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PART OF A HIGHLIGHT ON ORCHID BIOLOGY

Transitions between self-compatibility and self-incompatibility and the evolution of reproductive isolation in the large and diverse tropical genus *Dendrobium* (Orchidaceae)

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- **Background and Aims** The evolution of interspecific reproductive barriers is crucial to understanding species evolution. This study examines the contribution of transitions between self-compatibility (SC) and self-incompatibility (SI) and genetic divergence in the evolution of reproductive barriers in *Dendrobium*, one of the largest orchid genera. Specifically, it investigates the evolution of pre- and postzygotic isolation and the effects of transitions between compatibility states on interspecific reproductive isolation within the genus.
- **Methods** The role of SC and SI changes in reproductive compatibility among species was examined using fruit set and seed viability data available in the literature from 86 species and ~2500 hand pollinations. The evolution of SC and SI in *Dendrobium* species was investigated within a phylogenetic framework using internal transcribed spacer sequences available in GenBank.
- **Key Results** Based on data from crossing experiments, estimations of genetic distance and the results of a literature survey, it was found that changes in SC and SI significantly influenced the compatibility between species in interspecific crosses. The number of fruits produced was significantly higher in crosses in which self-incompatible species acted as pollen donor for self-compatible species, following the SI × SC rule. Maximum likelihood and Bayesian tests did not reject transitions from SI to SC and from SC to SI across the *Dendrobium* phylogeny. In addition, postzygotic isolation (embryo mortality) was found to evolve gradually with genetic divergence, in agreement with previous results observed for other plant species, including orchids.
- **Conclusions** Transitions between SC and SI and the gradual accumulation of genetic incompatibilities affecting postzygotic isolation are important mechanisms preventing gene flow among *Dendrobium* species, and may constitute important evolutionary processes contributing to the high levels of species diversity in this tropical orchid group.

Key words: Character reconstruction, *Dendrobium*, evolution, Orchidaceae, post-mating barriers, reproductive isolation, self-compatibility, self-incompatibility, speciation.

INTRODUCTION

Speciation can be viewed as a dynamic process in which previously interbreeding groups of individuals acquire reproductive isolation, thus impeding or limiting the homogenizing effect of gene flow among diverging lineages (Mayr, 1942; Dobzhansky, 1970; Grant, 1981; Morjan and Rieseberg, 2004; Lexer and Widmer, 2008). The understanding of the events that cause speciation is a primary goal of evolutionary biology and would in principle require disentangling which reproductive barrier acted in the early phase of species divergence, i.e. which was directly responsible for the reduction of gene flow among formerly interbreeding populations (Coyne and Orr, 1989). This goal could be directly achieved only through the investigation of isolating mechanisms among divergent intraspecific populations (Etterson *et al.*, 2007; Scopece *et al.*, 2010; Pinheiro *et al.*, 2013) because well-established species may have gathered changes that can potentially mask the relative contributions of various isolating mechanisms during incipient species formation (Kay, 2006). However, divergent populations will rarely become new species and, as a consequence, studies on speciation are complicated by a surplus of speculations either on the future (in the case of diverging populations) or on the past (in the case of well-established species) (Butlin *et al.*, 2008).

To overcome this problem, Coyne and Orr (1989) proposed an approach based on the measurement of the strength of reproductive isolating mechanisms across large groups of taxa that vary in divergence time and on its comparison with genetic distances considered as a proxy of time divergence. Such an approach, however, requires a huge experimental effort and, consequently, so far only few studies have employed it in plants (Moyle *et al.*, 2004; Archibald *et al.*, 2005; Scopece *et al.*, 2007, 2008; Jewell *et al.*, 2012). Such studies often rely on literature data, thus suffering from an intrinsic lack of specific experimental design or of appropriate sample sizes.

Furthermore, these studies often encompass few isolating mechanisms, whilst reproductive isolation can be achieved through a combination of many different pre- or postzygotic barriers (Ramsey *et al*., 2003; Lowry *et al*., 2008). Despite these shortcomings, such studies have contributed significantly to more general hypotheses on speciation in the plant kingdom. Indeed, only by employing a similar approach was it possible to shed light on the evolutionary rates of different types of isolating mechanisms and to show that prezygotic mechanisms generally evolve rapidly, whereas the evolution of postzygotic mechanisms appears to be more gradual (Coyne and Orr, 1989; Moyle *et al*., 2004). Particularly in Mediterranean orchids, it has been shown that, excluding the groups in which mechanisms such as allopolyploidy have a dominant role (e.g. Hedrén, 1996, 2001; Trávníček *et al*., 2010), postzygotic isolation evolves gradually (Scopece *et al*., 2007) and that late-acting mechanisms (such as hybrid sterility and inviability) evolve faster than early-acting ones (fruit formation, embryo mortality) (Scopece *et al*., 2008). These evolutionary patterns suggest an overall difference in the genetic background of different reproductive barriers, with prezygotic ones likely due to few genes and postzygotic ones with a multigenic basis (e.g. Coyne and Orr, 1998; Edmands, 2002).

Reproductive isolation can also evolve as a by-product of evolutionary processes related to different ecological requirements (Bomblies *et al*., 2007). For instance, shifts between different compatibility states can have a direct impact on reproductive isolation among species and populations (reviewed by Brandvain and Haig, 2005). In this context, studies performed with self-incompatible (SI) and self-compatible (SC) species of plants have shown a strong asymmetrical pattern of reproductive incompatibility, in which SI species pollen grows in SC species styles, but SC species pollen is inhibited in SI species styles, the so called SI × SC rule (Lewis and Crowe, 1958; de Nettancourt, 1977; Murfett *et al*., 1996; Hiscock *et al*., 1998; Brandvain and Haig, 2005). Barriers in SI species styles may have a multigenic origin associated with the *S* locus (Murfett *et al*., 1996; Hiscock *et al*., 1998), which plays a role in many SI systems (Charlesworth and Charlesworth, 1979; Hiscock and Dickinson, 1993; Igic *et al*., 2006). In SI species, the potential for sexual conflicts is higher because pollen tubes are normally unrelated to the sporophytic tissues through which they grow. In contrast, SC species often lack such incompatibilities since pollen tubes approach genetic identity with the sporophytic tissues through which they grow by constant cycles of selfing (Brandvain and Haig, 2005). Since changes in compatibility states intensely affect the levels of reproductive isolation between populations, transitions between SC and SI may accelerate the accumulation of reproductive barriers among distinct lineages, contributing to speciation (Hiscock *et al*. 1998; Brandvain and Haig, 2005).

The evolution of reproductive isolation has been extensively studied in Mediterranean deceptive orchids (Cozzolino and Scopece, 2008; Xu *et al*., 2011; Zitari *et al*., 2012; Scopece *et al*., 2013). Abundant sympatric populations composed of species showing different ecological attributes (food versus sexual deceptive systems) have provided an interesting biological model to investigate the evolutionary mechanisms underlying speciation in groups with different levels of pollinator specialization (Cozzolino and Scopece, 2008). Unfortunately, no such model is available for tropical regions, where most of the orchid species occur. For this reason, using literature data, we investigated the evolution of pre- and postzygotic reproductive isolation and the potential effect of the SI × SC rule on speciation patterns in *Dendrobium*, one of the largest orchid genera in the tropical region. Crossing compatibility among *Dendrobium* species was studied by Wilfret (1968) and Johansen (1990), who performed a large amount of crossing experiments in order to understand taxonomic affinities and self-incompatibility systems within the genus. By combining large and diverse datasets (crossing experiments, DNA sequences and breeding system information), this study specifically asks about patterns of evolution of pre- and postzygotic isolation and explores the effect of changes between compatibility states on the evolution of reproductive isolation within the genus *Dendrobium*.

MATERIALS AND METHODS

Plant group and dataset origin

Dendrobium is one of the largest orchid genera in the tropical region, with ~1200 species (Adams, 2011). Most species are distributed in tropical Asia, Australasia and Australia, and many endemic species are reported along its distribution range (Cribb and Govaerts, 2005; Wood, 2006; Zhu *et al*., 2009). The genus shows extensive morphological variation, which challenges classification systems and taxonomic decisions regarding species and infrageneric boundaries (Adams, 2011). Several authors have studied the genus from a phylogenetic perspective (Clements, 2003; Burke *et al*., 2008, 2013; Yuan *et al*., 2009; Li *et al*., 2012; Xiang *et al*., 2013), providing a sequence dataset for the nuclear internal transcribed spacer (ITS) region and a phylogenetic background for comparative studies. The ornamental value and medicinal properties of many species place the genus as a target for a large array of studies regarding the development of improved cultivars (reviewed by Kamemoto *et al*., 1999), gene expression (Xu *et al*., 2006), phytochemistry and physiology (reviewed by Ng *et al*., 2012). Furthermore, the genus *Dendrobium* encompasses SC and SI species (Kerr, 1909; Johansen, 1990; Kamemoto *et al*., 1999).

In order to examine interspecific compatibility and the breeding system of *Dendrobium* species, extensive crossing experiments were performed by Wilfret (1968) and Johansen (1990), who used a total of 86 species (Table 1) and carried out ~2500 hand pollinations. Self, intraspecific and interspecific pollinations were performed by both authors, and the number of flowers used in pollination experiments, the number of fruits produced (fruit set) and the proportion of viable seeds (seed viability) were measured. Interspecific crosses from both datasets are summarized in Supplementary Data Table S1.

Asymmetry in reproductive compatibility and transitions between SC and SI

To examine the role of SC and SI changes in reproductive compatibility among species, differences in fruit set and seed viability were tested using results of interspecific crosses from the Wilfret (1968) and Johansen (1990) datasets. Specifically, interspecific crosses were tested for differences in fruit set and seed viability when SC species and SI species acted as pollen

Table 1. *Total number of* Dendrobium *species analysed in this study. Crossing experiments results were available for species indicated by W (dataset from Wilfret, 1968) and J (dataset from Johansen, 1990)*

Species	Compatibility system	Dataset of origin[1]	Types of cross performed[2]	GenBank number[3]
D. aciculare[†]	SI	J	SE, intra, **inter**	
D. acinaciforme[†][§]	SI	J	SE, **inter**	HQ114253[#]
D. aggregatum[*][†]	SI	W	SE, **inter**	
D. albosanguineum	SI	J	SE	EU477498[#]
D. aloifolium[†][§]	SI	J	SE, **inter**	AY239951[#]
D. alterum[†]	SI	J	SE, **inter**	
D. aphyllum[†][§]	SI	J	SE, intra, **inter**	KF143430[#]
D. arachnites[†]	SI	W	SE, **inter**	
D. bellatulum[§]	–	W	**inter**	KF143431[#]
D. bicameratum	SI	–	–	HM054581[#]
D. bigibbum[†]	SC	W	inter	
D. bilobulatum[†]	SC/SI	J	SE, intra, **inter**	
D. blumei	SI	J	SE	
D. brevimentum[†]	SI	J	SE, **inter**	
D. brymerianum	SC	J	SE	KF143432[#]
D. bullenianum[†]	SI	W	SE, **inter**	
D. capillipes	SI	–	–	KF143433[#]
D. cariniferum[†][§]	SI	W, J	SE, **inter**	JN388583[#]
D. chrysotoxum[†][§]	SI	W, J	SE, intra, **inter**	KF143444[#]
D. compactum	SI	J	SE	KF143445[#]
D. concinnum	–	J	inter	
D. crepidatum	SC			KF143446[#]
D. crumenatum[†][§]	SI	W, J	SE, **inter**	HM590370[#]
D. crystallinum[†][§]	SC/SI	J	SE, **inter**	HQ114243[#]
D. dalbertsii[†]	SC	W	SE, **inter**	
D. delacourii[†]	SC	W	SE, **inter**	
D. densiflorum	SC	–	–	KF143451[#]
D. denudans	SI	J	SE	KF143452[#]
D. devonianum[†][§]	SI	J	SE, intra, **inter**	KF143453[#]
D. disticum[†]	SI	J	SE, **inter**	
D. dixanthum[†][§]	SC	W	SE, **inter**	KF143454[#]
D. draconis[†][§]	SC/SI	W, J	SE, **inter**	HM054628[#]
D. ellipsophyllum[†][§]	SI	J	SE, **inter**	KF143455[#]
D. erostelle	SI	J	SE	
D. exile	SC	J	SE	KF143457[#]
D. falconeri	SI	J	intra, **inter**	KF143458[#]
D. farmeri[†][§]	SI	W, J	SE, intra, **inter**	HM054631[#]
D. fimbriatum[†][§]	SI	W	SE, **inter**	JN388588[#]
D. formosum[†][§]	SI	W, J	SE, **inter**	AY239967[#]
D. friedericksianum[§]	–	W	**inter**	EU477505[#]
D. gibsonii[†][§]	SI	J	SE, **inter**	HQ114256[#]
D. gouldii[†]	SC	W	SE, **inter**	
D. grantii	–	W	**inter**	
D. gratiosissimum[†][§]	SI	J	SE, **inter**	KF143464[#]
D. griffithianum[†]	SI	J	SE, **inter**	
D. hancockii	SC	–	–	KF143467[#]
D. hendersonii[†]	SI	J	SE, intra	
D. hercoglossum	SC	–	–	KF143472[#]
D. heterocarpum[†][§]	SC	W, J	SE, **inter**	KF143473[#]
D. hildebrandii[†]	SC	W	SE, **inter**	
D. indivisum[†][§]	SI	J	SE, **inter**	AY239972[#]
D. infundibulum	SC/SI	J	SE	KF143477[#]
D. jenkinsii	SC	–	–	KF143479[#]
D. keithii[†]	SI	J	SE, intra, **inter**	
D. kingianum	SI	–	–	EU430386[#]
D. lamellatum	SI	J	SE	
D. leonis[†][§]	SI	W, J	SE, intra, **inter**	AY239978[#]
D. leptocladum	SI	–	–	HM590373[#]
D. linawianum	SI	–	–	HM590371[#]
D. lindleyi[†][§]	SI	J	SE, intra, **inter**	JN388568[#]
D. linguella[†]	SI	W, J	SE, intra, **inter**	
D. lituiflorum[†][§]	SI	W	SE, **inter**	AB593602[#]
D. loddigesii	SC	–	–	HM590374[#]
D. maccarthiae	–	W	inter	

(continued)

Table 1. *Continued*

Species	Compatibility system	Dataset of origin[1]	Types of cross performed[2]	GenBank number[3]
D. macrophyllum[†§]	SC	W	SE, **inter**	AY239979[#]
D. macrostachyum[†§]	SC	W	SE, **inter**	HM054696[#]
D. mannii	SI	J	SE	
D. monile[**†§]	SI	W	SE, **inter**	KF143489[#]
D. moschatum[†§]	SI	W, J	SE, intra, **inter**	KF143492[#]
D. mucronatum	SI	J	SE	
D. nathanielis[†]	SI	J	SE, **inter**	
D. nobile[†§]	SC	J	inter	HQ114219[#]
D. officinale	SI	–	–	HQ114245[#]
D. pachyglossum[†]	SI	J	SE, **inter**	
D. pachyphyllum[†]	SI	J	SE, **inter**	
D. panduriferum[†]	SI	J	SE, **inter**	
D. parcum	SI	J	SE	
D. parishii[†§]	SI	W, J	SE, intra, **inter**	HM590378[#]
D. pendulum	SC	J	SE	KF143498[#]
D. phalaenopsis[†]	SC/SI	W, J	SE, **inter**	
D. planibulbe[†]	SI	J	SE, **inter**	
D. podagraria	SI	J	SE	
D. primulinum[†§]	SI	W, J	SE, **inter**	HQ114242[#]
D. pulchellum[†§]	SI	J	SE, intra, **inter**	KF143503[#]
D. salaccense[†§]	SC	J	SE, **inter**	KF143506[#]
D. secundum[†§]	SI	J	SE, intra, **inter**	AY239993[#]
D. senile[†§]	SI	W, J	SE, **inter**	EU477509[#]
D. setifolium[†]	SI	J	SE, **inter**	
D. sinense	SI	–	–	KF143511[#]
D. speciosum	SI	–	–	AY239998[#]
D. spectabile[†]	SC	W	SE, **inter**	
D. stratiotes[†]	SC	W	SE, **inter**	
D. strebloceras[†]	SC	W	SE, **inter**	
D. stuposum	SC	–	–	KF143516[#]
D. subulatum	SI	J	SE	
D. sulcatum	SC	–	–	KF143517[#]
D. tetrodon	SC	J	SE	
D. thyrsiflorum[†§]	SI	J	SE, intra, **inter**	KF143519[#]
D. tortile[†]	SC	W, J	SE, inter	EU477511[#]
D. undulatum[†]	SC	W	SE, **inter**	
D. unicum[§]	–	J	intra, **inter**	KF143523
D. virgineum[†]	SI	J	SE, **inter**	
D. wardianum	SC	–	–	JN388600[#]

[1] *Dendrobium* species not used in crossing experiments (–) by Wilfret (1968) or Johansen (1990) were not included in the correlation between reproductive isolation and genetic distances.

[2] Reciprocal interspecific crosses are indicated in bold.

[3] ITS sequences used to calculate genetic distances among species and to build the phylogenetic tree where transitions between SC and SI were mapped (Fig. 1).

[†] Species used to investigate the transitions between SC and SI and the evolution of reproductive isolation.

[#] Species used to infer the phylogenetic hypothesis.

[§] Species used to test the association between reproductive isolation and genetic distances.

*Synonym of *D. lindleyi*.

**Synonym of *D. moniliforme*.

SC, self-compatible species; SI, self-incompatible species; SC/SI species, both; –, unknown; SE, self-pollination; intra, intraspecific pollination; inter, interspecific pollination.

donors and pollen receptors, and between interspecific crosses involving SC species × SC species and SI species × SI species. Information regarding the SC and SI status of most species was collected from the studies of Wilfret (1968) and Johansen (1990). Further information was retrieved from other reproductive biology studies conducted on *Dendrobium* species (Kerr, 1909; Slater and Calder, 1988; Bartareau, 1995; Wood, 2006; Li *et al*., 2009*a*, *b*; Vasudevan and Staden, 2010; Huda and Wilcock, 2012; Pang *et al*., 2012). Quantitative differences in fruit set and seed viability were tested using the Mann–Whitney *U*-test with the statistical package SPSS 13.0 (SPSS Inc., Chicago, IL).

Transitions between SC and SI in *Dendrobium* species were examined in a phylogenetic framework. The phylogenetic inference was based on ITS sequences found in GenBank for the 61 taxa for which the compatibility state was known (Table 1). All sequences used in this study were previously analysed and published in peer-reviewed journals, thus increasing our confidence in the sequence-specific names. Of the 61 taxa for which sequence data were obtained, there were representatives from all the main phylogenetic clades found by Xiang *et al*. (2013). Each ITS sequence accession was aligned using the ClustalW option in BioEdit v.7.1.9 (Hall, 1999). The resulting automated alignment was manually edited in BioEdit v.7.1.9 and then exported

Table 2. *Summary of interspecific crosses results obtained by Wilfret (1968) and Johansen (1990), used to test for differences in interspecific compatibilities when SC and SI species acted as pollen donors and pollen receptors, and vice versa (groups 1 and 2), and between interspecific crosses involving SI × SI species (group 3) and SC × SC species (group 4). The total number of crosses performed (number of crosses that produced fruits), number of pollinated flowers, mean number of fruits and mean seed viability are indicated*

Crossing group[1]	Pollen receptor	Pollen donor	Number of species	Crosses	Pollinated flowers	Mean fruits produced (s.d.)	Mean seed viability[2] (%)
1	SI species	SC species	39	137 (9)	171	0·07 (0·28)	10·68
2	SC species	SI species	38	164 (41)	219	0·34 (0·67)	10·72
3	SI species	SI species	16	304 (41)	919	0·41 (1·31)	22·78
4	SC species	SC species	46	154 (63)	218	0·48 (0·63)	42·86

[1] Significant differences were detected between groups 1 and 2 in fruit production ($P = 0{\cdot}000$) and between groups 3 and 4 in fruit production ($P = 0{\cdot}000$) and seed viability ($P < 0{\cdot}01$).

[2] Only crosses that produced fruits.

as a Phylip 4 file for maximum parsimony (MP) and maximum likelihood (ML) analyses, following Pessoa *et al.* (2012).

Homogeneity of the dataset was tested with the incongruence-length difference test (Farris *et al*., 1995) as implemented in PAUP 4.0 (Swofford, 2002). The MP analysis used the criterion of Fitch (1971), excluding uninformative characters, and with ACCTRAN optimization. Robustness of MP tree topologies was tested by bootstrap analysis (Felsenstein, 1985). Ten thousand addition sequence replicates were performed by stepwise addition and holding ten trees per replicate, and tree bisection and reconnection branch swapping on the best trees. The MP analysis was performed with PAUP. The ML analysis was conducted using RAxML v. 7.0.4 (Stamatakis, 2006) and RAxML-GUI v. 1.1 (Silvestro and Michalak, 2011). The GTR + Γ substitution model, which allows rate variation among sites, was determined using jModeltest v. 0.1.1 (Posada, 2008) under the Akaike information criterion (AIC). To find the optimal likelihood tree, we ran 100 independent tree searches on the ITS matrix. Support for individual branches was evaluated using non-parametric bootstrapping (Felsenstein, 1985) with 1000 thorough bootstrap replicates. Trees were rooted with *D. macrophyllum* and *D. salaccense*, which are members of the most basal clade in *Dendrobium* according to the phylogeny published by Xiang *et al.* (2013).

Ancestral character state reconstruction analysis was used to map the transitions between SC and SI in *Dendrobium* species. The SC? and SI? states were coded as binary data (0, 1) and optimized onto the best scoring ML tree under an MP criterion using the package Mesquite v. 2.75 (Maddison and Maddison, 2011). A polymorphic state (0and1) was used for species where the character is variable (i.e. SC? and SI? in the same species). The number and directionality of transitions between SC? and SI? states were quantified using the Summarize State Changes Over Trees function in Mesquite v. 2.75.

Following Escobar *et al.* (2010), ML and Markov chain Monte Carlo (MCMC) procedures were applied to test the significance of transitions between SC and SI in *Dendrobium* species, using the 1000 bootstrap trees from which we obtained support values of nodes. Analyses of bootstrap trees allowed assessment of the uncertainty of transitions in nodes not fully supported. The ML and MCMC analyses on bootstrap trees were performed with the BayesMultistates program (Pagel *et al.*, 2004) implemented in BayesTraits 1.0 v. 2.0 (http://www.evolution.rdg.ac.uk/BayesTraits.html). The MCMC analyses were run for 5 050 000 generations, a uniform prior distribution and a burn-in of 50 000 generations.

Three different models were compared using likelihood ratio tests, following Escobar *et al.* (2010): (1) the unrestricted model, in which the probability of the two types of transitions, from SI? to SC? (q_{IC}) and the converse (q_{CI}), were calculated; (2) a restricted model in which only SI? to SC? transitions were permitted (i.e. $q_{\mathrm{CI}} = 0$); and (3) an alternative, restricted model in which only SC? to SI? transitions were permitted (i.e. $q_{\mathrm{IC}} = 0$). The likelihood ratio test was used to compare the two likelihoods derived from unrestricted and each of the restricted models (unrestricted versus restricted $q_{\mathrm{CI}} = 0$, and unrestricted versus restricted $q_{\mathrm{IC}} = 0$). In the restricted models, ancestral states were fixed to SI or SC.

Reproductive isolation indices

Results from interspecific crosses were used to calculate two postmating reproductive isolation indices: one prezygotic (pollen–stigma incompatibility, $\mathrm{RI}_{\mathrm{prezygotic}}$) and one postzygotic (embryo mortality, $\mathrm{RI}_{\mathrm{postzygotic}}$). From Wilfret (1968) and Johansen (1990) we collected data on the proportion of fruits produced, which was used as the measure of pollen–stigma incompatibility, and on the proportion of viable seeds, used as the measure of embryo mortality. There was considerable overlap between the approaches employed in performing hand pollinations and estimating fruit and seed production. From the Wilfret (1968) and Johansen (1990) datasets we only selected bidirectional interspecific crosses and reproductive isolation was calculated as the average of crossing results where each species was used as both pollen donor and receiver. The traditional method of calculating reproductive isolation (Coyne and Orr, 2004) involves a comparison between interspecific crosses and intraspecific performances used as reference. However, the studies from which data were collected were focused on the investigation of SIy mechanisms and therefore mainly included intraspecific self-pollinations rather than intraspecific cross-pollinations, which would be the ideal reference for the calculation of reproductive isolation. To circumvent this issue, we assumed as reference the maximum hypothetic value of performance (i.e. 100 %). The pollen–stigma incompatibility isolation index was thus defined as $\mathrm{RI}_{\mathrm{prezygotic}} = 1 -$ (mean percentage of fruits in bidirectional interspecific crosses/total potential compatibility, i.e.100 %), where RI is the reproductive

Fig. 1. Evolutionary transitions between SC and SI in *Dendrobium*, using the best maximum likelihood tree based on ITS sequences. White and black branches indicate self-compatible and self-incompatible lineages, respectively. Grey branches indicate undetermined compatibility systems. *Species in which both SC and SI are present. White, grey and black diamonds indicate bootstrap support values above 50 % obtained by maximum parsimony, maximum likelihood and both methods, respectively.

isolation index. The embryo mortality isolation index was defined as $\mathrm{RI}_{\mathrm{postzygotic}} = 1 -$ (mean percentage seed viability in bidirectional interspecific crosses/total potential compatibility, i.e. 100 %). A few species pairs, present in both original datasets (Wilfret, 1968; Johansen, 1990), were averaged as a single data point to avoid duplication. All measures of isolation varied between 0 (no isolation) and 1 (complete isolation).

Correlations between reproductive isolation indices and genetic distances

Genetic distances were calculated from ribosomal ITS sequences available in GenBank, using 37 species (Table 1). Sequences were hand-aligned in BioEdit v. 7.1.9 and genetic distances were then calculated in PAUP 4.0 under the best-fit model of molecular evolution chosen with jModeltest v. 0.1.1.

TABLE 3. *Models testing transitions between SI and SC across the* Dendrobium *phylogeny, using ML and Bayesian MCMC methods, including the mean log-likelihoods for ML analyses and harmonic mean of log-likelihoods for MCMC analyses (lnL), the deviance of harmonic means between unrestricted and restricted models (Dev) with the respective probability* (P)*, the probability of SC-to-SI transitions* (q*01), the probability of SI-to-SC transitions* (q*10), the probability of self-compatibility at the root of the tree [Root* P*(0)] and the probability of self-incompatibility at the root of the tree [Root* P*(1)]*

Model	lnL	Dev (*P*)	q01	q10	Root *P*(0)	Root *P*(1)
ML unrestricted	–42·09		1000·00	596·33	0·50	0·50
ML 01 = 0	–63·96	43·74 (0·00)	0·00	6·65	0·00	1·00
ML 10 = 0	–54·13	24·08 (0·00)	12·18	0·00	1·00	0·00
MCMC unrestricted	–38·27		88·55	53·32	0·49	0·50
MCMC 01 = 0	–60·85	45·16 (0·00)	0·00	7·73	0·00	1·00
MCMC 10 = 0	–47·29	18·04 (0·00)	14·92	0·00	1·00	0·00

Because species of *Dendrobium* may be circumscribed very differently and receive different names in different studies, and to avoid the potential risk of estimating reproductive isolation and genetic distances not between but within the same *Dendrobium* species, potential synonyms were checked in the Plant List website (http://www.theplantlist.org/). Names were corrected for *D. aggregatum* (synonym of *D. lindleyi*) and *D. monile* (synonym of *D. moniliforme*), as both species were used to estimate correlations between reproductive isolation stages and genetic distances.

In order to test for significant associations between genetic distances and reproductive isolation, the non-parametric Kendall's τ rank correlation was used for full and strictly independent datasets. Since species were often involved in multiple crosses, most points in our dataset were not statistically independent. To circumvent this problem, we selected strictly phylogenetically independent species pairs to maximize the number of pairs that could be obtained from the available dataset (Felsenstein, 1985). All analyses were conducted using SPSS 13.0 (SPSS, Chicago).

RESULTS

Breeding system transitions

The transition between SC and SI and the evolution of reproductive isolation were analysed for 63 species, for which 759 interspecific crosses were performed (Table 1). Self-incompatibility was the predominant breeding system in 43 out of 63 species used in the crossing experiments (Table 1). Self-compatibility was observed in 16 species, and both states were present in only four species, specifically in individuals from different localities (Johansen, 1990; Table 1, Appendix 1).

The types of compatibility system significantly influenced the results of interspecific crosses (Table 2). The number of fruits produced was significantly higher in crosses where SI species acted as pollen donor for SC species, in comparison with the converse situation (Mann–Whitney $U = 9129{\cdot}0$; $P < 0{\cdot}001$). In this case, no significant differences were observed for seed viability (Mann–Whitney $U = 131{\cdot}5$; $P = 0{\cdot}184$). Furthermore, significant higher fruit set (Mann–Whitney $U = 17\,739{\cdot}0$; $P < 0{\cdot}001$) and seed viability values (Mann-Whitney $U = 820{\cdot}5$; $P < 0{\cdot}01$) were observed in crosses between SC species, in comparison with crosses between SI species (Table 2).

The ITS matrix comprised 61 species for which the compatibility system information was available (Table 1). The aligned matrix was 669 bp long after exclusion of regions of ambiguous alignment. A total of 386 (58 %) substitutions were parsimony-informative (225 were constant and 58 were parsimony-uninformative) with 198 of these (52 %) informative within *Dendrobium.* The topologies of MP and ML trees recovered in this study were very similar and, for this reason, only the result of the ML analysis is shown (Fig. 1). Ancestral state reconstruction suggested high levels of homoplasy, with frequent shifts between SC and SI across the tree (Fig. 1). Indeed, the number of character steps (23) and the consistency and retention indices (0·17 and 0·05, respectively) confirmed the homoplasious nature of the transitions between SC and SI in *Dendrobium* species. Ancestral states were difficult to interpret at several nodes (grey branches in Fig. 1), particularly when a node was poorly supported by bootstrap values (Fig. 1).

The number of transitions from SI to SC (minimum 12, maximum 21, average 15·48) was higher than the converse situation (minimum 2, maximum 9, average 6·1). However, ML and MCMC analyses suggested that the log-likelihood of the unrestricted model, allowing transitions from SI to SC (q_{IC}) and from SC to SI (q_{CI}), was significantly better than both restricted models tested (only transitions from SI to SC, $q_{\mathrm{CI}} = 0$; only transitions from SC to SI, $q_{\mathrm{IC}} = 0$). Thus, the best model describing changes in compatibility systems across the *Dendrobium* phylogeny was that in which both transitions between SI and SC were allowed (Table 3).

Impact of genetic distances on reproductive isolation patterns

Reproductive isolation indices were calculated for 310 interspecific bidirectional crosses involving 66 species (Appendix 1). The maximum reproductive isolation index (RI = 1) was observed for most crosses considering either fruit set (no fruit formation in 272 out of 310 crosses) or seed viability (0 % of seed viability in 22 out of 39 crosses that produced fruits).

ITS sequences were available for 36 species involved in the interspecific crosses. Consequently, the association between genetic distances and reproductive isolation was estimated for 105 species pairs. Using the full dataset, $\mathrm{RI}_{\mathrm{prezygotic}}$ was not correlated with genetic distance (Kendall's $\tau = 0{\cdot}085$; $P = 0{\cdot}272$) but $\mathrm{RI}_{\mathrm{postzygotic}}$ was positively correlated with genetic distance (Kendall's $\tau = 0{\cdot}326$; $P = 0{\cdot}026$) (Fig. 2). Using strictly independent species pairs, $\mathrm{RI}_{\mathrm{prezygotic}}$ was not correlated with genetic distance (Kendall's $\tau = 0{\cdot}645$; $P = 0{\cdot}079$), but also $\mathrm{RI}_{\mathrm{postzygotic}}$ was not correlated with genetic distance (Kendall's $\tau = 0{\cdot}086$; $P = 0{\cdot}822$).

DISCUSSION

The investigation of interspecific reproductive barriers is crucial to the understanding of species origin and evolution (Coyne and Orr, 2004). Because reproductive barriers can act at

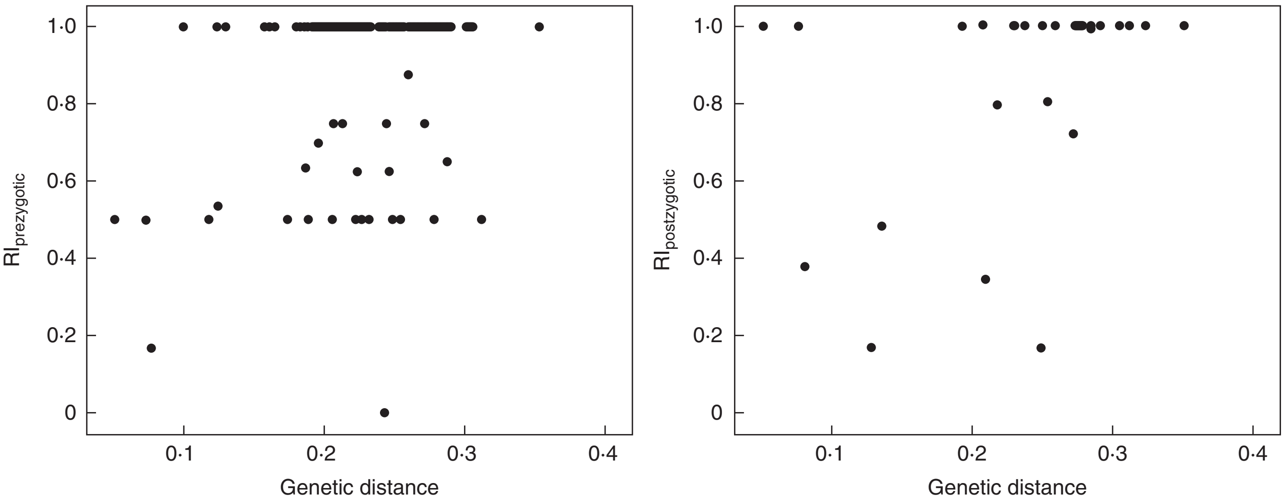

FIG. 2. Strength of pre- and postzygotic reproductive isolation versus genetic distance in *Dendrobium.*

different stages of the reproductive cycle, from mate recognition to offspring fertility, integrated approaches are critical to the understanding of the contribution of different barriers to the formation and maintenance of species boundaries. In this study, datasets from crossing experiments and DNA sequences were used to investigate the strength of two postmating (pre- and postzygotic) reproductive isolation mechanisms, the relative rates of reproductive barrier evolution and the role of transitions between SC and SI in speciation. The genetic distance among species was significantly correlated with interspecific seed production, suggesting a gradual accumulation of reproductive barriers, probably with a multigenic origin, as already observed in other food-deceptive orchids (Scopece *et al*., 2007). Transitions from SI to SC were the most common, suggesting the existence of an evolutionary advantage in self-pollination. This is in agreement with the traditional dead-end hypothesis (Stebbins, 1974; Busch and Delph, 2012), by which SCble lineages often evolve from SIble ancestors in flowering plants, and SCble lineages with a prevalence of self-pollination are thought to show limited potential for adaptation due to the accumulation of deleterious mutations. However, reverse changes from SC to SI could not be rejected by our analysis. In fact, the dead-end hypothesis only applies when SCble lineages display high levels of self-pollination, which is not a common mating system observed in orchids (Squirrell *et al*., 2001; Tremblay *et al*., 2005). Strong and significant asymmetrical patterns of reproductive isolation between SI and SC species follow the SI × SC rule, suggesting that changes in the compatibility system may be linked with the evolution of reproductive isolation during speciation events. Our data not only inform us about the strength and direction of reproductive barriers among *Dendrobium* species, but also contain important information about the potential ecological scenarios and genetic mechanisms underlining the diversification of this species-rich plant genus.

Relationship between reproductive isolation and time divergence

The few studies investigating relationships between reproductive isolation and genetic divergence in plants showed contrasting patterns ranging from consistently positive associations to weak or no correlation, depending on the plant lineage investigated (Moyle *et al*., 2004; Scopece *et al*., 2007; Jewell *et al*., 2012). Our data show a significant correlation between postzygotic but not prezygotic isolation and genetic distance, confirming previous results in the same plant family but in a phylogenetically distant lineage (Scopece *et al*., 2007). This finding suggests that, also in the genus *Dendrobium*, postzygotic isolation between species increases with increasing time since species divergence, in agreement with traditional theories of speciation in which reproductive isolation is considered a by-product of gradual genetic divergence (Presgraves, 2002). Furthermore, a gradual evolution of postzygotic reproductive isolation suggests that it can be shaped by the amount of genome divergence between parental species (Coyne and Orr, 1989; Moyle *et al*., 2004; Scopece *et al*., 2007), achieved through the accumulation of differences in many genes of small individual effects (Coyne and Orr, 1998; Edmands, 2002). Differently, prezygotic isolation has been generally found to evolve more rapidly and erratically than postzygotic isolation (Coyne and Orr, 1998; Scopece *et al*., 2007). In agreement, we found no correlation between prezygotic isolation and genetic distance between *Dendrobium* species. The stage of prezygotic isolation investigated here is likely linked to biochemical processes involving the recognition between pollen and stigma and to the consequent triggering of female gametophyte development (Zhang and O'Neill, 1993). This process, typical of the orchid family, is likely to be affected by ecological factors or by the action of natural selection in sympatry, which may explain why closely related species pairs often display strong prezygotic isolation in spite of presumed recent divergence (e.g. *D. cariniferum* × *D. unicum* and *D. gibsonii* × *D. moschatum*; Fig. 1).

To avoid phylogenetic constraints, we also selected a set of phylogenetically independent species pairs. The correlations, however, showed a lack of significance in both pre- and postzygotic isolation. This pattern is likely due to the reduced sample size (only six independent species pairs) and to the small interval of genetic distance in which the independent pairs fall. In addition, it should also be noted that several closely related species (e.g. *D. acinaciforme* × *D. leonis*) show elevated postzygotic isolation, which suggests the existence of mechanisms other than the mere accumulation of incompatibilities in shaping postzygotic barriers.

Directionality of SC and SI transitions and its effect on reproductive isolation in Dendrobium

The inferred phylogeny based on available ITS sequences indicates that SC may be the ancestral breeding system in *Dendrobium*. Accordingly, most species of section *Grastidium*, which is the ancestral group within *Dendrobium* (Xiang *et al.*, 2013) are reported to be SC species (Catling, 1990; Wood, 2006), such as *D. macrophyllum*, which was used to root our tree (Fig. 1). Likelihood and Bayesian tests could not reject transitions from SC to SI (Table 3), and the breakdown of SI occurred independently in different clades (Fig. 1). In addition, most of the transitions occurred from SI to SC, in agreement with the intuition that SI is lost more frequently than gained (Takebayashi and Morrell, 2001; Igic *et al.*, 2006; Escobar *et al.*, 2010). According to Davis *et al.* (2013), inferences of character evolution using trees with fewer than 300 terminal taxa have low power and should be interpreted with caution. Thus, we will not discuss the results of SC and SI transitions in relation to the directionality of changes, but only the transitions between SC and SI observed in different clades over the tree, a result that probably would not change if additional species were analysed in future studies. For instance, SC and SI species occur within the same clades in the phylogeny published by Xiang *et al.* (2013), which includes 192 accessions of 109 *Dendrobium* species.

A breakdown in SI has been shown to evolve in populations in which sexual reproduction is limited by mate availability, such as small or colonizing populations (Levin, 2012; Barrett, 2013), as found in epiphyte species (Gentry and Dodson, 1987; Vasquez *et al.*, 2003; Tremblay *et al.*, 2005, 2006). Fragmented distribution and pollen limitation are common ecological attributes of orchid populations (Gentry and Dodson, 1987; Larson and Barrett, 2000; Tremblay and Ackerman, 2001; Tremblay *et al.*, 2005; Phillips *et al.*, 2011). Population studies have shown that the colonization of new paths by epiphytic orchids is often due to a small number of individuals (Tremblay *et al.*, 2006), with subsequent population expansion resulting from *in situ* reproduction (Trapnell and Hamrick 2005; Trapnell *et al.*, 2013).

The founding of new populations by long-distance seed dispersal combined with further divergent selection in these novel selective environments has been proposed as an important mechanism of orchid speciation (reviewed by Phillips *et al.*, 2012). The observation that most *Dendrobium* species are epiphytes suggests that founding events from long-distance seed dispersal followed by the breakdown of SI may be an important mechanism of speciation in the genus. In this scenario, genetic incompatibilities and reproductive isolation barriers would accumulate faster by exposing the newly founded populations to different selective environments (Phillips *et al.*, 2012) and changes to SC (Squirrell *et al.*, 2002). Furthermore, outbreeding depression was observed in some crosses between individuals from distant populations (e.g. *D. aciculare*, *D. devonianum*, *D. lindleyi*; Johansen, 1990), suggesting that genetic incompatibilities that accumulate among divergent populations may reduce compatibility, triggering early stages of speciation, as observed in other plant groups (Scopece *et al.*, 2010), including orchids (Pinheiro *et al.*, 2013). Further population-level studies are thus necessary to shed light on these speciation stages, using sister species with different compatibility systems or, even better, different populations in species with SC? and SI? systems, e.g. in *D. aphyllum*, *D. crystallinum* and *D. draconis*.

Our results show that transitions between SC and SI in *Dendrobium* are followed by asymmetrical patterns of reproductive isolation (Table 2). This finding strongly agrees with the unilateral incompatibility explained by the SI × SC rule, which states that pollen from SI individuals fertilizes ovules of SC plants but the reciprocal cross fails (Hiscock *et al.*, 1998; Brandvain and Haig, 2005). Additional support for this hypothesis came from crosses between SC species, which showed significantly higher fruit and seed set compared with crosses between SI species. According to the SI × SC rule, pollen tubes from SI species are normally unrelated to the sporophytic tissues through which they grow, a situation not observed for SC species (Lewis and Crowe, 1958; Hiscock and Dickinson 1993; Murfett *et al.*, 1996). Thus, SI styles contain barriers to fertilization that are not retained in SC styles (Hiscock and Dickinson, 1993), decreasing overall fruit and seed set, as observed in crosses between SI species, in agreement with the results observed here (Table 2). According to the observed patterns, a higher frequency of interspecific hybridization would be expected between SC species, and genomic studies using new sequencing technologies (Twyford and Ennos, 2012) may shed light on this question.

Unilateral incompatibility was also observed in crosses between SI species (e.g. *D. bilobulatum* × *D. leonis*, *D. cariniferum* × *D. virgineum*, *D. devonianum* × *D. crystallinum*) and between SC species (e.g. *D. phalaenopsis* × *D. delacourii*, *D. undulatum* × *D. macrophyllum*, *D. strebloceras* × *D. stratiotes*) (Appendix 1), suggesting that variable SI systems and different degrees of outcrossing are present in SI and SC species. Usually orchid species show very low levels of selfing (Tremblay *et al.*, 2005), even considering only SC species (Squirrell *et al.*, 2001; Soliva and Widmer, 2003; Jacquemyn *et al.*, 2006; Pinheiro *et al.*, 2011). By showing high levels of outcrossing, asymmetrical incompatibilities between SC species are also expected to occur because the occurrence of pollen–pistil conflicts is proportional to the number of partners involved in pollination (Brandvain and Haig, 2005). Thus, reproductive isolation is expected between outcrossers and inbreeders, or in species in which SC was recently acquired (Lewis and Crowe, 1958; Brandvain and Haig, 2005). Future studies using non-model organisms should examine divergence time estimates between species pairs in which transitions in compatibility systems are present and absent. If divergence time estimates are shorter for lineages experiencing transitions between SC and SI, the reproductive isolation expected by the SI × SC rule may play a role in speciation events (Brandvain and Haig, 2005).

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of Table S1: full details of the interspecific reciprocal crosses performed by Wilfret (1968) and Johansen (1990).

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