heterozygosities, and to test for departure from Hardy–Weinberg equilibrium and for linkage disequilibrium between all pairs of loci. Ten SSRs were polymorphic, with the number of observed alleles per locus ranging from two to 12 with an average of 6.4 alleles per locus. The observed heterozygosity for the polymorphic loci ranged between zero and 0.95 with an average of 0.377 (Table 1). We found an absence of polymorphism at locus EPP49 in the Imbituba population. In the same population, two loci (EPP8 and EPP17) showed a significant departure from Hardy–Weinberg equilibrium ($P < 0.001$), due to heterozygote deficiency. The small size of the Imbituba population and fragmentation history of the region may be causing the observed low levels of diversity and the Hardy–Weinberg disequilibrium for some loci in this population. No linkage disequilibrium between any pair of loci was detected.

The primers proved to be useful in revealing levels of diversity in both populations and thus can be used to explore the genetic structure of fragmented populations of *E. puniceoluteum* across its actual geographical range. Historical demographic patterns such as bottlenecks and range contraction will be compared with information on reproductive success and seed dispersal ability in order to identify the evolutionary processes that are shaping their actual genetic patterns of populations.

Table 2 Cross-species and genera amplification of 10 microsatellite primers from *Epidendrum puniceoluteum* within the subtribe Laeliinae. Successful amplification with a single band visualized with expected allele size (+), successful amplification with more than one band visualized, with at least one band with the expected allele size (++), weak amplification of a band with the expected allele size (W) and failed amplification (–) are indicated

Species	EPP8	EPP10	EPP12	EPP17	EPP18	EPP49	EPP56	EPP86	EPP89	EPP96
Epidendrum xanthinum	+	W	+	+	+	+	++	++	+	+
Epidendrum secundum	+	W	+	–	++	++	++	+	+	+
Pseudolaelia cipoensis	+	–	+	+	W	+	–	++	+	W
Cattleya eldorado	W	–	W	–	W	+	+	+	+	W
Prosthechea vespa	+	–	+	–	+ +	+	++	+	+	W

Cross-species and cross-genera amplification of the microsatellite primers were performed on *Epidendrum* species and related members from subtribe Laeliinae (Table 2). Amplification was performed on single individual of each tested species using the same amplification conditions used for *E. puniceoluteum*. Several positive amplifications of PCR products with the expected allele sizes occurred across all tested species. These primers therefore should be useful for population studies both in *E. puniceoluteum* and in related species and genera, contributing to the knowledge about diversification processes and conservation in South American orchids.

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Capítulo 3

Cross-amplification and characterization of microsatellite loci for the Neotropical orchid genus *Epidendrum*

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Short Communication

Cross-amplification and characterization of microsatellite loci for the Neotropical orchid genus *Epidendrum*

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Abstract

In this study we tested the cross-amplification of 33 microsatellite loci previously developed for two closely related Neotropical orchid genera (*Epidendrum* and *Laelia*). A set of ten loci were polymorphic across five examined species (20 individuals each) with 2 to 15 alleles per locus. The mean expected and observed heterozygosity (average across species) ranged from 0.34 to 0.82 and from 0.27 to 0.85, respectively. In addition we tested all loci in 35 species representative of the genus *Epidendrum*. Of these, 26 loci showed successful amplification. Cross-application of these loci represent a potential source of co-dominant markers for evolutionary, ecological and conservation studies in this important orchid genus.

Key words: Epidendrum, Orchidaceae, short tandem repeat, cross-amplification.

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Epidendrum L. is the largest of the orchid genera in the Neotropical region, with approximately 1500 species (Hágsater and Arenas, 2005). Species of this genus display extensive variation in morphological features and growth habits (epiphytic, lithophytic and terrestrial). Moreover, they offer an interesting opportunity for exploring the influence of human activities on natural environments, since they occur within several types of threatened vegetation (Amazon and Atlantic Rainforests, savannas, coastal sand dunes, 'tepuis', and 'páramos').

Microsatellite markers are the current choice for most studies on evolution, ecology and conservation, due to their high levels of polymorphism and high reproducibility. Studies increasingly aim at comparing genetic, demographic, behavioural, and breeding system parameters among related species. To address these questions, researchers require 'universal' genetic markers that can easily be transferred between species (Barbara *et al.*, 2007). The capacity to transfer and apply the same set of microsatellite loci in different species can significantly facilitate studies among closely related and endemic taxa. This is important where resources for undertaking conservational genetic studies are limited, thus making it less cost-effective to develop specific microsatellite loci for many species of the same taxa.

Although, positive cross-amplification of some microsatellite loci have been previously reported for a few species of *Epidendrum* (Cortés-Palomec *et al.*, 2008; Pinheiro *et al.*, 2008a, 2008b), to date, there is no precise and standardized information about amplification-efficiency or polymorphism in these loci. The aim of this study is to report the potential of cross-species transferability of microsatellite markers across the genus *Epidendrum* in order to identify a set of polymorphic loci available for inquiries assessing the effect of landscape fragmentation on gene flow, species delimitation, origin and the maintenance of reproductive barriers among species of this genus.

Total genomic DNA was extracted from silica gel-exsiccated leaves according to the Pinheiro *et al.* (2008a) protocol. We tested three sets of microsatellite markers previously described for *Epidendrum fulgens* (nine loci - Pinheiro *et al.*, 2008a), *E. puniceoluteum* (ten loci - Pinheiro *et al.*, 2008b) and *Laelia speciosa* (14 loci - Cortés-Palomec *et al.*, 2008), the latter a closely related genus belonging to the subtribe Laeliinae, the same subtribe as that of *Epidendrum* (Hágsater and Arenas, 2005). Altogether, we tested 33 microsatellite loci (Table S4) for cross-amplification in 35 species belonging to different sections of genus *Epidendrum*.

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For each microsatellite locus, the forward primers were synthesized with a 5'-M13 tail according to the Schuelke (2000) method, involving three primer polymerase chain reactions (PCRs), including a universal M13 primer labelled with a fluorescent dye, 6-FAM (Applied Biosystems). All PCR amplifications were performed in an Applied Biosystems 2700 thermocycler according to Pinheiro *et al.* (2008a, 2008b). The conditions were maintained constant for all loci so as to maximize standardization. Microsatellite alleles were resolved on a 3130 Genetic Analyzer (Applied Biosystems) and sized in accordance with LIZ (500) standard by using GENEMAPPER v. 3,7 software (Applied Biosystems).

We initially tested the potential of cross-amplification for all loci with one sample from each of the 35 *Epidendrum* species. Furthermore, we focused our effort on five of those species belonging to different phyletic sections of the genus *Epidendrum* (Hágsater and Arenas, 2005): *E. denticulatum, E. secundum, E. campestre, E. densiflorum* and *E. rigidum*. We sampled 20 individuals from each species of a single population (Table S1). GENEPOP software (Raymond and Rousset, 1995; web version 3.4) was used to calculate observed (H_O) and expected (H_E) heterozygosity, and to test for departure from Hardy - Weinberg equilibrium (HWE) as well as for link-

Table 1 - Size range of the PCR products, number of observed alleles (A), expected and observed heterozygosity (H_E/H_O), and the significance of the test for departure from Hardy-Weinberg equilibrium, for the ten selected microsatellite loci (indicated by rows) in each of the five *Epidendrum* species. The size range of the original alleles described by the authors is indicated in parentheses below each locus.

Locus	Species	size range	A	H_E/H_O
EPP08	*E. campestre*	211-219	3	0.19/0.20
(219-223)	*E. densiflorum*	211-229	5	0.71/0.36
	E. denticulatum	211-213	2	0.51/1.00*
	E. rigidum	201-215	4	0.64/0.05*
	E. secundum	213-221	4	0.28/0.30
	Mean		3.6	0.47/0.38
EPP18	*E. campestre*	274-314	11	0.88/0.89
(288-324)	*E. densiflorum*	284-290	3	0.68/0.70
	E. denticulatum	288-328	15	0.92/0.84
	E. rigidum	284-312	4	0.64/0.55
	E. secundum	284	monomorphic	-
	Mean		8.3	0.62/0.60
EPP49	*E. campestre*	no amplification	-	-
(182-186)	*E. densiflorum*	162-187	9	0.79/0.32*
	E. denticulatum	176-190	6	0.78/0.70
	E. rigidum	170-186	3	0.46/0.10
	E. secundum	176-186	6	0.83/0.63
	Mean		6	0.71/0.44
EPP56	*E. campestre*	136-154	3	0.14/0.10
(136-144)	*E. densiflorum*	148-152	2	0.10/0.10
	E. denticulatum	132-166	10	0.87/0.85
	E. rigidum	152-156	2	0.51/0.00*
	E. secundum	122-162	9	0.78/0.28*
	Mean		5.2	0.48/0.27
EPP86	*E. campestre*	217-231	8	0.83/0.85
(215-239)	*E. densiflorum*	217-227	6	0.81/0.80
	E. denticulatum	217-223	4	0.71/0.60
	E. rigidum	217-235	7	0.83/1.00*
	E. secundum	215-229	8	0.85/1.00
	Mean		6.6	0.81/0.85

Locus	Species	size range	A	He/Ho
EFF26	*E. campestre*	190-204	8	0.76/0.84
(199-205)	*E. densiflorum*	196-202	4	0.66/0.95
	E. denticulatum	164-202	5	0.64/0.65
	E. rigidum	196-204	5	0.66/0.75
	E. secundum	192-204	6	0.77/0.88
	Mean		5.6	0.70/0.81
EFF45	*E. campestre*	280-284	3	0.50/0.35
(288-294)	*E. densiflorum*	288-294	3	0.49/0.53
	E. denticulatum	278-294	5	0.32/0.25
	E. rigidum	288-340	5	0.81/0.35*
	E. secundum	288-294	4	0.67/0.55
	Mean		4	0.56/0.41
EFF58	*E. campestre*	210-212	2	0.46/0.68
(210-212)	*E. densiflorum*	210-216	4	0.66/0.95
	E. denticulatum	212	monomorphic	-
	E. rigidum	210-212	2	0.51/1.00
	E. secundum	212	monomorphic	-
	Mean		2.7	0.34/0.54
Lspe-1	*E. campestre*	219-221	2	0.36/0.35
(350-390)	*E. densiflorum*	215-225	4	0.49/0.35
	E. denticulatum	471-493	3	0.34/0.28
	E. rigidum	225-233	2	0.49/0.00*
	E. secundum	462-488	9	0.85/0.85
	Mean		4	0.51/0.37
Lspe-3	*E. campestre*	250-266	8	0.83/0.79
(224-250)	*E. densiflorum*	250-288	12	0.88/0.40*
	E. denticulatum	262-304	15	0.93/1.00
	E. rigidum	268-286	5	0.78/0.25*
	E. secundum	244-260	6	0.69/0.90
	Mean		9.2	0.82/0.67

Significant departures from HWE: *p < 0.05.

age disequilibrium at each locus, by applying the Bonferroni correction to account for multiple comparisons.

Among the 33 loci tested, 26 showed positive amplification and PCR products with the expected allele sizes throughout most of the 35 species tested (Table S2). The percentage of cross-amplification was 78% on an average, thus higher than the mean value reported for monocot species (60% - Barbara *et al.*, 2007).

A total of ten polymorphic loci exhibited the features so desired for use as co-dominant molecular markers in the five examined species (Table 1), with the number of alleles per locus ranging from two to 15 (overall mean 5.6 alleles) Expected and observed heterozygosity ranged from 0.34 to 0.82 and 0.27 to 0.85, respectively (an average of 0.60 and 0.53, respectively) (Table 1). For each sampled population of the five species, we found sporadic cases of departure from HW equilibrium ($p < 0.05$): for loci EPP8 (in *E. denticulatum* and *E. rigidum*), EPP49 (in *E. densiflorum*), EPP56 (in *E. rigidum* and *E. secundum*), EPP86 (in *E. rigidum*), EFF45 (in *E. rigidum*), Lspe-1 (in *E. rigidum*) and Lspe-3 (in *E. rigidum*). Interestingly, six out of ten loci in *E. rigidum* departed significantly from HW equilibrium due to heterozygotic deficiency. Such deviations could be caused by inbreeding and/or Wahlund effects arising from secondary population subdivision. Although null alleles cannot be ruled out, there was no evidence of scoring error due to 'stuttering' or 'large allele dropout', when using MICRO-CHECKER software (van Oosterhout *et al.*, 2004. Three loci in *E. campestre*, five loci in *E. denticulatum,* five loci in *E. rigidum* and seven in *E. secundum* exhibited linkage disequilibrium ($p < 0.001$). Loci that were monomorphic or not amplified in most of the five *Epidendrum* species are listed in Table S3.

This study unveiled evidence that cross-transferability of developed microsatellite loci can increase the availability of markers to address both ecological and evolutionary questions in *Epidendrum*. The markers tested here showed to be of great potential for the use in comparing multiple co-occurring *Epidendrum* species in different ecological communities, thus contributing to knowledge on diversification processes and conservation among neotropical orchids.

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Supplementary Material

The following online material is available for this article:

Table S1 - Geographical region, sample size and Biome of species sampled.

Table S2 - Cross-species amplification of 26 loci tested for 30 additional *Epidendrum* species. Size range of the PCR products and unsuccessful amplifications are indicated (-).

Table S3 - Size range of the PCR products, number of observed alleles (A), expected heterozygosity (He), observed heterozigosity (Ho), and the significance of the test for departure from Hardy - Weinberg equilibrium (HWE - Significant departures from HWE: $p < 0{,}001$), for the microsatellite loci (indicated by rows) that were not detected as polymorphic (monomorphic), or not amplified (na) in most of the five *Epidendrum* species. The size range of the original alleles described by the authors is indicated in parentheses on the bottom of each locus.

Table S4 - Primer names, sequences and Genbank Accession numbers of 33 orchid species SSR loci.

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Table S1. Geographical region, sample size and Biome of species sampled.

Species	**N**	**Population**	**Biome**
E. denticulatum	20	Araruama, Rio de Janeiro, RJ	Atlantic Rainforest
E. densiflorum	20	Ilha do Cardoso, Cananéia, SP	Atlantic Rainforest
E. rigidum	20	Ilha do Cardoso, Cananéia, SP	Atlantic Rainforest
E. secundum	20	Serra do Cipó, Conceição do Mato Dentro, MG	Cerrado
E. campestre	20	Serra do Cipó, Conceição do Mato Dentro, MG	Cerrado

Table S2. Cross-species amplification of 26 loci tested for 30 additional *Epidendrum* species. Size range of the PCR products and failed amplifications are indicated (-).

Locus	**epp_8§**	**epp_10§**	**epp_18§**	**epp_86§**	**epp_49§**	**epp_56§**	**epp_89§**	**epp_96§**	**eff_06Ψ**	**eff_26Ψ**	**eff_29Ψ**	**eff_43Ψ**	**eff_45Ψ**
species													
E. xanthinum	213	250-264	306-308	221-227	170-179	-	291	292-294	372	200-202	193	150	288
E. cattilus	214-216	306	280-290	219-241	156-165	119-146	-	-	-	200-202	198	150	286
E. calanthum	215	-	290-331	217-223	156-165	119	-	-	372	-	205	150	-
E. funkii	215-219	252-266	278	223-241	157-166	159	-	-	-	200-202	-	150	282-286
E. myrmecophorum	213-219	278	284-286	219-225	152-161	144-154	-	-	-	200-202	-	150	288
E. purpureum	213	268	284-300	241	-	141-153	289	300	370	198-	203	150	288
E. ibaguense	215	-	290-304	221-223	156-165	-	-	-	375	200-202	-	150	-
E. radicans	215	246	288-290	223-225	168-178	129-139	-	-	368	200-202	179-181	150	288
E. incisum	215	245	288-290	217-219	-	-	-	-	377	200-202	191	150	289
E. cinnabarinum	210	221	286	221-227	-	-	281-285	294-298	366-370	196-200	193-191	150	289
E. martianum	213-219	-	289-291	225	-	140-151	-	-	378	200-202	-	150	278
E. flexuosum	219	223	290	223	161-169	-	-	-	368	200-202	-	150	279
E. ramosum	213-219	250-254	290	219-223	152-160	140-151	-	-	-	200-202	-	150	288
E. saxatile	219	290	290	242	169-172	144-147	-	-	-	200-202	-	150	305-316
E. cristatum	215-219	236	284-286	219-225	-	133-144	-	-	372	200-202	-	150	289-291
E. purpurascens	219	254-261	304-306	225-227	169-172	-	-	-	-	200-202	-	150	-
E. ciliare	212-219	303,69	290-308	217-223	-	141-152	-	-	-	200-202	-	150	-
E. nocturnum	215-219	-	295-321	219-225	-	141-152	-	-	391	200-202	-	150	319-321
E. cooperianum	219	-	290	215-219	159-161	141-152	-	-	-	200-202	-	150	-
E. warasii	219	261	297	219-221	156-166	139-150	-	-	377	200-202	-	150	286
E. avicola	213-221	250-252	290-308	217-219	-	-	-	-	330	200-202	-	150	-
E. schlechterianum	212-219	290	290-308	219-227	167-169	241-246	-	-	-	200-202	-	150	-
E. coronatum	220-222	-	289-307	219	161-164	-	-	-	373	200	-	150	279
E. filicaule	219	-	290-306	225-229	139-149	-	-	-	377	200-202	-	150	-
E. chlorinum	219	-	290-306	217-219	-	-	259	-	-	200-202	-	150	321-323
E. tridactylum	212-219	-	290-306	-	188-198	-	-	-	-	200-202	-	150	-
E. vesicatum	215	-	290-308	227-229	166-168	-	-	-	-	200-202	-	150	295-301
E. latilabre	219-221	261	313	221-223	152-161	-	-	-	-	200-202	-	150	-
E. fulgens	210	265-275	286-308	219-243	152-160	-	284	-	366	199-203	201-211	150-152	291-295
E. puniceoluteum	211-219	271-273	290-310	221-227	160-184	136-144	288	294	370	197	219-225	150	289-291

§Markers isolated by Pinheiro et al (2008b); ΨMarkers isolated by Pinheiro et al (2008a); *Markers isolatated by Cortés-Palomec et al (2008).

Table S2. Continue

Locus	eff_58Ψ	eff_61Ψ	eff_70Ψ	eff_51Ψ	Lspe_1*	Lspe_3*	Lspe_4*	Lspe_6*	Lspe_8*	Lspe_9*	Lspe_10*	Lspe_11*	Lspe_14*
species													
E. xanthinum	212	265-271	329	335	218	249	214	172-175	221-247	219-229	-	205	254
E. cattilus	210	264	322-328	369-371	218-220	258-260	214	172-175	221	231-	-	205	254
E. calanthum	208	265	-	376-383	229	253	214	172-175	221	223-231	172-197	205	253
E. funkii	210	265	325-329	-	220	247	214	172-175	242	229-235	-	205	255
E. myrmecophorum	212-216	265	327-329	375-378	-	237-242	214	172-175	207-211	229-231	-	205	254
E. purpureum	212	265-270	328-341	375-377	215-218	233-237	214	172-175	207-211	232	172	205	254
E. ibaguense	208	265	300	-	218-220	269-271	214	172-175	208-210	231	172	205	254
E. radicans	210	265-272	308	374-376	221-227	258-260	214	172-175	243	231	-	205	252
E. incisum	210	266-275	329-331	376-382	242	241	214	172-175	221-247	229-233	-	205	254
E. cinnabarinum	210-212	266-270	325	376	-	-	214	172-175	244-246	232	-	205	252
E. martianum	210-215	262-265	320-325	-	218	260	214	172-175	226-230	229-231	197	205	258
E. flexuosum	208	265	336	-	225	250-252	214	172-175	-	231	275	205	253
E. ramosum	210-212	264-266	327-330	-	220	242	213-214	172-175	246	232	-	205	246
E. saxatile	210	266	339	-	226	234-236	214	172-175	203-205	229-231	196	205	209
E. cristatum	212	266	324-326	370	218-220	258	213	172-175	207	231	-	205	253
E. purpurascens	210	266	323-336	-	218	248-252	213-220	172-175	207-209	226-231	-	205	253
E. ciliare	222	264-266	325-329	339-340	218	259	214	172-175	214-218	227-232	219	205	259
E. nocturnum	211	264-266	329-336	372-380	217	239-242	214	172-175	220	227-233	-	205	253
E. cooperianum	216	-	323-326	373	219-222	261-263	213	172-175	237	231	-	205	253
E. warasii	216	264-266	318-330	372	218	264-272	214-218	172-175	219	231-237	196	205	246-253
E. avicola	210-213	264-266	323-325	272	-	253	213	172-175	220	232	-	205	254
E. schlechterianum	210	264-266	328	375	228-234	248	213-214	172-175	220	232	-	205	253
E. coronatum	210	262-266	318-323	373	226-228	242	213	172-175	238	231	152-172	205	249-256
E. filicaule	219	264-266	329-330	335	218	264-274	214	172-175	221	233-239	172	205	253
E. chlorinum	215	264-266	323-325	-	218	251	214	172-175	218-220	229-232	-	205	254
E. tridactylum	210-213	270-277	321-329	335	218-224	236	213	172-175	192	231-238	197-219	205	253
E. vesicatum	216	264-266	-	335	218-220	260-266	213	172-175	219-226	231	120-172	205	254
E. latilabre	210	264-266	328-338	374	216-226	268-270	213	172-175	247	231	-	205-209	253
E. fulgens	210-212	266	343-345	371-375	215	256-266	214	172-175	221-247	232	142	205	253
E. puniceoluteum	210-212	264-266	333-347	373-377	215-218	251-272	214	172-175	247	232	-	205	253

§Markers isolated by Pinheiro et al (2008b); ΨMarkers isolated by Pinheiro et al (2008a); *Markers isolatated by Cortés-Palomec et al (2008).

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Table S3. Size range of the PCR products, number of observed alleles (A), expected heterozygosity (He), observed heterozigosity (Ho) and the significance of the test for departure from Hardy–Weinberg equilibrium (HWE - Significant departures from HWE: P< 0,001), for the microsatellite loci (indicated by rows) that were not successful polymorphic (monomorphic), or not amplified (na) in most of the five *Epidendrum* species. The size range of the original alleles described for the authors is indicated in parentheses on the bottom of each locus.

Locus	***Species***	**size range**	**A**	**He**	**Ho**	**HWE**
EPP10	*E. campestre*	na	-	-	-	-
(234–250)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	252-274	9	0.836	0.900	ns
	E. rigidum	na	-	-	-	-
	E. secundum	246-278	10	0.903	0.833	***
EPP89	*E. campestre*	na	-	-	-	-
(284-290)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	279-291	6	0.200	0.432	***
	E. rigidum	na	-	-	-	-
	E. secundum	na	-	-	-	-
EPP96	*E. campestre*	286-302	5	0.643	0.765	ns
(291-299)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	282-310	9	0.849	0.500	***
	E. rigidum	na	-	-	-	-
	E. secundum	286-308	8	0.795	0.450	***
EFF29	*E. campestre*	na	-	-	-	-
(185–229)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	193-225	13	0.920	0.850	***
	E. rigidum	na	-	-	-	-
	E. secundum	na	-	-	-	-
EFF43	*E. campestre*	150	monomorphic	-	-	-
(148–160)	*E. densiflorum*	150	monomorphic	-	-	-
	E. denticulatum	150-154	3	0.405	0.400	***
	E. rigidum	150	monomorphic	-	-	-
	E. secundum	150	monomorphic	-	-	-
EFF70	*E. campestre*	353-369	7	0.800	0.800	ns
(321–349)	*E. densiflorum*	na	-	-	-	
	E. denticulatum	na	-	-	-	
	E. rigidum	na	-	-	-	
	E. secundum	na	-	-	-	

Significant departures from HWE: ns – not significant; ***$P < 0.05$.

Table S3. Continued.

Locus	*Species*	size range	No. of alleles	He	Ho	HWE
Lspe-4	*E. campestre*	213	monomorphic	-	-	-
(176–189)	*E. densiflorum*	212	monomorphic	-	-	-
	E. denticulatum	213	monomorphic	-	-	-
	E. rigidum	na	-	-	-	-
	E. secundum	213	monomorphic	-	-	-
Lspe-6	*E. campestre*	na	-	-	-	-
(176–185)	*E. densiflorum*	172	monomorphic	-	-	-
	E. denticulatum	172	monomorphic	-	-	-
	E. rigidum	173-175	2	0.097	0.100	ns
	E. secundum	172	monomorphic	-	-	-
Lspe-8	*E. campestre*	na	-	-	-	-
(222–239)	*E. densiflorum*	na	-	-	-	-
	E. denticulatum	247-251	5	0.773	0.150	***
	E. rigidum	246	monomorphic	-	-	-
	E. secundum	na	-	-	-	-
Lspe-9	*E. campestre*	229	monomorphic	-	-	-
(190–206)	*E. densiflorum*	219-235	6	0.767	0.400	-
	E. denticulatum	na	-	-	-	-
	E. rigidum	na	-	-	-	-
	E. secundum	na	-	-	-	-
Lspe-11	*E. campestre*	204	monomorphic	-	-	-
(183–184)	*E. densiflorum*	204	monomorphic	-	-	-
	E. denticulatum	204	monomorphic	-	-	-
	E. rigidum	204	monomorphic	-	-	-
	E. secundum	204	monomorphic	-	-	-
Lspe-14	*E. campestre*	208	monomorphic	-	-	-
(221–233)	*E. densiflorum*	243-251	5	0.792	0.368	***
	E. denticulatum	252	monomorphic	-	-	-
	E. rigidum	259	monomorphic	-	-	-
	E. secundum	252	monomorphic	-	-	-

Significant departures from HWE: ns – not significant; ***$P < 0.05$.

Table S4: Primer names, sequences and Genbank Accession numbers of 33 orchid species loci.

Name	Forward sequence	Reverse sequence	Genbank Accession
epp_8§	F: TGTTCAAGAACAACATCGGACT	R: TCTTGCTGGTTGGCATTATCT	EU326290
epp_10§	F: GGAGGCCAATGTGATGAAAC	R: TCGAATAAGCTCCTGCATCC	EU326291
epp_12§	F: GTCGGTGAGGGTCCAGAAA	R: CACCATCTTCTCTCCCCTGAG	EU326292
epp_17§	F: AGCACATCCGGGCCTAACTA	R: TGCCTGGCATCCATAATGAC	EU326293
epp_18§	F: TGCATACGTAACAACTGGAGGT	R: GGAAGGTCATTCTAACCAGGAA	EU326294
epp_49§	F: GCAAAGGGAGACGATTTGAG	R: AGCATTTTTCGCCCTTAACA	EU326295
epp_56§	F: ACGCTCTTTGGCTGGAACT	R: CTCACATGCCTTTAGCCTCAC	EU326296
epp_86§	F: CAGCCTTTAGGCATTCTTGG	R: GCTCATTGGCCTTAGTGACC	EU326297
epp_89§	F: TTCTTGTTGTCGCCTTCGAT	R: TCAGAGAGCTCGTCCGACA	EU326298
epp_96§	F: TCTAACATGCGAAGGCAAAA	R: TTTGGTTGTTAAGCCCCATT	EU326299
eff_06Ψ	F: TCAAGCCTATCATAAGTGCTCCA	R: CCTTGTTGCAACTGGGTGTT	EU363791
eff_26Ψ	F: TGTCCTAAGTCAAGTGGGGTTT	R: TCCGAGTCTGTCGGTCTTTT	EU363792
eff_29Ψ	F: TCCGCTGATTTGAGTTTGCT	R: CTGGTCCCGTAAGATCAATCAC	EU363793
eff_43Ψ	F: TGCCCCACAGACAATTAAGC	R: CCTCGATGGAACCCCATAAT	EU363794
eff_45Ψ	F: TTGGGTTTCGTCTCACATCA	R: CCCTCAGTATCCGCCACTT	EU363795
eff_51Ψ	F: CTTGTCTACGTGAGGGCACTG	R: TCAACAACGTGAAAAGCCATC	EU363796
eff_58Ψ	F: TGAATGCTTATACTCTCCCATCA	R: AAGTGGCAAAGCACCATGTA	EU363797
eff_61Ψ	F: TGTCCCCTATATTCTGATGGTG	R: AGGGTTTTAGGTCAAAGTGCTC	EU363798
eff_70Ψ	F: CGCGAGATTGTTCCAAACC	R: GCTCCACGCAAAACCTTTTTA	EU363799
Lspe_1*	F: AGAGAAAGCCCTTGTGTTGG	R: TCAGCTCTTCCGATTCTGGT	EF439820
Lspe_2*	F: GCAGATCCCACCATGAACTC	R: AATGTTGGAAATCGGTAGCA	EF439821
Lspe_3*	F: GCTTCAAGCAAGTGCAGAAA	R: AAGACAGGCCAACCAGAGAA	EF439822
Lspe_4*	F: GCATCGTTGAAGTTGCCAAT	R: TTTAGGGATCACCACCTTGG	EF439824
Lspe_5*	F: CCCACACAACCCCTGAACTA	R: ATGATTCGGGTTGACGAAAG	EF439825
Lspe_6*	F: GAAGCCCGTCGTCAGAAGTA	R: AAAAGAAGACCCCGAGCCTA	EF439826
Lspe_7*	F: CTTGAGGTGGGGAGTGATGT	R: GGCTTAGCTGTTTGGAATCG	EF439827
Lspe_8*	F: AAGCTCCTAGTGCCTGCTTG	R: CATGTGGCTCTGGATTGTTG	EF439828
Lspe_9*	F: GGGGAAGAAATGCAAACTAGC	R: CACAGGCATACGCACACAT	EF439829
Lspe_10*	F: TGGTTTGCTAAGTATTTCTTCAAGTT	R: TATGCAAAGCTTCCCCAAGT	EF439830
Lspe_11*	F: TGAATGCAAAATCCAATTGCT	R: GAAATATTCAAGCACGATGATCC	EF439831
Lspe_12*	F: GGGGAAACAGAAGAAGGAAGA	R: AGACCTGGAGCAACTTTCCA	EF439832
Lspe_13*	F: TCTCTAAATACCATAAGTGGAGTGAAA	R: TTCAACCGAGAGCCACCTAC	EF439835
Lspe_14*	F: TGAGACGAAAAACCCATTCTT	R: GGTTACCAGCCATTCCCTTT	EF439836

§Markers isolated by Pinheiro et al (2008b); ΨMarkers isolated by Pinheiro et al (2008a); *Markers isolatated by Cortés-Palomec et al (2008).

Capítulo 4

Chloroplast microsatellite markers for the Neotropical orchid genus *Epidendrum*, and cross-amplification in other Laeliinae species (Orchidaceae)

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TECHNICAL NOTE

Chloroplast microsatellite markers for the Neotropical orchid genus *Epidendrum*, and cross-amplification in other Laeliinae species (Orchidaceae)

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Abstract One of the most significant challenges confronting orchid researchers is the lack of specific molecular markers, mainly for species in the Neotropics. Here we report the first set of specific chloroplast microsatellite primers (cpSSR) developed for Neotropical orchids. In total, nine polymorphic cpSSR loci were isolated and characterized in four species occurring in the Brazilian Atlantic Rainforest: *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum*. Levels of intraspecific polymorphism were characterized using two populations for each species, with 13–20 individuals each. Allele numbers varied from two to three per locus, while the number of haplotypes ranged from three to six per species. Extensive differentiation among the taxa was detected. All markers were successfully cross-amplified in eight other different genera. These cpSSRs markers will enable novel insights into the evolution of this important Neotropical genus.

Keywords Orchidaceae · *Epidendrum* · Marker development · Chloroplast microsatellites · Cross-amplification · Populations genetics

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Noncoding chloroplast (cp) DNA markers are a valuable resource in plant phylogenetics and evolution. The uniparentally inherited nature of the cpDNA genome is particularly useful to detect historical demographic processes, such as range contractions, expansions, and fragmentation (Cozzolino et al. 2003; Hedrén et al. 2008). Of particular interest for population level studies are chloroplast microsatellites or simple sequence repeats (cpSSR), also known as chloroplast simple sequence repeats (Provan et al. 2001) due to the high amounts of polymorphisms recovered. When genus-specific cpSSR primers are not available, de novo sequencing of noncoding chloroplast regions is the most effective and efficient way to discover chloroplast microsatellites in wild species (Ebert and Peakall 2009).

One of the most significant challenges confronting orchid researchers is the lack of specific molecular markers (Peakall 2007). Specific cpSSRs for orchid species belonging mainly to temperate regions have been developed in the last few years (Fay and Cowan 2001; Hedrén et al. 2008; Ebert et al. 2009). On the other hand, there is a

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Table 1 Description of chloroplast universal primers used to search for microsatellite regions in *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum*

Primer pairs (reference)	T_a (°C)	*E. cinnabarinum*		*E. denticulatum*		*E. fulgens*		*E. puniceoluteum*		Genbank accession no.[a]
		pb	SSR	pb	SSR	pb	SSR	pb	SSR	
trnH^{GUG} (1)–*psbA* (2)	55	837	T_9	839	T_{11}	839	T_{11}	839	T_{11}	GQ890570–GQ890573
3′rps16 (3)–*5′trnK*^{UUU} (3)	55	513	(C_{11}) (T_{12})	512	(C_{11}) (T_{12})	512	(C_{10}) (T_{11})	523	(C_{11}) (T_9)	GQ890574–GQ890577
trnS^{UGA} (4)–*trnfM*^{CAU} (4)	48	931	T_{13}	930	T_{12}	929	T_{11}	930	T_{12}	GQ890578–GQ890581
*rpS4*R2 (5)–*trnT*^{UGU}R (5)	50	476	A_{10}	531	A_9	520	A_9	531	A_9	GQ890582–GQ890585
trnT^{UGU}A (6)–*trnL*^{UAA}B (6)	48	549	T_{12}	475	T_9	551	T_{10}	496	T_9	GQ890586–GQ890589
ndhJ (3)–*trnL*^{UAA}E (6)	60	589	T_9	603	T_9	516	T_{10}	618	T_9	GQ890590–GQ890593
trnL^{UAA}C (6)–*trnF*^{GAA}F (6)	55	1017	(A_9) (A_{10})	1018	(A_9) (A_9)	1019	(A_9) (A_9)	1002	(A_9) (A_9)	GQ890594–GQ890597
ndhF (3)–*rpl32*R (3)	48–58	mu								
trnD^{GUC}F (4)–*trnT*^{GGU} (4)	48–58	mu								
psaI (3)–*accD* (3)	58	516	–	728	–	727	–	728	–	GQ890598–GQ890601
*psbM*F (5)–*trnD*^{GUC}R (4)	48–58	mu								
*rpl32*F (3)–*trnL*^{UAG} (3)	58	805	–	801	–	791	–	801	–	GQ890602–GQ890605
trnC^{GCA}F (4)–*psbM*R (5)	48–58	mu								
rpL16F71 (7)–*rpL16R1516* (7)	48–58	mu								
atpI (3)–*atpH* (3)	48–58	na								

Annealing temperature (T_a), size of the amplified product (base pairs) and microsatellite motif (SSR) are included

References 1, Tate and Simpson (2003); 2, Sang et al. (1997); 3, Shaw et al. (2007); 4, Demesure et al. (1995); 5, Shaw et al. (2005); 6, Taberlet et al. (1991); 7, Small et al. (1998)

-, microsatellite region absent; mu, region not sequenced due to amplification with multiple bands; na, amplification failed

Table 2 Characteristics of chloroplast microsatellite loci in *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum*, including locus name and region from which it was isolated, primer sequences, repeat type (SSR), allele size range, no. of alleles (A) and expected heterozygosity (*H*E) for each population

Locus (region)	Primer sequences[a]	Species (population code)	SSR	Size	*A*	H_E
Epcp-01 (*trnH–psbA*)	*F:* TTTTGAACATAGAAAGCAATCC	*E. cinnabarinum* (PI)	T_6–T_{10}	150–154	2	0.282
	R: GATTCGGATAGAGAAGCAAAA	*E. cinnabarinum* (RR)	T_{10}	154	1	0.000
		E. denticulatum (AL)	T_{10}–T_{11}	154–155	2	0.458
		E. denticulatum (PC)	T_{11}–T_{13}	155–157	2	0.439
		E. fulgens (IT)	T_{11}–T_{12}	155–156	2	0.133
		E. fulgens (ST)	T_{11}	155	1	0.000
		E. puniceoluteum (CO)	T_{10}–T_{11}	154–155	2	0.198
		E. puniceoluteum (GU)	T_{11}	155	1	0.000
Epcp-02 (*rps16–trnK*)	*F:* TTCTTGCTTCTTTTTGTGGA	*E. cinnabarinum* (PI)	C_8–C_{11}	267–270	3	0.666
	R: ATTTGTTTGATACGCCATTG	*E. cinnabarinum* (RR)	C_8	267	1	0.000
		E. denticulatum (AL)	C_{11}	270	1	0.000
		E. denticulatum (PC)	C_{10}	269	1	0.000
		E. fulgens (IT)	C_8–C_{10}	267–269	2	0.133
		E. fulgens (ST)	C_8	267	1	0.000
		E. puniceoluteum (CO)	C_8–C_{11}	267–270	3	0.444
		E. puniceoluteum (GU)	C_{11}	270	1	0.000
Epcp-03 (*rps16–trnK*)	*F:* GTGCTAATTCAACGCAAA	*E. cinnabarinum* (PI)	T_{12}	218	1	0.000
	R: TTAAAAGCCGAGTACTCTACC	*E. cinnabarinum* (RR)	T_9	215	1	0.000
		E. denticulatum (AL)	T_{12}	218	1	0.000
		E. denticulatum (PC)	T_9	215	1	0.000
		E. fulgens (IT)	T_{10}–T_{11}	216–217	2	0.133
		E. fulgens (ST)	T_{11}	217	1	0.000
		E. puniceoluteum (CO)	T_9–T_{11}	215–217	2	0.105
		E. puniceoluteum (GU)	T_9	215	1	0.000
Epcp-04 (*trnS–trnfM*)	*F:* TGCATCATGAAGGGATTTGA	*E. cinnabarinum* (PI)	T_{13}	113	1	0.000
	R: ACATGTCGACTCCATGTCCA	*E. cinnabarinum* (RR)	T_{13}	113	1	0.000
		E. denticulatum (AL)	T_{12}	112	1	0.000
		E. denticulatum (PC)	T_{12}–T_{13}	112–113	2	0.439
		E. fulgens (IT)	T_{11}	111	1	0.000
		E. fulgens (ST)	T_{11}	111	1	0.000
		E. puniceoluteum (CO)	T_{11}–T_{12}	111–112	2	0.280
		E. puniceoluteum (GU)	T_{12}	112	1	0.000

Table 2 continued

Locus (region)	Primer sequences[a]	Species (population code)	SSR	Size	*A*	H_E
Epcp-05 (*rpS4–trnT*)	*F:* TGTCGGGTCACCGATCTATT	*E. cinnabarinum* (PI)	A_{11}	141	1	0.000
	R: GGAACTTCAAGCGAAGTTTACG	*E. cinnabarinum* (RR)	A_{10}	140	1	0.000
		E. denticulatum (AL)	A_9	139	1	0.000
		E. denticulatum (PC)	A_9	139	1	0.000
		E. fulgens (IT)	A_9	139	1	0.000
		E. fulgens (ST)	A_9	139	1	0.000
		E. puniceoluteum (CO)	A_9	139	1	0.000
		E. puniceoluteum (GU)	A_9	139	1	0.000
Epcp-06 (*trnL–trnF*)	*F:* CCTAGCCCCTGAATTTCTTAG	*E. cinnabarinum* (PI)	A_{10}	191	1	0.000
	R: CTTCCAATCCAATCTCATTTG	*E. cinnabarinum* (RR)	A_9	190	1	0.000
		E. denticulatum (AL)	A_9	190	1	0.000
		E. denticulatum (PC)	A_9	190	1	0.000
		E. fulgens (IT)	A_9–A_{10}	190–191	2	0.133
		E. fulgens (ST)	A_9	190	1	0.000
		E. puniceoluteum (CO)	A_9	190	1	0.000
		E. puniceoluteum (GU)	A_9	190	1	0.000
Epcp-07 (*trnL–trnF*)	*F:* TGAGATTGGATTGGAAGAAGA	*E. cinnabarinum* (PI)	A_9	220	1	0.000
	R: TGAGGGTTCAAGTCCCTCTA	*E. cinnabarinum* (RR)	A_9–A_{10}	220–221	2	0.133
		E. denticulatum (AL)	A_9	220	1	0.000
		E. denticulatum (PC)	A_9	220	1	0.000
		E. fulgens (IT)	A_9	220	1	0.000
		E. fulgens (ST)	A_9	220	1	0.000
		E. puniceoluteum (CO)	A_9	220	1	0.000
		E. puniceoluteum (GU)	A_9	220	1	0.000
Epcp-08 (*trnT–trnL*)	*F:* AGTGCATCTTTGAATAGTGGA	*E. cinnabarinum* (PI)	T_{12}	90	1	0.000
	R: TCAATGAAATGAGAATTCAAAA	*E. cinnabarinum* (RR)	T_9	87	1	0.000
		E. denticulatum (AL)	T_9	87	1	0.000
		E. denticulatum (PC)	T_9	87	1	0.000
		E. fulgens (IT)	T_9–T_{10}	87–88	2	0.133
		E. fulgens (ST)	T_{10}	88	1	0.000
		E. puniceoluteum (CO)	T_9–T_{10}	87–88	2	0.280
		E. puniceoluteum (GU)	T_9	87	1	0.000

Table 2 continued

Locus (region)	Primer sequences[a]	Species (population code)	SSR	Size	*A*	H_E
Epcp-09 (*ndhJ–trnL*)	*F:* TAGGATGATGCACGGGAAA	*E. cinnabarinum* (PI)	T_9–T_{10}	241–242	2	0.384
	R: GGGGGTTTTTATCATTGAGGA	*E. cinnabarinum* (RR)	T_9–T_{10}	241–242	2	0.505
		E. denticulatum (AL)	T_9	241	1	0.000
		E. denticulatum (PC)	T_9–T_{10}	241–242	2	0.439
		E. fulgens (IT)	T_{11}	243	1	0.000
		E. fulgens (ST)	T_{10}–T_{11}	242–243	2	0.400
		E. puniceoluteum (CO)	T_9–T_{10}	241–242	2	0.280
		E. puniceoluteum (GU)	T_9	241	1	0.000

Locus Epcp-05 and Epcp-07 were monomorphic for all populations tested. Genbank accession n°s GQ890606–GQ890614

complete lack of specific cpSSRs for species-rich Neotropical orchid groups, limiting the options for population level research on those taxa.

The target group for the present study is the genus *Epidendrum,* the largest (1500 species) and most widespread (South United States to North Argentina) Neotropical orchid genus (Hágsater and Soto Arenas 2005). *Epidendrum* is famous for its taxonomic uncertainties regarding taxa delimitation in many species complexes, as many of those taxa show an impressive morphological diversification. Studies in *Epidendrum* are mainly limited to the description of new species, and the evolutionary processes involved in species radiation of this genus are poorly understood.

Here, we report on the development of a set of polymorphic chloroplast microsatellite markers for *Epidendrum cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum* that will be useful in addressing questions on evolutionary processes shaping the phylogeographic and genetic structure of these species, thus serving evolutionary and conservation purposes.

Total genomic DNA was extracted from silica gel-exsiccated leaves from four *Epidendrum* target species following the protocol of Pinheiro et al. (2008). cpSSR regions were isolated from *E. cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum* by sequencing 15 noncoding regions of chloroplast DNA, based on polymorphism levels described in Shaw et al. (2005, 2007). Briefly, the chloroplast DNA fragments of each species were amplified by polymerase chain reaction (PCR) using universal chloroplast primer pairs described in Table 1. All PCR were carried out in a total volume of 20 µl containing: 10 ng template, 1× Bioline PCR buffer, 2 mM Bioline $MgCl_2$, 100 µM dNTPs, 20 pmol forward primer, 20 pmol reverse primer, and 2U *Taq* polymerase (Bioline, London, UK). Reactions were performed in a PE Applied Biosystems 9700 thermocycler by using a standard cycling program: 95°C for 3 min, 38 cycles of 94°C for 30 s, T_a (annealing temperature—Table 1) for 30 s, 72°C for 30 s and a final elongation step at 72°C for 10 min. Products were purified (QIAquick, West Sussex, UK) and sequenced using the BigDye terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and a 3730 DNA Analyzer (Applied Biosystems). Forward and reverse sequences were edited in SeqMan 5.01 software (Lasergene 7.0, DNASTAR Inc.) and multiple sequence alignments were generated with MegAlign software (Lasergene 7.0, DNASTAR Inc.) using the ClustalW option. Based on sequence alignments, specific primers were designed to match regions conserved across the four *Epidendrum* species, flanking cpSSRs with nine or more uninterrupted mononucleotide repeats using Primer 3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

To analyze the polymorphisms of the isolated cpSSR loci, a total of 130 samples were collected from eight natural populations (two for each species) distributed along the Brazilian Atlantic Rainforest (Table 2). Genomic DNA was extracted as described before. For each SSR, the forward primers were synthesized with a 19-bp long 5M13 tail (5′-CACGACGTTGTAAAACGAC-3′) following Schuelke (2000). PCR amplifications, genotyping and allele scoring were performed according to Pinheiro et al. (2008). To ascertain the basis of the observed polymorphism, we sequenced a large proportion of the different length variants for each locus. This allowed us to separately analyze mononucleotide repeat variation from insertion-deletion (indel) variation.

Each locus was characterized for levels of diversity using the number of alleles detected and the gene diversity (H_E) according to Nei (1978). CpSSR length variation was combined to define the haplotype of each individual, and levels of diversity of each population were characterized using the number of haplotypes and the analysis of molecular variance (AMOVA), using the software Arlequin 3.01 (Excoffier et al. 2005). Furthermore, cross-genera amplification tests were performed with these loci on eight related genera from subtribe Laeliinae (Table S2), using the same amplification conditions described above.

Seven regions out of the 15 universal chloroplast regions tested contained microsatellite loci (Table 1). The *rps16*/*trnK*^{UUU} and *trnL*^{UAA}/*trnF*^{GAA} regions contained two microsatellite loci each. In total, primers were designed for nine loci (Table 2), and all of them were polymorphic within and/or among species. Between one and three alleles were detected per polymorphic locus, and genetic diversity ranged between 0 and 0.66 (Table 2). The sequencing of polymorphic alleles revealed that the polymorphisms were, indeed, restricted to length variation occurring in the mononucleotide repeats. The number of haplotypes ranged from three to six per species. Unique haplotypes were found for different species and populations (Fig. S1). The analysis of molecular variance (AMOVA) across all populations and species revealed extensive and significant ($P < 0.001$) differentiation among the four species (32.5%), among populations within species (51.6%) and within populations (15.9%). All loci were successfully amplified in the related genera tested for their cross-amplification potential, showing PCR products with the expected allele sizes across all tested species (Table S2).

The results showed that these loci provide cpSSR markers with polymorphisms at different levels, useful in species delimitation, inter and intraspecific phylogeographic studies and for characterization of historical demographic processes. The extensive difference in chromosome numbers and hybridization events reported for many *Epidendrum* species (Hágsater and Soto Arenas 2005) imposes many challenges for molecular marker-based research on this genus. In such groups, results obtained with nuclear markers alone are often difficult to interpret due to independent events of polyploidization and hybridization (Hedrén et al. 2008). Differences in ploidy levels and chromosome numbers do not affect results obtained with organellar markers. Furthermore, when results from nuclear marker loci are combined with results from chloroplast markers, the direction and extension of introgression can be measured (Lexer et al. 2005), facilitating the depiction of complex scenarios of hybridization and species radiation. Chloroplast markers are tools that can overcome these challenging characteristics of *Epidendrum*, and the loci described and characterized here should be useful for population studies both in *Epidendrum* species and in related genera, thus contributing to the knowledge on diversification processes and conservation strategies in South American orchids.

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Table S1: *Epidendrum* species used to develop the chloroplast microsatellite loci, including population origin, number of individuals sampled (n) and voucher information.

Species	Locality – State (code)	n	Voucher[a]
E. cinnabarinum	Pirambú – SE (PI)	13	F. Pinheiro *et al*. 578
	Serraria – PB (RR)	20	F. Pinheiro *et al*. 579
E. denticulatum	Alcobaça – BA (AL)	16	F. Pinheiro *et al*. 580
	Rio de Janeiro – RJ (PC)	14	F. Pinheiro *et al*. 581
E. fulgens	Itajaí – SC (IT)	15	F. Pinheiro *et al*. 582
	Porto Alegre – RS (ST)	16	F. Pinheiro *et al*. 583
E. puniceoluteum	Ilha Comprida – SP (CO)	19	F. Pinheiro *et al*. 584
	Pontal do Sul – PR (GU)	17	F. Pinheiro *et al*. 585

[a]Vouchers are deposited in the Herbarium SP (Instituto de Botânica, São Paulo, Brazil).

SE, Sergipe; PB, Paraíba; BA, Bahia; RJ, Rio de Janeiro; SC, Santa Catarina; RS, Rio Grande do Sul; SP, São Paulo; PR, Paraná States.

Table S2: Cross-species and genera amplification of nine chloroplast microsatellite loci developed for *Epidendrum* species, within the subtribe Laeliinae. The size of amplified alleles is indicated.

	Epcp-01	Epcp-02	Epcp-03	Epcp-04	Epcp-05	Epcp-06	Epcp-07	Epcp-08	Epcp-09
Prostechea vespa	143	260	275	107	139	193	222	84	241
Encyclia patens	142	261	275	108	138	190	220	90	241
Hoffmansegella crispata	143	268	274	110	140	190	220	90	242
Hadrolaelia purpurata	143	267	274	110	139	191	222	89	242
Cattleya eldorado	142	266	272	112	140	190	222	88	242
Brassavola tuberculata	143	222	276	109	140	189	223	92	241
Sophronitis cernua	145	265	273	110	140	188	220	88	188
Pseudolaelia cipoensis	142	276	274	108	140	189	221	89	240

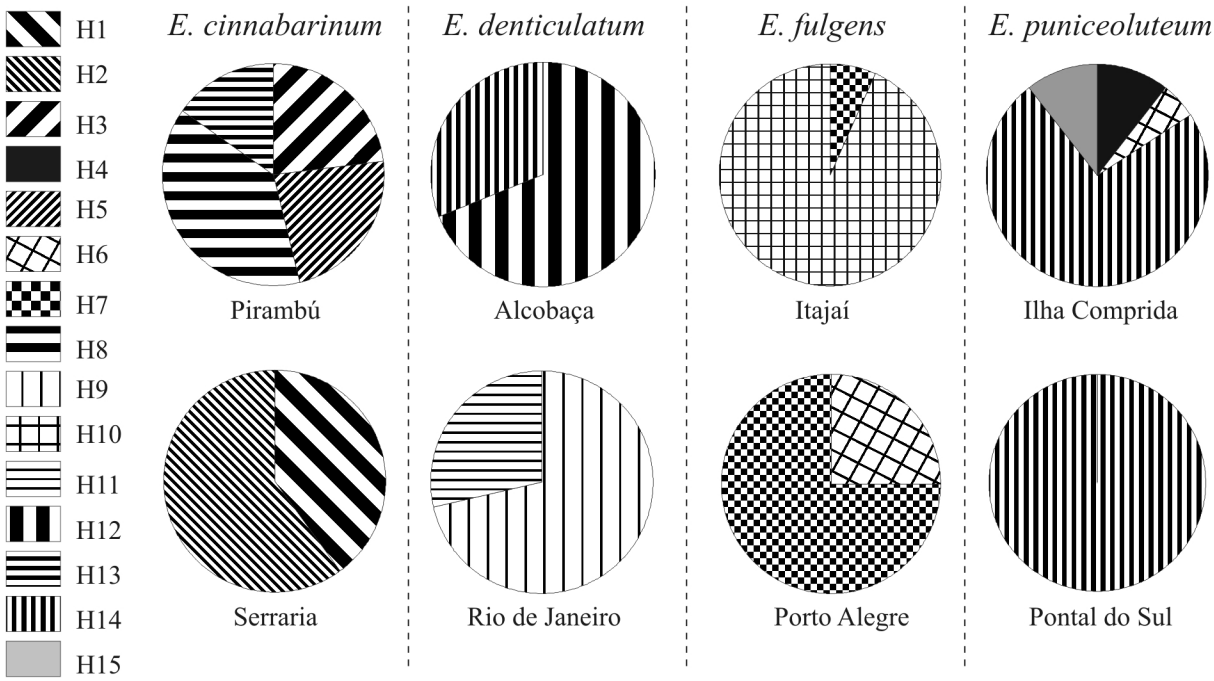

Figure 1: Haplotype frequences found in natural populations of *E. cinnabarinum*, *E. denticulatum*, *E. fulgens* and *E. puniceoluteum.* Population names are indicated.

Capítulo 5

Hybridization and introgression in the Neotropical orchids, *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae)

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Hybridization and introgression in the Neotropical orchids, *Epidendrum fulgens* and *E. puniceoluteum* (Orchidaceae)

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Keywords: Orchidaceae, *Epidendrum*, hybridization, introgression, reproductive barriers, orchid evolution

Running title: HYBRIDIZATION IN NEOTROPICAL ORCHID SPECIES

Abstract

The hypothesis of gene flow between species with extensive differences in chromosome numbers has rarely been tested, mainly because species with different ploidy levels are commonly assumed to be reproductively isolated from one another due to strong post-zygotic barriers. In the present study, a broad-scale survey of molecular variation was carried out across the geographic zone of range overlap of *E. fulgens* and *E. puniceoluteum* along the coast of Brazil. To test the strength of their reproductive barriers, we examined the distribution of genetic variation within and among sympatric and allopatric populations of these two species. Nine specifically developed nuclear microsatellite loci and five chloroplast microsatellite loci were used to genotype 463 individuals from eight populations across the geographical range of both species. All six sympatric populations analyzed presented hybrid zones, indicating that hybridization between *E. fulgens* and *E. puniceoluteum* is a common phenomenon. Bayesian assignment analysis detected the presence of F_1 and F_2 individuals and signs of introgression as well, demonstrating a high potential for interspecific gene flow. Introgression occurs preferentially from *E. fulgens* to *E. puniceoluteum*. Pure parental individuals from both species display strong genotype-habitat associations, indicating that environment-dependent selection can be at work in all hybrid zones. This study suggests that hybridization and introgression could play an important role in the diversification of *Epidendrum*, and indicates the importance to investigate hybrid zones for better understanding of the reproductive barriers and speciation processes in Neotropical orchid species.

Introduction

Natural hybrid zones offer exciting opportunities to investigate speciation and species evolution through genetic exchange (Barton & Hewitt 1985; Arnold 2006). Hybrid zones are of special interest for studying the genetics and evolution of reproductive isolation (RI) among co-occurring species, because they facilitate the study of all possible components of RI *in situ*, i.e. under natural conditions (Barton &

Hewitt 1985; Cruzan & Arnold 1993; Rieseberg et al. 1999; Lexer et al. 2005, Moccia et al. 2007).

Hybrid zones between cytotypes with different ploidy levels are of particular interest for studies of the ecology and evolution of reproductive interactions in mixed-ploidy species complexes (Petit et al. 1999). The hypothesis of gene flow between species with extensive differences in chromosome numbers has rarely been tested (Petit et al. 1999), mainly because species with different ploidy levels are commonly assumed to be reproductively isolated from one another due to strong post-zygotic barriers (Coyne & Orr 2004). Recent theoretical and empirical studies have provided important evidence for the evolution of isolating mechanisms in diploid-polyploid contact zones, including the possibility of introgression across species boundaries (Menken et al. 1995; Ramsey & Schemske 1998; Petit et al. 1999; Aagaard et al. 2005).

Many hybrid zones of animals and plants are maintained by a balance of dispersal into the centre of the zone of contact on the one hand, and intrinsic (endogenous) selection against hybrids on the other (Barton & Hewitt 1985). In more complex hybridization scenarios involving exogenous selection and introgression across "mosaic" hybrid zones (Rieseberg et al. 1999), the examination of genotype-habitat associations is of interest (Johnston et al. 2001). Divergent selection associated with differences in habitat can have a strong impact on patterns of RI and gene flow between divergently adapted species in diploids and polyploids alike, which will affect the impact and outcome of interspecific introgressive hybridization (Seehausen et al. 2008). Tests for genotype-habitat associations are greatly facilitated by the availability of species-informative molecular markers originating from different genomic compartments, most typically the nuclear and plastid DNA genomes in the

case of plants (Soliva & Widmer 2003; Lexer et al. 2005; Moccia et al. 2007). Although population dynamics in mixed-polyploidy complexes is known to be more complex than in diploids (Ramsey & Schemske 1998; Petit et al. 1999), genetic analysis based on allele sharing (e.g. Pritchard et al. 2000; Anderson & Thompson 2002) is feasible as long as the homology of DNA-based markers can be established.

Whether or not interspecific hybridization is important as a mechanism generating biological diversity in Orchidaceae is a matter of controversy. Several orchid hybrid zones have recently been documented and investigated with molecular genetic markers, providing evidence either for or against a potential creative role of hybridization in orchid evolution (Aagaard et al. 2005; Cozzolino et al. 2006; Moccia et al. 2007; Cortis et al. 2009; Scopece et al. 2008). In the case of neotropical orchids, only limited and indirect evidence is available for the strength of RI between related species. Intermediacy in morphological characters, sexual compatibility between putative parental species and hybrid viability have all been used to infer the role of hybridization in orchid diversification in the neotropics (Van der Pijl & Dodson 1966; Pansarin & Amaral 2008). Unfortunately, the genomic composition of putative hybrids remains unknown for most of these cases (but see Azevedo et al. 2006). Since information about genomic ancestry and admixture in neotropical orchid hybrid zones is unavailable, the evolutionary processes underlying hybrid zone dynamics and genetic cohesion of parental species in species-rich neotropical biomes remains obscure.

Epidendrum (subtribe Laeliinae) is the largest orchid genus in the Neotropics, comprising ca. 1500 species (Hagsater & Soto Arenas 2005). The genus is composed of species complexes with many taxonomic uncertainties due to extensive morphological variability and wide variation in chromosome numbers. Hybridization

has often been hypothesized as a driving force responsible for the origin of chromosomal and morphological variation in *Epidendrum* (Dunsterville 1979; Hágsater & Soto Arenas 2005, Pinheiro et al. 2009b). The existence of hybrid zones between *Epidendrum* species has been postulated by many authors based on morphological and distributional data (Van der Pijl & Dodson 1966; Dunsterville 1979; Hagsater & Soto-Arenas 2005; Pansarin & Amaral 2008). The main evidence used to describe these hybrid swarms was the presence of individuals showing intermediacy in flower morphology relative to their putative parental species. Reproductive compatibility was reported for many co-occurring *Epidendrum* species, based on controlled interspecific crosses (Dunsterville 1979; Pansarin & Amaral 2008). In addition, a lack of pollinator specificity among *Epidendrum* species (Van der Pijl & Dodson 1966; Fuhro 2006; Pansarin & Amaral 2008) indicates weak or absent pre-pollination barriers, thus making hybridization in natural populations feasible. Human activities have sometimes been reported as being responsible for the origin of *Epidendrum* hybrid swarms, as many species experienced contact following habitat disturbance (Dunsterville 1979; Hagsater & Soto-Arenas 2005; Pansarin & Amaral 2008). To our knowledge, the numerous possible instances of hybridization among *Epidendrum* species have never been tested with rigorous molecular genetic methods.

Epidendrum fulgens and *E. puniceoluteum* are two enigmatic *Epidendrum* species that co-occur in many localities along the Brazilian sandy coastal plains in restinga vegetion (see Material and Methods for details) between the Brazilian states of Rio de Janeiro and Rio Grande do Sul (24°50.886′S to 28°12.538′S, Fig. 1). The two species are well differentiated for floral morphological traits and flower color: orange sepals and petals with yellow labellum in *E. fulgens*, red petals, sepals and

labellum with a yellow labellum callus in *E. puniceoluteum* (Pinheiro & Barros 2006). Phylogenetic analyses (Pinheiro et al. 2009b) indicated that *E. fulgens* and *E. puniceoluteum* are very closely related species, despite their different chromosome numbers ($2n$ = 2x = 24 and $2n$= 4x = 52, respectively). The existence of hybrids between the two species has been suspected ever since specimens with intermediate morphological characters were found in herbarium collections and natural populations where these species co-occur in sympatry (Fig. S1) (Pinheiro & Barros 2006). Experimental crosses between two species, carried out on cultivated individuals, produced viable seeds and plants, suggesting that hybridization is possible (Pinheiro et al., unpubl. res.).

In the present study, a broad-scale survey of molecular variation was carried out across the geographic zone of range overlap of *E. fulgens* and *E. puniceoluteum* along the coast of Brazil. Specifically, we aimed to answer the following questions related to the genetics and evolution of RI between these neotropical orchids: (1) What is the genomic composition of hybrids when estimated with species-informative sets of nuclear and plastid DNA microsatellites, and what do the genomic patterns tell us about the likelihood of hybridization and gene flow across ploidy levels? (2) How strong are genotype-habitat associations in zones of sympatry, and what do they teach us about the role of ecology in currently maintaining the species barrier? (3) Which types of pre- and postzygotic barriers are likely to be most important in RI between these species? Our results allow us to discuss the role of hybridization in diversification of this large and successful group of neotropical orchids. The data also challenge the widely-held view of "instant isolation" among species with different ploidy levels (Coyne & Orr 2004).

Materials and Methods

Plant species and habitat characteristics

E. fulgens and *E. puniceoluteum* are species pollinated by butterflies, following a model of pollination by deceit, as there is no reward (nectar) for the pollinators (Moreira et al. 2008, Pinheiro unpubl. res.). Fuhro (2006) observed 29 butterfly species acting as potential pollinators of *E. fulgens*, indicating a non-specific pollination system. Both species are self-compatible, but pollinator are necessary for pollen transfer. In addition, flowering phenologies and the identities of their pollinators overlap extensively (Fuhro 2006, Pinheiro unpubl. res.).

The two orchid species typically inhabit restinga vegetation, composed of a mosaic of different coastal plant communities along the Brazilian seashore (Araujo 1992). The structure and dynamics of this vegetation is affected by a sharp gradient of abiotic factors (e.g., salt spray, sand movement, soil moisture content, water availability, etc) that decreases in intensity with increasing distance from the beach (Araujo 1992; Scarano 2002). Following Araujo (1992), restinga vegetation can be subdivided into several vegetation zones, based on their floristic composition. *E. fulgens* and *E. puniceoluteum* are mainly found in two different vegetation zones: sand dunes that are not subject to inundation, composed of shrubby vegetation, corresponding to *vegetation zone 2* (according to Araujo 1992); and sedge swamp communities flooded during most of the year, located in depressions between successive beach ridges, corresponding to *vegetation zone 5* according to Araujo (1992). Vegetation zone 5 is located further inland than vegetation zone 2, but both are adjacent to each other (Fig. 2). Based on field observations, individuals of *E.*

fulgens are more abundant on vegetation zone 2, and *E. puniceoluteum* can be found more oftenly on vegetation zone 5.

Population sampling

A total of six putative hybrid zones were sampled, in which individuals of *E. fulgens*, *E. puniceoluteum* and putative hybrids were collected (Fig. 1). To test for habitat and genotype associations, the type of habitat in which individuals were collected, zone 2 or zone 5 according to Araujo (1992) was recorded. In addition, samples were collected from one allopatric population of *E. fulgens* and one allopatric population of *E. puniceoluteum*, to be analyzed as reference populations (Figure 1). In total 463 individuals were sampled (Table 1). Individuals were collected nonexhaustively but randomly with a minimum distance of 10 m. In order to extract a maximum of information regarding the detection of hybridization, we avoided collecting individuals stemming from vegetative reproduction (individuals growing in clusters with many stems close to each other). Sample sizes, names and geographical coordinates of the sampled populations are given in Table S1. For each plant, leaf material for DNA extraction was collected and dried in silica gel. Total genomic DNA was extracted as described by Pinheiro et al. (2008a).

Molecular markers and genotyping assays

Nine microsatellite nuclear markers were used in this study, six isolated from *E. fulgens* (markers EFF26, EFF29, EFF43, EFF45, EFF61, EFF70; Pinheiro et al. 2008a) and three isolated from *E. puniceoluteum* (markers Epp10, Epp18, Epp86; Pinheiro et al. 2008b). The proportion of microsatellites that successfully cross-amplified between the two species was carefully evaluated, as it contains important

information about homology of the loci: our expectation was that *all or most* loci isolated from *E. fulgens* should amplify in *E. puniceoluteum*, as chromosome counts (Introduction) indicate that the latter was derived from an allo-polyploidization event involving the former as one parent. For exactly the same reason, ca. *half* of the loci isolated from *E. puniceoluteum* should not be present in *E. fulgens*.

Five plastid microsatellite loci (Epcp01, Epcp02, Epcp04, Epcp08, Epcp09; Pinheiro et al. in press) were screened for identifying and characterizing plastid DNA haplotypes. All PCR amplifications were performed in an Applied Biosystems 2700 thermocycler following protocol described by Pinheiro *et al*. (2008a). The conditions were maintained constant for all loci to maximize standardization. Microsatellite alleles were resolved on a 3130 DNA Sequence Analyzer and were sized with LIZ (500) standard by using GENEMAPPER v3.7 software (Applied Biosystems).

Data analysis

Nuclear admixture analysis and assignment tests. The classification of individuals based on morphological characters was difficult, especially in sympatric populations, due to extensive morphological variability found in both species and putative hybrids (Fig. S1). Thus, individuals were classified as *E. fulgens*, *E. puniceoluteum* and hybrids using nuclear molecular markers, analyzed by Bayesian assignment tests. To achieve this, allopatric populations of each species were used as reference samples of pure individuals of *E. fulgens* and *E. puniceoluteum*. To estimate nuclear admixture proportions and patterns of introgression, two different Bayesian clustering approaches were carried out, as implemented in the programs STRUCTURE version 2.2 (Pritchard *et al*. 2000) and NEWHYBRIDS version 1.1 beta (Anderson and Thompson 2002). Analyses were performed separately for each hybrid zone,

including specimens from allopatric populations used as reference samples from each species. In the model implemented in STRUCTURE, the posterior probability (q) describes the proportion of an individual genotype originating from each of K categories. The K = 2 model was used, corresponding to the assumption of two species contributing to the gene pool of the sample. The NEWHYBRIDS model assumes that samples are drawn from two pure parental individuals and hybrids. Under this model, q describes the posterior probabilities that each individual belongs to each of the six genotypic classes that originate after two generations of hybridization: parental purebreds, F1, F2 and the two first back-crosses categories. With STRUCTURE, calculations were carried out under the admixture model allowing for correlated allele frequencies. A burn-in of 50 000 steps followed by run lengths of 300 000 were used in each program. The choice of the optimal threshold value (Tq) for the q associated with the classification of each individual into purebred or hybrid was based on simulations performed as described in Vähä and Primmer (2006) and Burgarella et al. (2009) (details of the simulations are available from the corresponding author upon request). With STRUCTURE, a value of q higher or equal to the threshold indicates a purebred genotype and a value of q lower than the threshold indicates an admixed (hybrid) genotype. With NEWHYBRIDS, the threshold value was applied to each category (parental purebred, F1, F2 and backcrosses) separately, by assigning only the individuals with $q \geq Tq$ and taking the others as unassigned.

Genetic diversity of sampled loci and populations. Describing patterns of genetic diversity was not an important goal of this paper, but characterizing the studied loci and populations is important in any speciation genetics study. In order to characterize

the nuclear microsatellite loci in the two studied species and their hybrids, the number of alleles (*A*), variance in allele size (Var), expected heterozygosity (H_E), observed heterozygosity (H_O), fixation index and inbreeding coefficient (Weir and Cockerham, 1984) were calculated for each locus using the program FSTAT (Goudet 1995). In addition, departures from Hardy–Weinberg equilibrium (HWE) for each locus within each species and hybrids were tested using GENEPOP (Raymond & Rousset 1995). The microsatellite data set was tested for genotyping errors due to stuttering, short allele dominance and null alleles using a Monte Carlo simulation of expected allele-size differences using MICRO-CHECKER (Van Oosterhout *et al*. 2004). Subsequently, each population studied was characterized using the number of alleles (A), variance in allele size, H_E, H_O and allelic richness calculated by FSTAT. Departures from HWE for each population were identified using exact tests in GENEPOP. *F*-statistics were estimated for both species in each mixed population and in the whole set of individuals, excluding hybrids, following the weighted analysis of variance method of Weir & Cockerham (1984) using FSTAT. In order to explore the existence of clones in both species and hybrids we performed an analysis using Gimlet software (Valière 2002).

In addition, populations Ilha Comprida, Ilha do Cardoso, Pontal do Sul, Florianópolis, Imbituba and Torres were characterized for levels of diversity at plastid DNA markers, using the number of haplotypes detected in each sample, H_E and F_{ST} among species and within each species estimated by ARLEQUIN 3.01 (Excoffier et al. 2005).

Plastid DNA haplotype network. A median-joining (MJ) network (Bandelt *et al*. 1999) was constructed based on plastid DNA haplotypes using the program NETWORK v.

4.5.1.0 (www.fluxus-engineering.com). It uses maximum parsimony criteria to reconstruct all possible shortest least complex phylogenetic trees. Following the user manual, we excluded singleton haplotypes to simplify the network. The default settings were used for all other parameters. To test for a possible role of cyto-nuclear incompatibilities in determining patterns of hybridization across the hybrid zone (Arnold 1993), nuclear admixture proportions, based on STRUCTURE results, and plastid DNA haplotype data were compared using the nonparametric Spearman rank correlations with the software SPSS 13.0 for Windows (SPSS Inc.).

Results

Genetic composition of hybrid zones

Based on the results from simulations (Table S2 and S3), STRUCTURE was used to classify individuals among the two parental species and hybrids in the hybrid zones, using a threshold of q=0.90. NEWHYBRIDS was used to classify hybrids, which were previously classified by STRUCTURE, in classes (F1, F2 and backcrosses), using a threshold value of q=0.75 (details are available upon request).

Bayesian admixture analysis indicated that the allopatric populations of *E. fulgens* and *E. puniceoluteum*, used as reference populations, were composed exclusively of purebreds (STRUCTURE threshold q-value $\geq$ 0.900 or $q \leq$ 0.100, respectively) (Fig. 3, S2). For most individuals of *E. fulgens* classified in the field as pure, species status was confirmed with STRUCTURE with a threshold of $q > 0.90$ (Fig. 3). In contrast, many individuals classified in the field as *E. puniceoluteum* showed intermediate q values ($0.90 \leq q \geq 0.10$) with STRUCTURE), indicating hybrid ancestry. The existence of hybrids was confirmed in all six sympatric populations

(Figs. 3 and S2). A total of 205 individuals were classified as hybrids (Table 1), ranging from 27(39.71%) in Ilha do Mel) to 53(64.63%) in Ilha do Cardoso.

One hundred and four individuals out of 205 were unequivocally assigned to different genotypic classes (F1, F2 and backcrosses) with NEWHYBRIDS using *q*-values ≥ 0.75 (Fig. 3, S2). In total, 32 F1, 67 backcrosses with *E. puniceoluteum* and five F2 genotypes were identified, (Fig. 3, S2). The hybridization patterns recovered indicate unidirectional introgression across the species barrer, and variation in hybrid genomic composition among populations. Whereas F1 were frequent in the Ilha do Cardoso population, backcrosses with *E. puniceoluteum* were more abundant in the remaining hybrid zones (Fig. 3 and S2). No backcrosses towards *E. fulgens* were detected in any of the studied populations. There is no clear pattern about specific morphological features on the depicted hybrid classes, because F1 and backcrossed individuals showed both intermediate characteristics between the parental species and more similar morphological traits with *E. puniceoluteum* (Fig. 3 and S2).

Habitat associations among purebred individuals and hybrids

Individuals classified as pure *E. fulgens* and pure *E. puniceoluteum* based on nuclear markers differed strongly in their habitat preferences (Fig. 2). All individuals classified as pure *E. puniceoluteum* were collected in the swamp habitat (zone 5), pure *E. fulgens* specimens were found growing on sand dunes (zone 2), while hybrids were found in both habitats (Fig 2). In the Florianópolis population, where the swamps were restricted to few and small patches, pure *E. puniceoluteum* individuals were not identified. In the sand dune habitat, *E. fulgens* plastid DNA haplotypes were found in individuals classified as *E. fulgens* and as hybrids based on the nuclear genetic data, whereas haplotypes specific to *E. puniceoluteum* were found exclusively in hybrids

(Fig. 2). In the swamp habitat, haplotypes specific to *E. fulgens* were found in both species and hybrids; whereas haplotypes specific to *E. puniceoluteum* were found in pure individuals of *E. puniceoluteum* and hybrids only (Fig. 2).

Variability at nuclear microsatellite loci

All nine nuclear microsatellites were polymorphic, with up to 30 alleles per locus, expected heterozygosities (H_E) from 0.005 to 0.908 and observed heterozygosities (H_O) from 0.005 to 0.917 (Table 2). The variance in allele size ranged from 0 to 85.2. Three loci displayed significant deviation from HWE in *E. fulgens* and two in *E. puniceoluteum*, possibly reflecting occasional departures from random mating, as tests for null alleles in the MICRO-CHECKER software were not significant. No locus displayed consistent departures from HWE across all two species and hybrids (Table 2). The microsatellites showed great and significant genetic differentiation between the two species in the entire dataset and in each locality (whole dataset: $\theta = 0.39$, P-value = 0.001; minimum $\theta = 0.31$, Imbituba; maximum $\theta = 0.46$, Ilha do Cardoso). Intraspecific differentiation (Table 2) was much lower in *E. fulgens* than in *E. puniceoluteum* ($\theta = 0.03$ and $\theta = 0.127$, respectively), and inbreeding coefficients (Table 2) was low in both species ($f = 0.05$ and $f = 0.06$, respectively).

Genetic Diveristy in Epidendrum *populations*

Genetic diversity evaluated at the population level was always higher in *E. fulgens* than in *E. puniceoluteum*, regardless of whether it was estimated via variance in allele size (Var), allelic richness (AR), expected heterozygosities (H_E), or observed heterozygosities (H_O) (Table 1), which probably reflects differences in population sizes found between the species (higher in *E. fulgens*). *E. fulgens* and *E.*

puniceoluteum from Imbituba displayed significant departures from HWE due to heterozygote deficits. Three hybrid zones deviated from HWE, indicating departures from random mating. No instances of clonality (=duplicated genotypes) were found in any of the populations, thus indicating an important role for sexual reproduction in the maintenance of *Epidendrum* hybrid zones.

Plastid DNA diversity and haplotype network

A total of 11 haplotypes were identified based on the five plastid microsatellite analyzed. Four haplotypes were removed from the subsequent analysis because they were restricted to single individuals (singletons). Genetic diversity in populations (Table 1) ranged from 0.0 (one haplotype) to 0.455 (four haplotypes). Great differentiation among species ($\theta = 0.89$) and and low genetic structure within species were detected (*E. fulgens:* $\theta = 0.03$ and *E. puniceoluteum*:$\theta = 0.05$). The median-joining analysis, based on seven haplotypes, resulted in a network with two major groups (Fig. 4), one of which contained five haplotypes typical of *E. fulgens* (individuals with $q \geq 0.900$), whereas the other one consisted of haplotypes from *E. puniceoluteum* (individuals with $q \leq 0.100$). Hybridization occurred in both directions (*E. fulgens* pollen and seeds, and *E. puniceoluteum* pollen and seeds), since all types of haplotypes could be observed in hybrid individuals (Table 3). Haplotypes H2, typical from *E. fulgens*, and H11 typical from *E. puniceoluteum*, were present in F1, F2 and backcrossed individuals. There was no evidence of plastid DNA introgression into *E. fulgens*, whereas two individuals of *E. puniceoluteum* carried haplotypes typical of *E. fulgens* (H2), thus indicating introgression of the plastid DNA molecule. A wide range of nuclear admixture proportions was detected in hybrids carrying *E. fulgens* (q = 0.051-0.988) and *E. puniceoluteum* haplotypes (q = 0.014-0.884),

indicating extensive variation in hybrid ancestry (Table 3), and a significant correlation was detected between nuclear and plastid genomic composition (Spearman´s r = -0.695, P = 0.000), suggestive of cyto-nuclear incompatibilities.

Discussion

Hybridization in Epidendrum *sympatric populations*

Hybridization was detected in all sympatric populations analyzed (Fig. 3, S2), confirming previous hypothesis of hybridization between *E. fulgens* and *E. puniceoluteum* based on floral morphology (Pinheiro & Barros 2006). Nuclear and plastidial microsatellite loci, revealed admixture proportions and genetic architecture of all hybrid zones (Fig. 3, S2), and were able to depict species origin of plastid DNA variants (Fig. 4). The nuclear data indicate F1 and backcrossing towards *E. puniceoluteum* as the main classes in the hybrid zones. Hybridization occurs in both directions, and hybrid F1s can act as pollen donors and receptors to produce backcrossed individuals with *E. puniceoluteum* (Table 3). Pure individuals from both species showed several fixed chloroplast haplotypes (Fig. 4; Table 3), suggesting that those hybrid zones have arisen by a secondary contact, after enough time to accumulate the amount of differences observed. Different habitat associations were detected between parental genotypes (Fig. 2), suggesting that niche divergence contributes to species cohesion as a potential barrier limiting gene flow.

The dataset not only describe the architecture of the hybrid zones, they also contain important information about the main ecological processes shaping the current gene flow between *E. fulgens* and *E. puniceoluteum*, the strength of pre and post zygotic barriers between the parental species and how hybridization play a role in *Epidendrum* diversification.

Hybridization between different ploidy levels

The extensive amount of recombinant hybrids classes (F2 and backrossing) found between *E. fulgens* and *E. puniceoluteum* indicate that differences in ploidy levels (2*n* = 2x = 24 and 2*n*= 4x = 52, respectively) are not enough to prevent introgression. Thus the presence of introgressive hybridization between *E. fulgens* and *E. puniceoluteum* challenge the widely-held view of "instant isolation" among species with different ploidy levels (Coyne and Orr 2004). Indeed this pattern has being showed for other plant groups, reviewed by Ramsey and Schemske (1998), as well for other orchids species (see discussion below).

Our data showed backcrossing only towards the tetraploid species: *E. puniceolutem*. Our results are similar with those obtained for two studies on orchids hybridization in the genus *Dactylorhiza*: a) *D. incarnata* ssp. *cruenta* (2n=2x=40) and *D. lapponica* (2n=4x=80) (Aagaard et al. 2005); b) *D. maculata* ssp. *fuchsia* (2n=2x=40) and *D. maculata* ssp. *maculata* (2n=4x=80) (Ståhlberg and Hedrén 2009). In these studies hybrid zones are composed by first generation and backcrosses towards the polyploid parental species. However, backcrossing is thought to occur more often between a triploid hybrid and the diploid ancestor, rather than the tetraploid ancestor (Ramsey and Schemske, 1998). Why our results and those with *Dactylorhiza* deviated from the expected pattern is difficult to explain (Aagaard et al. 2005; Ståhlberg and Hedrén 2009). Moreover, cross-amplification tests using nuclear microsatellite loci developed specifically for *E. fulgens* and *E. puniceoluteum* shows that all loci developed for *E. fulgens* (diploid) can be successfully amplified in *E. puniceoluteum* (tetraploid), but only 60% of the markers developed for *E. puniceoluteum* could be amplified in *E. fulgens* (Pinheiro et al. 2009a). All together

these information could indicate that *E. puniceolutem* is an allotetraploid having *E. fulgens* as one parent. The presence of genomic elements of *E. fulgens* in the genome of *E. puniceoluteum*, could facilitate chromosome pairing between F1 hybrids and *E. puniceoluteum*, but not the opposite. The same pattern was found in hybrid zones between the diploid *Dacthylorhiza incarnata* and its putative allotetraploid derivative *D. lapponica*, were introgression was found towards the allotetraploid species *D. lapponica* (Aagaard et al. 2005). The role of a possible existence of a triploid bridge between *E. fulgens* and *E. puniceoluteum* in gene exchange and introgression needs further clarification, since this process is widespread in many plant groups (Ramsey and Schemske, 1998).Undergoing cytogenetic studies in the meiotic behavior and GISH analyses (genome *in situ* hybridization) of both parental species and hybrids will help to clarify chromosome homologies and parentage.

Overcome of pre and post reproductive isolation

E. fulgens and *E. puniceoluteum* are food deceptive orchids pollinated by many species of butterflies (Fuhro 2006; Moreira et al. 2008, Pansarin & Amaral 2008, Pinheiro unp. res.). Food deception is a pollination strategy that can attract a guild of locally available pollinators, and pollinator sharing is common, as observed for orchid species in Mediterranean region (Cozzolino et al. 2004). Low values of inbreeding coefficients and fixation indexes (Table 2) were observed, indicating a low genetic structure among populations within species. These patterns are in accordance with expected for species without reward, because this mechanism reduces the chances for geitonogamous pollination, promoting outcrossing and reducing population differentiation (Soliva & Widmer 2003, Cozzolino & Widmer 2005). In

addition, sexual reproduction plays a major role in parental species reproduction and in hybrid formation as well, as clone individuals were not found.

Epidendrum hybrid zones seem to have weaker post zygotic barriers to gene flow than other food deceptive orchids, as a large proportion of hybrids is constituted by backcrossed individuals. Hybridization and respective breakdown of species boundaries were observed for many co-occurring orchid species in the Mediterranean region (Cozzolino et al. 2004; Moccia et al. 2007 and references therein). In food deceptive orchids, where pollinator specificity is weak or absent (Moccia et al. 2007; Scopece et al. 2008), gene flow among co-occurring species is limited by late postzygotic reproductive barriers, such as chromossomic rearrangements (Cozzolino et al. 2004) promoting F1 hybrid infertility and preventing backcrossing (Moccia et al. 2007; Scopece et al. 2008). This scenario suggests that pre and post mating barriers are not the unique forces acting in the maintenance of the *Epidendrum* hybrid zones.

The continued backcrossing and introgression can be facilitated by intrinsic features of Orchidaceae, playing a role in the overcome of apparently strong post zygotic reproductive barriers, such as hybrid inviability and sterility. The high number of ovules and pollen grains in the flowers, and the complete lack of endosperm in the seed, improve the possibilities of seed production in nearly sterile hybrids. Once formed, F1 and backcrossed hybrids can act as source of pollen and seed for several years. *Epidendrum* species are long lived perennials plants, which could also provide to nearly sterile hybrids greater chances of reproduction (van der Pijl & Dodson 1966).

Habitat and genotype associations and differences among hybrid zones

Pure parental individuals from both species display strong association with different habitats in restinga vegetation (zone 2: sand dunes and zone 5: swamps), indicating that environment-dependent selection can be at work in all hybrid zones. Similar patterns have been observed in *Ranunculus* and *Iris* (He *et al*. 1999; Johnston et al. 2001), and can be explained by competitive displacement from the more optimal (i.e. drier) habitat. In this system, higher flood tolerance in one species permits coexistence with another competitive dominant that is intolerant to flooding (Johnston et al. 2001). Previous studies have suggested that selection in hybrid zones is dependent on environmental factors (Cruzan & Arnold 1993; He *et al*. 1999; Johnston et al. 2001; Ståhlberg and Hedrén 2009). Tolerance of salinity stress and flooding are characteristics playing an important role in hybrid zones on coastal habitats (Van Zandt et al. 2003; Johnston et al. 2001). The importance of these abiotic factors in the *Epidendrum* hybrid zones needs further investigations. Reciprocal transplant experiments between pure individuals of *E. fulgens* and *E. puniceoluteum* will provide a measure of divergent natural selection occurring in sand dunes and swamps, (Schluter 2000).

Extensive differences in the genetic architecture of *Epidendrum* hybrid zones were detected by nuclear markers, including the predominance of F1 and F2 individuals in Ilha do Cardoso, and the high frequency of backcrossed hybrids, towards *E. puniceoluteum* in the other localities. In addition, the presence of chloroplast haplotypes from both parental species in natural hybrids indicates that hybridization between *E. fulgens* and *E. puniceoluteum* has occurred in both directions (Fig. 2, 4), but the frequency of plastid DNA haplotypes was different among the sympatric areas (Table 1). In different species, the genetic architecture of hybrid zones can differ substantially across different localities, with hybrid zones

composed exclusively by F1 individuals (Moccia et al. 2007) and others that are dominated by backcrossed genotypes (Cruzan & Arnold 1993; Lexer et al. 2005).

Several local ecological factors might explain this discrepancy in the direction of gene flow among hybrid zones, such as different frequencies with which parental individuals occur among hybrid zones (Rieseberg 1995), the presence of different rewarding plants that can act as models for these deceptive orchids (Fuhro 2006) and age of the hybrid zones (Moccia et al 2007). *E. fulgens* and *E. puniceoluteum* populations are located in restinga vegetation localities based on young marine sand deposits (Holocene sedimentary barriers) formed at 5100 years B.P. (Lessa et al. 2000). Historically, the variation in the sea level promoted drastic modifications in the floristic composition of many localities (Ybert et al. 2003), changing the amount of sand dunes and/or swamps available. In Ilha do Cardoso, the predominance of F1 and F2 individuals suggests a relative young age of this hybrid zone. Some studies (Chung *et al*. 2005) suggested that the occurrence of only F1 hybrids in a hybrid zone might be evidence for recent hybrid formation where not enough time has elapsed to produce advanced generation hybrids. In addition, this locality showed the higher amount of hybrid individuals detected among all other hybrid zones. Highly dynamics changes in habitat availability can be ruled out as the main cause of the absence of pure individuals of *E. puniceoluteum* in Florianópolis as well. The swamps, where *E. puniceoluteum* is more abundant, were small and patchy in this population. The influence of the sand dunes is strong on this part of the Brazilian coast (Araujo 1992), and the wind intensively change the position of the sand dunes, which tend to cover flooded areas. *E. fulgens* is not affected as this species is more abundant on sand dunes. The main part of hybrids showed plastid DNA haplotypes from *E. puniceoluteum*, which is less abundant and more suitable for act as pollen recipient,

and thus as female parent, in hybrid formation in Florianópolis population (Table 1). The collapse of a species assemblage to a hybrid swarm can be associated with a reduction in population densities caused by loss of environmental heterogeneity, generating a deep impact on the hybrid zones composition (Seehausen et al. 2008).

Gene flow and species cohesion

E. fulgens shows higher levels of gene flow among populations considering the nuclear and plastid markers ($\theta = 0.03$ for both markers) and no signs of introgression were detected in this species. Those results are in agreement with the discussed and predicted by Petit and Excoffier (2009), showing that the rate of introgression is most often negatively correlated with the rate of intraspecific gene flow. Gene flow among populations of *E. puniceoluteum* is less effective considering the nuclear markers ($\theta = 0.127$), and introgression is common in almost all hybrid zones. Under a scenario where species ranges are dynamic, if a species expands its range and meets a closely related species with which reproductive barriers are still incomplete, asymmetric introgression will take place from the local species to the colonizing species (Currat et al. 2008). According to our results, *E. puniceoluteum* could be the colonizing species, and *E. fulgens* the local species, where the enhanced gene flow is important for its cohesive evolution. The phylogenetic ancestral position of *E. fulgens* relative to *E. puniceoluteum* (Pinheiro et al. 2009b) and the wider geographic distribution of *E. fulgens* (Fig. 1) are additional evidences of the colonizing nature of *E. puniceoluteum* populations.

Introgression and increase of ecological amplitude

Hybrid genotypes occupied sand dunes and swamps, with a large overlapping with their parental species in all sympatric zones (Fig. 2). The extensive variation in nuclear admixture proportions found in hybrid individuals (Table 3) seems to be connected with the ability to grow in different habitats, extending the ecological amplitude found in hybrids relative to the parental species (Lexer et al. 2005). The restinga vegetation is composed mainly by plant species with broad ecological plasticity, as these communities experience a wide array of adverse environmental conditions, such as flooding, drought, constant wind, high salinity and lack of nutrients (Scaranno 2002). Positive interactions among plants (nurse and pioneer species) play an important role in the structuring and functioning of coastal swamps and sandy vegetation (Scaranno 2002). Hybridization generating introgression towards *E. puniceoluteum* can be another positive interaction occurring in this ecosystem. In this context, introgression of genome elements from *E. fulgens* into *E. puniceoluteum* allows introgressed individuals to grow in swamps and sand dunes.

Gene flow might favor the transfer of adaptation among species with different cytotypes. Rieseberg et al. (1996) demonstrated that, although interactions between coadapted genes strongly constrain the genomic composition of hybrids in diploid *Helianthus* (Asteraceae), a non negligible proportion of these gene combinations might be favorable to the adaptive evolution of hybrids. In theory, it is thus possible that such unidirectional 'transfer of adaptation' from diploids to tetraploids by the production of advanced generation hybrids inflates tetraploid adaptability to diploid environments (Maceira et al. 1993). Transplant experiments can be used to test the hypothesis of increase of ecological amplitude in hybrids, using fitness measures taken from individuals with the same genetic architecture (reproduced vegetatively) growing in sand dunes and swamps (Schluter 2000).

Conclusions and future remarks

The framework produced by the joint analysis of nuclear and plastidial molecular markers offer a new frontier of hypothesis to be tested, considering the hybrid zones analyzed. Hybridization is a common evolutionary process in *Epidendrum*, with hybrids showing extensive ecological amplitude and acting as bridges for gene flow between different species. The extensive amount of hybrids detected in all hybrid zones, coupled with the presence of many backcrossed individuals, can explain the taxonomic difficulties in delimit species in *Epidendrum*, as pointed out by other authors (Dunsterville 1979; Hagsater & Soto Arenas 2005; Pansarin & Amaral 2008). Our results indicate that *Epidendrum* species are good models to study the evolution of reproductive barriers and speciation in the Neotropical region.

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Table 1: Characterization of populations of *Epidendrum fulgens, E. puniceoluteum* and hybrids, with nine nuclear and five plastid microsatellite markers, including the number of individuals sampled, number of alleles (A), allelic richness (AR), variance in allele size (Var), as well as expected (H_E) and observed (H_O) heterozygosities, the within population inbreeding coefficient *f* for nuclear microsatellites, the frequence of plastid DNA haplotypes found in each sample and the respective H_E for each population.

	Nuclear microsatellites						**Plastid microsatellites**	
Species (sample size)	A	AR	Var	H_E	H_O	*f*	Haplotypes (frequency)	H_E
Alopatric								
E. fulgens (25)	98	6.84	19.89	0.724	0.702	0.030	H2 (25)	0.000
E. puniceoluteum (27)	48	3.01	8.22	0.418	0.414	0.008	H11 (27)	0.000
Sympatric								
Ilha Comprida - SP								
E. fulgens (18)	75	5.97	14.67	0.642	0.616	0.041	H2 (18)	0.000
Hybrids (30)	77	7.44	11.82	0.621	0.605	0.025**	H2 (19), H9 (1), H11 (10)	0.388
E. puniceoluteum (21)	28	2.27	5.71	0.552	0.547	0.010	H2 (2), H9 (1), H11 (18)	0.161
Ilha do Cardoso - SP								
E. fulgens (20)	70	5.45	15.69	0.604	0.586	0.036	H2 (20)-	0.000
Hybrids (53)	81	7.99	13.68	0.631	0.614	-0.026**	H2 (29), H11 (24)	0.404
E. puniceoluteum (9)	16	1.71	1.19	0.373	0.136	0.639*	H11 (9)	0.000
Ilha de Superagui - PR								
E. fulgens (17)	77	6.38	20.89	0.649	0.586	0.084	-	-
Hybrids (27)	72	7.14	11.19	0.585	0.617	-0.054	-	-

Table 1: continued

	Nuclear microsatellites						Plastid microsatellites	
Species (sample size)	A	AR	Var	H_E	H_O	f	Haplotypes (frequency)	H_E
E. puniceoluteum (6)	18	2.00	3.88	0.512	0.500	0.027	-	-
Ilha do Mel - PR								
E. fulgens (23)	71	5.55	15.16	0.617	0.652	0.084	-	-
Hybrids (27)	82	7.95	14.08	0.620	0.664	-0.056	-	-
E. puniceoluteum (18)	31	2.48	9.10	0.446	0.412	0.076	-	-
Florianópolis - SC								
E. fulgens (35)	110	7.01	18.57	0.740	0.736	0.003	H2 (31), H3 (2), H6 (2)	0.044
Hybrids (20)	87	8.97	14.56	0.665	0.594	0.107*	H2 (3), H3 (1), H6 (1), H11 (15)	0.245
Imbituba - SC								
E. fulgens (38)	105	6.85	16.54	0.759	0.689	0.093**	H1 (3), H2 (34), H5 (1)	0.040
Hybrids (38)	93	8.58	8.29	0.684	0.663	0.028	H1 (5), H2 (12), H5 (1), H11 (20)	0.455
E. puniceoluteum (11)	28	2.62	3.03	0.472	0.375	0.222**	H11 (11)	0.000
Overall = 463 individuals								

Departures from Hardy–Weinberg equilibrium are indicated by asterisks (*P < 0.05, **P < 0.005)

Table 2: Genetic variability at nine nuclear microsatellite loci in *Epidendrum fulgens*, *E. puniceoluteum* and hybrids, including locus name, repeat type, number of alleles (A), and expected (H_E) and observed (H_O) heterozygosity for each locus.

	E. fulgens						**Hybrids**						***E. puniceoluteum***					
Locus	**A**	**Var**	H_E	H_O	θ	f	**A**	**Var**	H_E	H_O	θ	f	**A**	**Var**	H_E	H_O	θ	f
Epp10	22	18.6	0.903	0.806*	0.021	0.096	20	18.2	0.810	0.701**	0.032	0.136	9	8.4	0.508	0.378	0.094	0.220
Epp18	30	85.2	0.901	0.845**	0.026	0.073	24	24.2	0.804	0.748	0.024	0.068	12	1.7	0.167	0.183	0.082	-0.152
EFF26	7	1.0	0.524	0.506	0.158	0.043	9	2.1	0.725	0.823**	0.079	-0.132	6	0.5	0.123	0.043	0.498	0.224
EFF29	23	16.6	0.907	0.891	0.012	0.025	21	23.6	0.897	0.917	0.026	-0.020	17	15.5	0.717	0.708*	0.079	0.027
EFF43	7	1.4	0.349	0.351	0.052	0.018	7	0.2	0.137	0.132	0.044	0.024	2	0.3	0.095	0.091	0.025	0.019
EFF45	6	0.5	0.539	0.519	0.011	0.041	4	0.3	0.258	0.112**	0.037	0.562	2	0.2	0.103	0.099	0.067	0.020
EFF61	5	0.3	0.172	0.156	0.018	0.120	6	0.3	0.420	0.463	0.089	-0.103	2	0.2	0.401	0.383	0.198	-0.090
EFF70	22	17.6	0.908	0.891	0.007	0.006	21	15.2	0.834	0.863	0.009	-0.033	13	7.1	0.488	0.370**	0.040	0.093
Epp86	16	12.2	0.885	0.797**	0.015	0.072	16	6.4	0.829	0.855	0.021	-0.031	7	2.6	0.568	0.532	0.095	0.096
Overall	15.33	17.04	0.676	0.640	0.033	0.052	14.22	10.06	0.635	0.624	0.037	0.017	7.78	4.00	0.331	0.290	0.127	0.065

*P<0.05; **P<0.005, exact tests for departure from Hardy-Weinberg equilibrium.

Table 3: Plastid DNA haplotypes (H) found in 361 individuals from four hybrid zones, including indication about haplotype sharing among taxa, frequencies in both species and hybrids considering STRUCTURE and NEWHYBRIDS assignment probabilities and range of nuclear admixture proportions (Q) for plants carrying each haplotype (only with STRUCTURE admixture proportions).

		Frequencies (STRUCTURE assignment results)			STRUCTURE admixture range (Q)	Frequencies (NEWHYBRIDS assignment results)		
H	Haplotype group	*E. fulgens* (*N*=136)	Hybrids (N=141)	*E. puniceoluteum* (N=68)		F1	F2	BP
H1	*E. fulgens*	3	5	0	0.539-0.979			
H2	*E. fulgens*	128	63	2	0.051-0.991	18	4	30
H3	*E. fulgens*	2	1	0	0.624-0.990			
H5	*E. fulgens*	1	1	0	0.516-0.940			
H6	*E. fulgens*	2	1	0	0.598-0.988			
H9	*E. puniceoluteum*	0	1	1	0.014-0.265			
H11	*E. puniceoluteum*	0	69	65	0.012-0.884	14	1	37

For STRUCTURE assignment probabilities, were considered pure *E. fulgens*, pure *E. puniceoluteum* and hybrids individuals with posterior probabilities *q* of Q≥0.90, 0.90≤Q≥0.10 and Q≤0.10, respectively.
In NEWHYBRIDS assignment results, only hybrid classes with individuals showing posterior probabilities *q* of Q≥0.90 were included.

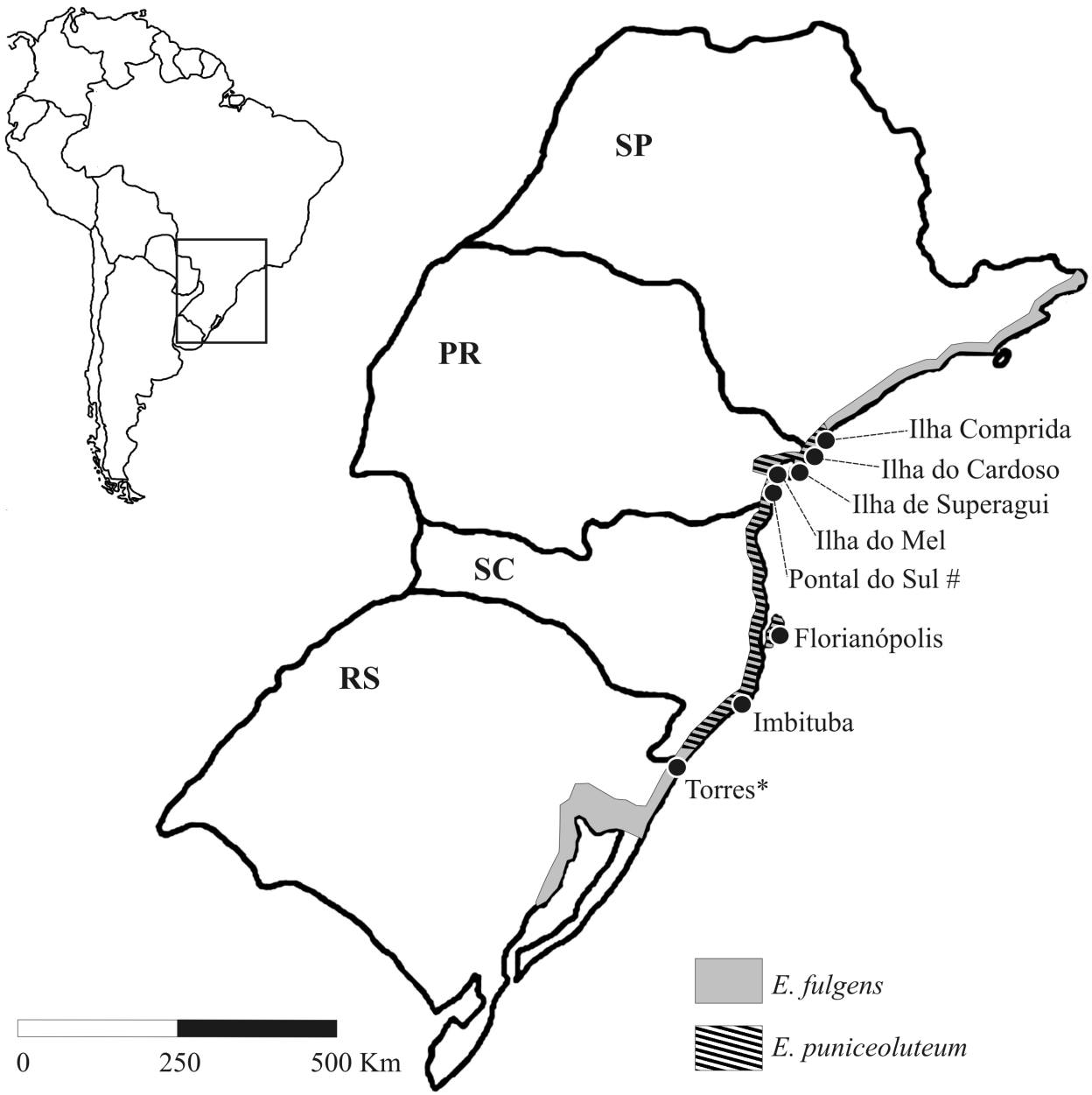

Figure 1: Distribution map of *Epidendrum fulgens* and *E. puniceoluteum* based on field collection and herbarium material, and location of the populations studied. Allopatric populations of *E. fulgens* (*) and *E. puniceoluteum* (#) are indicated. PR, Paraná; RS, Rio Grande do Sul; SC, Santa Catarina; SP, São Paulo states.

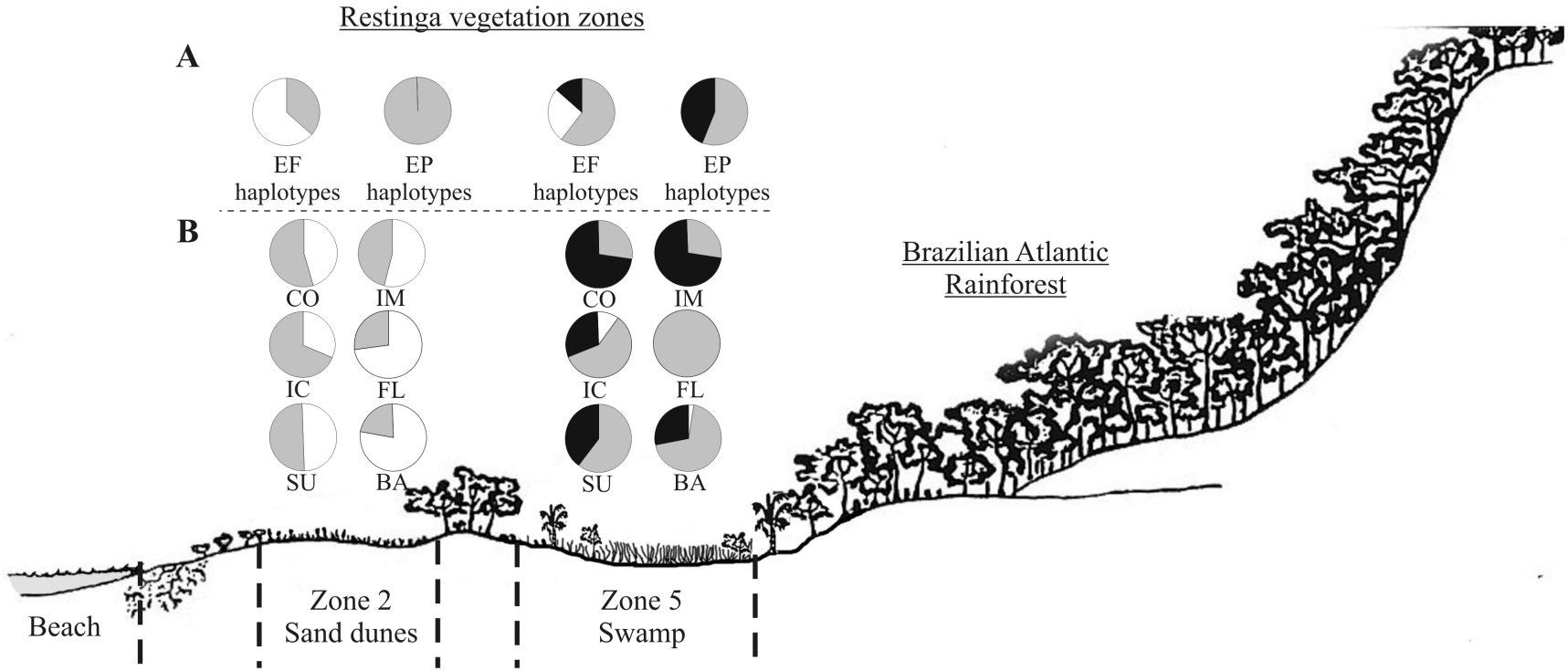

Figure 2: Schematic representation of a transect from the beach to the slopes of the Brazilian Atlantic Rainforest, indicating the location of vegetation zones 2 (sand dunes) and 5 (swamps) according to Araujo (1992), where the *Epidendrum* hybrid zones were sampled. A - Frequence of hybrids (grey) and pure individuals (*E. fulgens* - white; *E. puniceoluteum* - black) carrying the *E. fulgens* (EF) or *E. puniceoluteum* (EP) specific haplotypes, in each habitat. B - Frequence of pure parental species (*E. fulgens* - white; *E. puniceoluteum* - black) and hybrids (gray) in each population and habitat. See Table S1 for population codes, and Table 3 for specific haplotypes of each parental species. Pure parental species and hybrids were classified considering STRUCTURE assignment results.

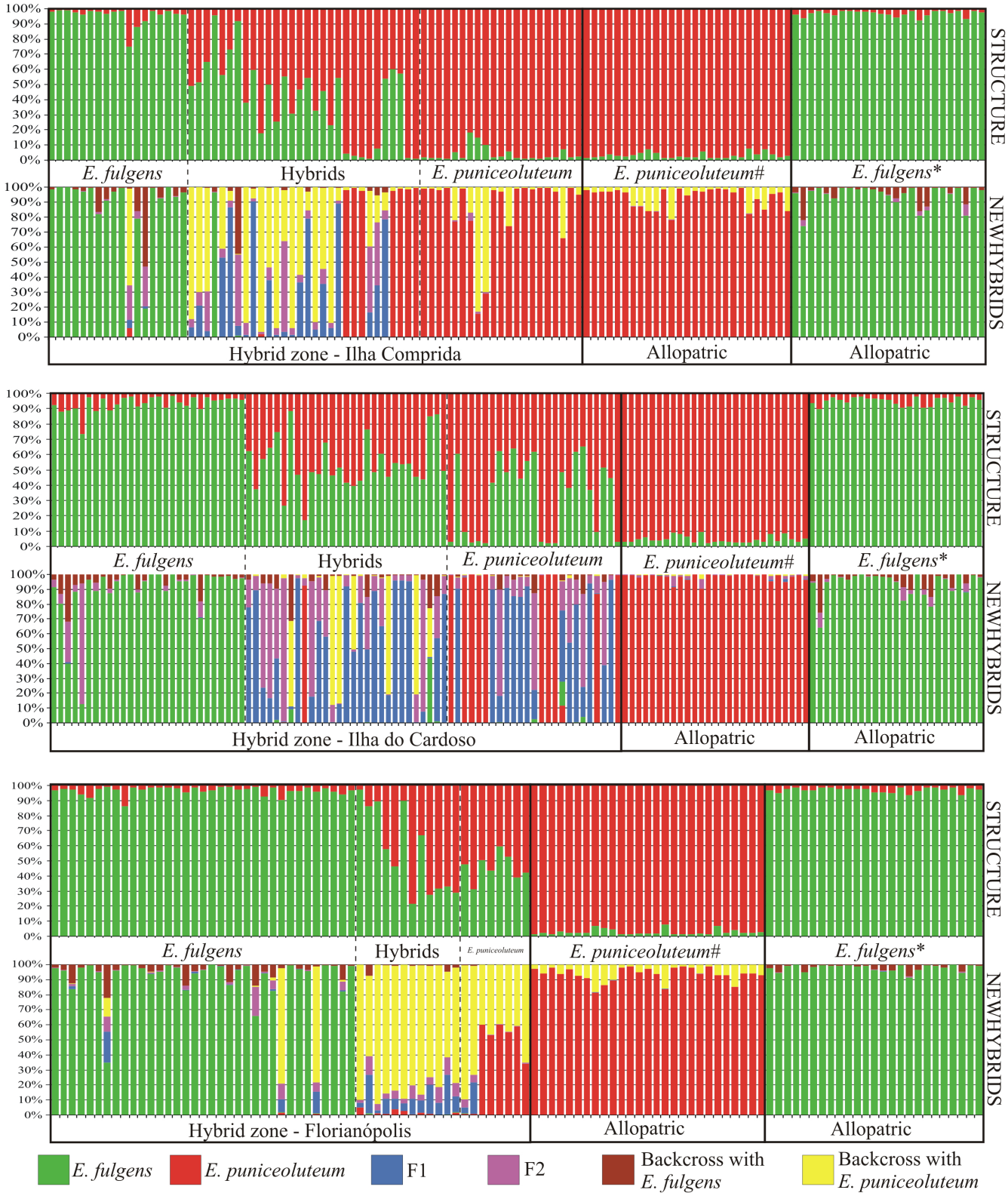

Figure 3: Posterior probabilities (*q*) for Ilha Comprida, Ilha do Cardoso and Florianópolis natural hybrid zones analyzed with STRUCTURE and NEWHYBRIDS. Hybrid zone and allopatric localities of *E. fulgens* in Torres(*) and *E. puniceoluteum* in Pontal do Sul (#) are delimited by solid lines. Individuals identified in the field, based on morphological characters, are delimited by dashed lines. Each vertical bar represents an individual. The proportion of color in each bar represents an individual's assignment probability, according to different categories (pure parental species, hybrid F1, F2 and backcrosses). See Figure 1 for details of geographic position of each locality.

Table S1: *Epidendrum* populations sampled for the present study, including population names (codes), sample size (n) and geographical coordinates.

Population	n	Coordinates
Allopatric		
Pontal do Sul	27	25°39.933′S, 48°26.895′W
Torres	25	29°22.583′S, 49°45.168′W
Sympatric		
Ilha Comprida (CO)	69	24°50.886′S, 47°41.802′W
Ilha do Cardoso (IC)	82	25°04.271′S, 47°54.324′W
Ilha de Superagui (SU)	50	25°27.537′S, 48°12.779′W
Ilha do Mel (IM)	68	25°30.159′S, 48°19.024′W
Florianópolis (FL)	55	27°37.667′S, 48°27.193′W
Imbituba (BA)	87	28°12.538′S, 48°41.133′W
Total	463	

Table S2: Results of STRUCTURE assignment analyses with 10 simulated samples of 60 individuals each (N = 600), without hybrids (simulated *HP* = 0%), and 10 simulated samples of 100 individuals each (N = 1000), including hybrids (simulated *HP* = 40%).

Method	Simulated *HP* (%)	*Tq*	Estimated HP (%)	Power		Accuracy		Type I error	Overall performance	
				Purebreds	Hybrids	Purebreds	Hybrids		Purebreds	Hybrids
STRUCTURE	0	0.9	4.5	0.955	—	1.000	—	0.045	0.955	—
	0	0.75	1.0	0.990	—	1.000	—	0.010	0.990	—
NEWHYBRIDS	0	0.9	0.1	0.975	—	1.000	—	0.025	0.975	—
	0	0.75	0.5	0.985	—	1.000	—	0.015	0.985	—
STRUCTURE	40	0.9	40.1	0.981	0.979	0.959	0.947	0.019	0.940	0.927
	40	0.75	28.5	1.000	0.712	0.839	1.000	0.000	0.839	0.712
NEWHYBRIDS	40	0.9	37.8	0.856	0.927	0.988	0.981	0.141	0.845	0.909
	40	0.75	38.8	0.955	0.94	0.960	0.969	0.044	0.916	0.910

Abbreviations: *HP*, hybrid proportion; *Tq*, threshold *q*-value.
With NEWHYBRIDS, for each individual the *Tq* was applied to the sum of posterior probability for all hybrid classes used as one estimate
See Table 3 for results of power, accuracy and overall performance of NEWHYBRIDS in the identification of hybrid classes (F1, F2 and backcrosses).

Table S3: Results of NEWHYBRIDS analyses with 10 simulated samples of 100 individuals each (N = 1000), considering only hybrid classes F1, F2, backcross with *E. fulgens* (BF) and backcross with *E. puniceoluteum* (BP).

Tq	Power				Accuracy				Overall performance			
	F1	F2	BF	BP	F1	F2	BF	BP	F1	F2	BF	BP
0.9	0.530	0.390	0.360	0.390	0.929	0.951	0.878	0.928	0.492	0.370	0.316	0.361
0.75	0.690	0.530	0.58	0.720	0.884	0.898	0.773	0.911	0.609	0.475	0.448	0.655

Abbreviations: *Tq*, threshold *q*-value.

Figure S1: Morphological variation of flowers in individuals of *E. fulgens*, *E. puniceoluteum* and hybrids. The classification of individuals is based on STRUCTURE results (see text for more details).

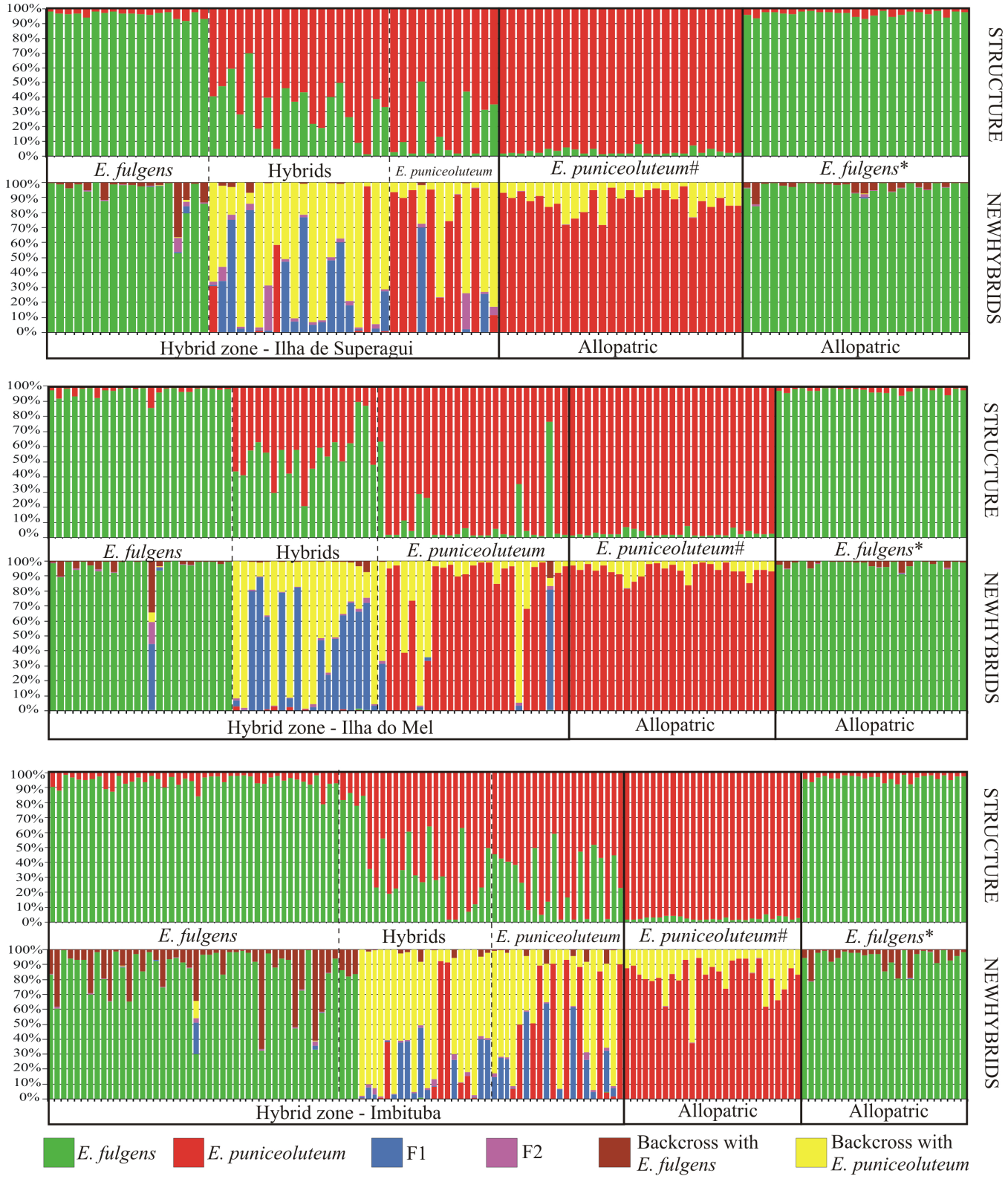

Figure S2: Posterior probabilities (*q*) for Ilha de Superagui, Ilha do Mel and Imbituba natural hybrid zones analyzed with STRUCTURE and NEWHYBRIDS. Hybrid zone and allopatric localities of *E. fulgens* in Torres(*) and *E. puniceoluteum* in Pontal do Sul (#) are delimited by solid lines. Individuals identified in the field, based on morphological characters, are delimited by dashed lines. Each vertical bar represents an individual. The proportion of color in each bar represents an individual's assignment probability, according to different categories (pure parental species, hybrid F1, F2 and backcrosses). See Figure 1 for details of geographic position of each locality.

Discussão Geral e Conclusões

A presente tese é constituída de cinco trabalhos que fazem parte de um amplo projeto, que tem como objetivo contribuir para o entendimento de questões relacionadas à evolução, delimitação e isolamento reprodutivo de espécies da família Orchidaceae. O conjunto de dados obtidos nestes trabalhos descreve um panorama inicial que contribuirá para o preenchimento de lacunas do conhecimento existentes em Orchidaceae, e auxiliará na compreensão de aspectos da história evolutiva e da dinâmica das populações de espécies do gênero *Epidendrum.*

Inicialmente, marcadores nucleares do tipo microssatélites foram isolados e caracterizados para *Epidendrum fulgens* e *E. puniceoluteum*, totalizando 19 *loci*. O número de alelos e a diversidade genética observada em ambas espécies foram elevadas, indicando que os *loci* possuem um elevado potencial em análises que tenham como objetivo detectar eventos de hibridação e introgressão. Estes marcadores, em conjunto com os *loci* caracterizados para outra espécie da subfamília Laeliinae (*Laelia speciosa* -Cortes-Palomec et al. 2008), mostraram uma taxa de amplificação cruzada entre espécies de *Epidendrum* (total de 35 espécies) de 78%, maior do que o valor previamente publicado para monocotiledôneas (60% - Barbará *et al.* 2007). Estes resultados indicam que os marcadores isolados possuem potencial bastante elevado para serem utilizados em outras espécies de *Epidendrum*, constituindo uma ferramenta molecular importante para estudos evolutivos no gênero.

A caracterização de *loci* de microssatélites de cloroplasto também foi uma etapa importante do trabalho, uma vez que abre novas possibilidades e incentiva estudos desta natureza no gênero *Epidendrum*. Foi observado elevado poder de discriminação de populações e espécies, indicando que estes marcadores plastidiais são bastante promissores para estudos de genética de populações e filogeografia. Além disso, estes marcadores também foram amplificados em diferentes gêneros da subtribo Laeliinae, da qual *Epidendrum* faz parte, revelando-se uma ferramenta molecular importante para estudos futuros com espécies de orquídeas neotropicais.

Todas as seis populações simpátricas de *E. fulgens* e *E. puniceoluteum*, analisadas com marcadores nucleares e plastidiais, revelaram a presença de híbridos. Os dados obtidos com microssatélites nucleares foram analisados com métodos de atribuição Bayesianos, e os resultados indicaram que os indivíduos híbridos possuem

grande variação genotípica, podendo ser caracterizados híbridos do tipo F1 e introgressão assimétrica na direção de *E. puniceoluteum*. Os marcadores de cloroplasto revelaram que ambas as espécies parentais podem receber e doar pólen para a formação da geração F1. A introgressão na direção de *E. puniceoluteum* ocorre quando indivíduos F1 doam ou recebem o pólen de indivíduos de *E. puniceoluteum*. A ausência de polinizadores específicos para cada uma das espécies contribui para a grande quantidade de híbridos observados nas populações naturais analisadas. A diferença cromossômica entre as espécies (*E. fulgens*: 2x = 24; *E. puniceoluteum*: 4x = 52) não constitui uma barreira eficiente ao fluxo gênico entre as espécies.

Indivíduos puros de *E. fulgens* e *E. puniceoluteum* revelaram preferência por diferentes tipos de hábitat: dunas para a primeira espécie e áreas encharcadas para a segunda. Indivíduos híbridos puderam ser encontrados em ambos os tipos de hábitat, sugerindo uma amplitude ecológica maior que a das espécies parentais. Indivíduos híbridos não foram encontrados em hábitats distintos daqueles em que as espécies parentais foram coletadas, indicando que os eventos de hibridação ainda não são suficientes para caracterizar eventos de especiação. O processo de hibridação na vegetação de restinga pode ser um processo evolutivo importante para as espécies deste tipo de ambiente, uma vez que promove o fluxo gênico entre espécies adaptadas a diferentes ambientes. A introgressão de elementos genômicos de *E. fulgens*, espécies adaptada às dunas arenosas, na direção de *E. puniceoluteum*, espécie adaptada a áreas encharcadas, produz indivíduos híbridos capazes de crescer nos dois tipos de ambientes. Estes indivíduos podem ser importantes para a sobrevivência de *E. puniceoluteum*, principalmente em períodos em que as áreas encharcadas diminuem de tamanho, tornando-se fragmentadas e ocupando uma área menor, em relação às dunas arenosas. Populações de *E. fulgens* são mais extensas e conectadas entre si do que populações de *E. puniceoluteum*, e o maior fluxo gênico entre populações de *E. fulgens* pode ser um mecanismo importante, que evita a existência de introgressão sobre esta espécie. A vegetação de restinga está submetida a uma dinâmica intensa que modifica a disponibilidade dos hábitas nela encontrados, sendo muito influenciada por fatores abióticos, como salinidade e escassez de água, e fatores históricos, como o aumento e a diminuição do nível de água do oceano. Neste contexto, a hibridação interespecífica constitui um fator importante para a manutenção da biodiversidade, uma vez que promove a troca de elementos genômicos

entre espécies distintas, tornando possível a coexistência de diferentes espécies neste tipo de vegetação.

O presente trabalho abre diversas possibilidades de estudos futuros. Uma delas é a execução de experimentos de transplantes recíprocos, utilizando *E. fulgens*, *E. puniceoluteum* e indivíduos híbridos, com o objetivo de verificar a existência de diferentes preferências por hábitat nas espécies parentais, e confirmar a presença de uma maior amplitude ecológica nos híbridos. Uma vez que as plantas são facilmente propagadas vegetativamente, o sucesso reprodutivo de indivíduos com o mesmo genótipo, em hábitas distintos, pode ser facilmente verificado, conferindo maior poder de inferência ao experimento. A existência de fluxo gênico entre espécies com ploidias distintas é um evento interessante, que será investigado utilizando análises citogenéticas, através de análises de comportamento meiótico e bandeamento cromossômico. Eventos de hibridação também podem originar novas linhagens e espécies, e esta questão será estudada através de medidas de sucesso reprodutivo de indivíduos híbridos, em comparação com as espécies parentais, através da realização de cruzamentos artificiais.

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Resumo

Epidendrum L. é o maior gênero de Orchidaceae da região Neotropical com cerca de 1500 espécies, e os processos de diversificação no grupo são pouco conhecidos. Apesar de existirem muitos relatos sobre hibridação no gênero, não há trabalhos que tenham testado essa hipótese em populações naturais. *Epidendrum fulgens* Brongn. e *E. puniceoluteum* F. Pinheiro & F. Barros são espécies que ocorrem ao longo do litoral brasileiro, freqüentemente em simpatria. Para testar a eficiência de suas barreiras reprodutivas, foi examinada a distribuição da variação genética dentro e entre populações simpátricas e alopátricas dessas duas espécies. Nove *loci* de microsatélites nucleares, e cinco *loci* de microssatélites de cloroplasto foram utilizados para genotipar 463 indivíduos de oito populações, ao longo de toda distribuição geográfica das espécies. A utilização de métodos de atribuição Bayesianos (programas STRUCTURE e NEWHYBRIDS) detectou a existência de grande quantidade de híbridos nas populações simpátricas. As zonas de hibridação são constituídas por híbridos F1, F2 e retrocruzamentos. A introgressão foi assimétrica, ocorrendo preferencialmente de *E. fulgens* para *E. puniceoluteum*. Na população da Ilha do Cardoso, foi detectada a predominância de indivíduos F1 e F2, enquanto nas demais localidades a maior parte dos indivíduos híbridos foi identificada como sendo retrocruzamentos na direção de *E. puniceoluteum*. Em Florianópolis, não foi possível observar a existência de indivíduos puros de *E. puniceoluteum*, apenas indivíduos exibindo fortes sinais de introgressão, revelando que o processo de hibridação pode interferir na integridade genética das espécies, levando um dos parentais à extinção. O presente estudo sugere que hibridação e introgressão podem ter papel importante na diversificação em *Epidendrum* e mostra a importância de investigar zonas de hibridação para melhor entender as barreiras reprodutivas e os processos de especiação nas espécies neotropicais de orquídeas.

Abstract

Among members of the genus *Epidendrum*, the largest orchid genus of the Neotropics, *E. fulgens* and *E. puniceoluteum* occur along the seashore in Brazilian Atlantic Rainforest in sympatric populations. To test the strength of their reproductive barriers, we examined the distribution of genetic variation within and among sympatric and allopatric populations of these two species. Nine specifically developed nuclear microsatellite loci and five chloroplast microsatellite loci were used to genotype 463 individuals from eight populations across species geographical range. All six sympatric populations analyzed present hybrid zones, indicating that hybridization between *E. fulgens* and *E. puniceoluteum* is a common phenomenon. Bayesian assignment analysis detected the presence of F_1 and F_2 individuals, and signs of introgression as well, demonstrating a high potential for interspecific gene flow. The introgression patterns are assimetrical, with differences among populations. Introgression occurs preferentially from *E. fulgens* to *E. puniceoluteum*. In Florianópolis population the hybridization seems to lead a species erosion, where pure individuals of *E. puniceoluteum* where not found. This study suggests that hybridization and introgression could play an important role in the diversification of *Epidendrum*, and indicated the importance to investigate hybrid zones for better understanding reproductive barriers and speciation processes in Neotropical orchid species.