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## Evidence for progenitor–derivative speciation in sexually deceptive orchids

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• **Background and Aims** Sexually deceptive orchids of the genus *Ophrys* use mimicry of pollinator females to attract specific pollinators. Pollinator shifts may drive speciation in *Ophrys*, since novel pollinators may in principle act as isolating factors immediately. It is thus possible that evolution of novel species occurs rapidly and with a progenitor–derivative pattern. The aims of this study are to compare genetic structure and diversity among widespread and geographically restricted *Ophrys* taxa, to test whether genetic structure is associated with specific pollinators, and to investigate whether any widespread species may have acted as a progenitor for the evolution of more restricted taxa.

• **Methods** Genetic differentiation and diversity were investigated in *O. leucadica* and *O. cinereophila*, the two taxa of the *Ophrys fusca sensu lato* complex widespread in the Aegean, and three geographically restricted taxa from Rhodes, *O. attaviria*, *O. parvula* and *O. persephona*, all differing in their specific pollinators. This was done using amplified fragment length polymorphism (AFLP) DNA fingerprinting, and sequencing of the low-copy nuclear gene *LEAFY* (*LFY*).

• **Key Results** All taxa were found to be separate genetic entities, with *O. leucadica* forming two geographic groups from the west and east of the Aegean. Genetic structure was significantly shaped by pollinators and geography, and comparison of sequence and AFLP data revealed ancestral polymorphisms shared among several taxa. Among the sampled taxa, *O. leucadica* harbours the greatest genetic differentiation and geographic structure, and the highest genetic diversity. Part of the genome of *O. parvula*, endemic to Rhodes, may be derived from *O. leucadica*.

• **Conclusions** Pollinators probably influence the genetic structure of the investigated *Ophrys* species. The genetic pattern identified is consistent with *O. leucadica* being the oldest of the sampled taxa, making *O. leucadica* a candidate progenitor species from which more restricted taxa such as *O. parvula* may have evolved.

**Key words:** AFLP, genetic diversity, genetic structure, low-copy nuclear genes, *Ophrys*, pollination, progenitor–derivative speciation, sexually deceptive orchids.

### INTRODUCTION

Many orchids are characterized by a high specificity of pollination (Schiestl and Schlüter, 2009). In particular, sexually deceptive orchids, the flowers of which mimic female bees to attract males as pollinators, can attain pollinator-mediated reproductive isolation by differential attraction of pollinator species (Paulus and Gack, 1990; Schiestl and Ayasse, 2002; Peakall *et al.*, 2010; Ayasse *et al.*, 2011; Gaskett, 2011). *Ophrys* is a European and Mediterranean genus of Orchidaceae that is pollinated using a mechanism of sexual deception (Kullenberg, 1961; Paulus and Gack, 1990; Paulus, 2006). *Ophrys* does not offer any reward or incentive for generalized pollinators, and species of this genus are predominantly characterized by pollination by one (or few) specific insect species (Paulus and Gack, 1990; Paulus, 2006). *Ophrys* flowers attract male pollinators by mimicry of key traits of their females and induce pollinator males to mate with the flower, resulting in pollen transfer. The most important trait mimicked by *Ophrys* flowers is the insect virgin female's

sex pheromone (Schiestl *et al.*, 1999, 2000), which elicits copulatory behaviour in males and explains the high specificity of pollinator attraction observed in *Ophrys* (Ayasse *et al.*, 2001; Vereecken, 2009). Accordingly, strong floral isolation among co-flowering *Ophrys* species has been reported, whereas post-zygotic mating barriers appear to be largely absent (Ehrendorfer, 1980; Cozzolino *et al.*, 2004; Scopece *et al.*, 2007; Schlüter *et al.*, 2009; Xu *et al.*, 2011; but see Gögler *et al.*, 2009).

In plant species with a high specificity of pollination, such as *Ophrys*, pollinator behaviour can serve as a pre-mating reproductive barrier. In this case, pollinator-mediated reproductive isolation is consistent with ecological speciation (Schluter and Conte, 2009) due to divergent selection on odour phenotypes (see Mant *et al.*, 2005b). As long as divergent selection acts on a small number of traits, this is expected to be a 'genic' speciation process (Wu, 2001; Wu and Ting, 2004), in which species differences are initially caused by only a few genes that are the targets of divergent selection, whereas gene flow is effective throughout the majority of the

genome. Such a scenario is especially likely in *Ophrys* because a small number of genes are expected to be responsible for differences in pollinator attraction among species (Schlüter and Schiestl, 2008; Schlüter et al., 2009, 2011), and strong floral isolation, linked to only a few genes of large effect, make rapid speciation by pollinator shift appear likely. Since speciation brought about by a pollinator shift within one population would not be expected to have any impact on pollinator specificity in other populations of the source species, one would expect speciation to follow a progenitor–derivative pattern (see, for example, Levin, 1993; Rieseberg and Brouillet, 1994; Gottlieb, 2003; Levin, 2004; Waser and Campbell, 2004; Crawford, 2010), in which a derivative species arises as a genetic sub-set of the progenitor species without affecting the progenitor. The derivative species should then (a) be monophyletic and genetically closely related to the progenitor (which might be paraphyletic); but (b) contain only a sub-set of the progenitor's genetic diversity, less genetic population structure and fewer private alleles (Perron et al., 2000). Moreover, (c) due to its local origin, the derivative species should be geographically restricted when compared with its more widespread progenitor. Furthermore, (d) in the case of *Ophrys*, progenitor and derivative should be isolated by their different pollinators. Therefore, a common *Ophrys* species may give rise to a number of local endemics that are genetically similar to the gene pool from which they are derived. The potential for rapid speciation implies that many *Ophrys* species may be of recent origin, making it difficult to obtain reliable phylogenetic hypotheses. This may be further complicated by the expectation of paraphyly for any species that acted as a progenitor for other species (e.g. Rieseberg and Brouillet, 1994). In practice, many markers commonly used to infer phylogenies do not harbour sufficient variation to obtain a well-supported estimate of relationships within *Ophrys* (Soliva et al., 2001; Bateman et al., 2003; Bernardos et al., 2005; Schlüter et al., 2007a; Devey et al., 2008). Nonetheless, *Ophrys* sect. *Pseudophrys* is well supported as a monophyletic group based on molecular data (Soliva et al., 2001; Bateman et al., 2003; Bernardos et al., 2005; Devey et al., 2008). Morphologically, this section is characterized by the direction of the trichomes on the labellum. This determines the orientation of male pollinators on the lip (Ågren et al., 1984; Pirstinger, 1996; Pirstinger and Paulus, 1996), which results in the attachment of pollinaria to an insect's abdomen rather than its head. Within section *Pseudophrys*, *O. fusca sensu lato* (*s.l.*) represents the most diverse species complex, relationships within which are poorly understood (but see Schlüter et al., 2007a).

The *O. fusca s.l.* group has a pan-Mediterranean distribution, containing a few widely distributed taxa and a large number of highly restricted or endemic taxa. In the Aegean, *O. leucadica* and *O. cinereophila* are two common members of the *O. fusca s.l.* group. *Ophrys leucadica* occurs throughout the Aegean, with the exception of Crete, and may be conspecific with *O. bilunulata* from the west Mediterranean, based upon morphology and pollination biology (Paulus, 2001b; Paulus and Salkowski, 2007). *Ophrys cinereophila* is distributed throughout the Aegean, but does not occur in the west Mediterranean (Delforge, 2006). *Ophrys attaviria*, *O. parvula* and *O. persephonae* have much more restricted

distributions, restricted to or centred around the east Aegean island of Rhodes (Paulus, 2001a; Kreutz, 2003; Paulus and Schlüter, 2007). All five study species co-occur on Rhodes, and differ in their pollinators (Supplementary Data Table S1, available online) and flower labellum size (which is correlated with pollinator body size; Paulus, 2006), but partially overlap in their flowering times (Kretzschmar et al., 2001; Paulus, 2001a; Paulus and Schlüter, 2007). The study species are exclusively pollinated by *Andrena* bees, all of which appear to share a common pheromone chemistry (Ayasse et al., 2011). Mimicry of *Andrena* pheromones by other *Ophrys* species has a genic basis (Schlüter et al., 2011) and results in strong pollinator-mediated reproductive isolation (Xu et al., 2011). It is noted that *Ophrys* species-level taxonomy is contended (compare, for example, Delforge, 2006; Pedersen and Faurholdt, 2007), the present study following the taxonomy of Paulus (2001a, b).

The pattern of widespread vs. restricted taxa may be indicative of the presence of a few progenitor species that gave rise to a number of derivative species. Specifically, *O. leucadica* or *O. cinereophila* may have acted as progenitors for any of the restricted species *O. attaviria*, *O. persephonae* or *O. parvula*. To evaluate this hypothesis, it is necessary to uncover the genetic structure in both the more widely distributed and more restricted *Ophrys* taxa. Here, amplified fragment length polymorphism (AFLP) markers (Vos et al., 1995) were used for this purpose. AFLP data were complemented with sequence data from the putatively single-copy gene *LEAFY* (*LFY*) (Montieri et al., 2004; Schlüter et al., 2007a) from *Ophrys* sect. *Pseudophrys* members. Using multiple lines of evidence from pollinator specificity, phylogenetic and population genetic data, the present study seeks to (a) elucidate the genetic structure and diversity of *O. leucadica* and *O. cinereophila* in the Aegean, and of restricted taxa from Rhodes; (b) test whether genetic structure is associated with pollinators or geography; (c) investigate the relationships among taxa; and (d) test whether any of the widespread taxa may have acted as a progenitor for the more restricted taxa found on Rhodes.

## MATERIALS AND METHODS

### *Plant material, DNA extraction and fluorescent AFLP reactions*

Plant material (Fig. 1, Table 1) was collected in the field and stored in silica gel. Where possible, plant individuals were photographed and representative vouchers deposited in the herbarium at Vienna University (WU), Austria. Populations were sampled so as to address questions at the taxon rather than at the within-population level (for sample sizes, see Table 1). Plants from all populations included in AFLP analyses were tested for specific pollinator attraction in the field, as described previously (Schlüter et al., 2009). Most of the sampled populations were small, with <50 individuals observed. DNA was extracted using a DNeasy plant mini kit (Qiagen, Vienna, Austria) and the manufacturer's protocol. The AFLP analyses followed the procedure of Vos et al. (1995), with modifications as detailed in Schlüter et al. (2007b). Six primer combinations of 5'-fluorophore-labelled *EcoRI* primers and unlabelled *MseI* primers were used: *MseI*-CTCG with *EcoRI*-ACT (6-FAM), -ATC (HEX) and -ACC (NED), and *MseI*-CTAG with

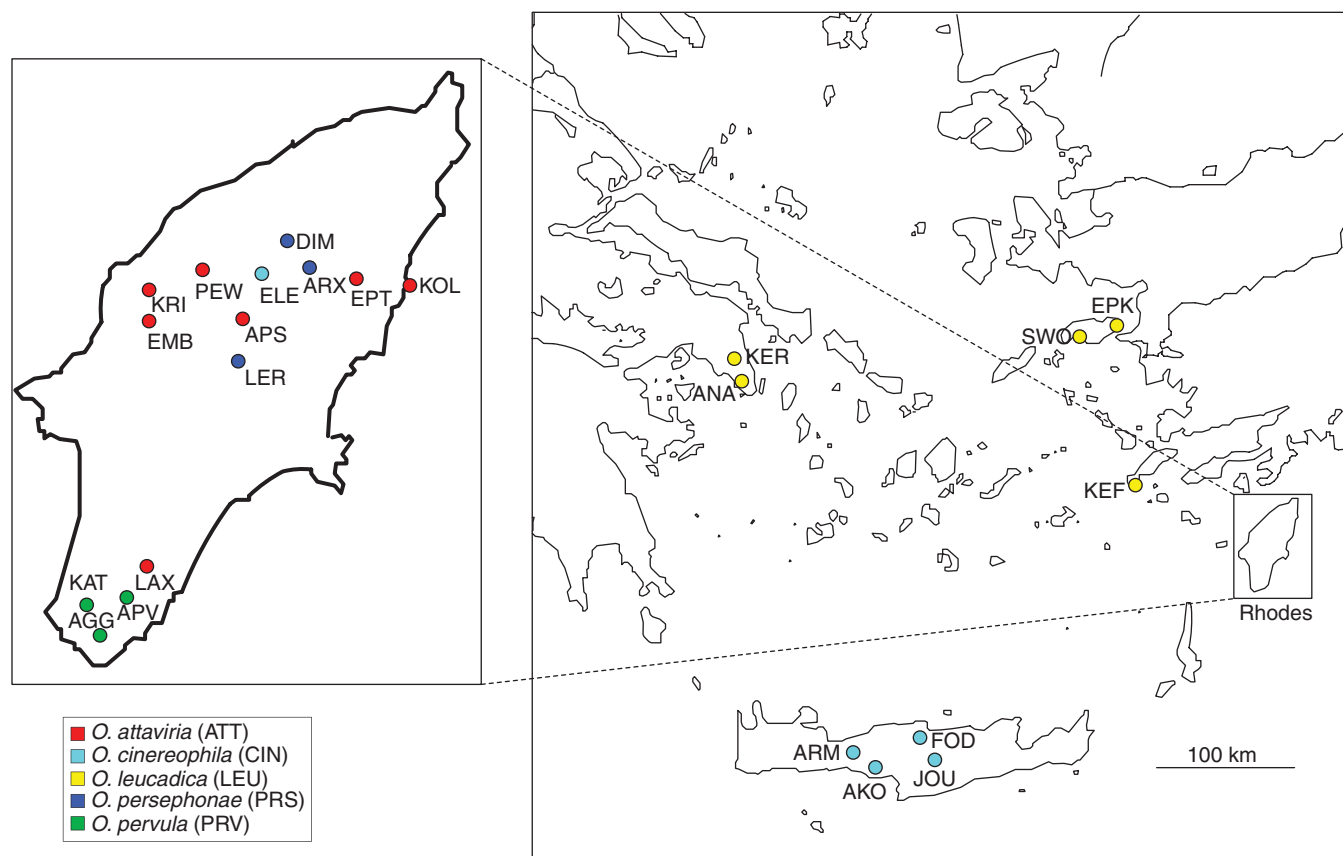


FIG. 1. Map of the Aegean indicating localities from which population samples were taken for AFLP analysis. *Ophrys bilunulata* was sampled from the west Mediterranean and is therefore not indicated on this map. Localities for different taxa are highlighted in different colours, as indicated in the figure. The inset shows details of the island Rhodes. Sampling localities conform to the three-letter codes shown in Table 1.

*EcoRI*-ACT (6-FAM), -AGG (HEX) and -AGC (NED). Genescan-500-ROX (Applied Biosystems, Vienna, Austria) was used as an internal size standard, and AFLP reactions, including appropriate negative and positive controls, were run on a 4 % denaturing polyacrylamide gel in an ABI Prism 377 DNA sequencer (Perkin Elmer Applied Biosystems, Vienna, Austria).

#### Scoring and data analysis

The AFLP banding patterns were scored manually using Genographer software v.1.6.0 (Benham *et al.*, 1999) as a visual aid. The data set was scored twice independently, coding bands as presence (1) or absence (0), explicitly scoring ambiguous bands as missing data (?). Both scorings were first analysed separately, and secondly as a combined data set. AFLP error rates were estimated as suggested by Bonin *et al.* (2004); the mean genotyping error rate among controls and the mean error rate among scorings (of the same fragments) were estimated applying (a) strict and (b) relaxed criteria. Strict error rates treated 1/? and 0/? band comparisons as errors, whereas these combinations were not treated as erroneous under relaxed criteria.

Maximum-likelihood-based reallocation tests were performed in AFLPOP (Duchesne and Bernatchez, 2002) to test if sampled individuals belonged to their respective putative

source populations. Bayesian analysis of population structure was carried out using BAPS 3.2 (Corander *et al.*, 2003), with AFLP data treated as diploid and coding the second allele at every locus as missing data. Clustering of individuals and admixture analysis were performed with the maximum number of populations set to ten. Structure 2.3.1 (Falush *et al.*, 2007) analyses were carried out using the admixture model with correlated allele frequencies, and AFLP data input as diploid, dominant data. Each analysis was run for 100 000 generations, discounting the first 50 % as a burn-in. Analyses were performed in triplicate for  $K = 2$  to  $K = 10$ , and the optimal  $K$  value was determined using the method of Evanno *et al.* (2005). Pairwise distance matrices were calculated from AFLP data using an average Jaccard coefficient taking into account missing data, and subjected to principal coordinate analysis (PCoA) in FAMD 1.29 (Schlüter and Harris, 2006). Counting of private bands was done in the same software.

Allele frequencies were estimated with the Bayesian method of Zhivotovsky (1999) in FAMD, using the non-uniform prior from among-population variation, and pairwise population distances calculated using the chord distance (Cavalli-Sforza and Edwards, 1967) in the multilocus formulation of Takezaki and Nei (1996). The chord distance was shown to outperform other population distance methods in recovering the true topology in simulations (Takezaki and Nei, 1996). Pairwise  $\Phi_{ST}$  values

TABLE 1. Plant samples used for AFLP analysis, where n is the number of individuals sampled from a population

Code	Region	Locality	n	Date	Collector	Population number
<i>O. attaviria</i> D. Rückbrodt & Wenker (ATT), n = 25						
APS	Rhodes	S. of Apollonia	5	25-04-2003	PMS	144
EMB	Rhodes	Embonas	3	24-04-2003	PMS	139
KRI	Rhodes	Kritinia	3	24-04-2003	PMS	137
KOL	Rhodes	Kolybia	1	23-04-2003	PMS	134
PEW	Rhodes	Profitis Elias (W side)	3	22-04-2003	PMS	126
EPT	Rhodes	Epta Piges	4	20-04-2003	PMS	117
LAX	Rhodes	Lachania	6	23-04-2003	PMS	136
<i>O. bilunulata</i> RISSO (BIL), n = 7						
CLD	Malaga	Coin Las Delicias	7	09-04-2004	HFP	198
<i>O. cinereophila</i> PAULUS & GACK (CIN), n = 24						
AKO	Crete	Akoumia	3	02-04-2003	HFP	114
ARM	Crete	Armeni	2	02-04-2003	HFP	112
ELE	Rhodes	W. of Eleoussa	8	22-04-2003	PMS	130
FOD	Crete	Fodele	5	01-04-2003	HFP	110
JOU	Crete	Jouchtas	6	30-03-2003	HFP	103
<i>O. leucadica</i> Renz (LEU), n = 25						
ANA	Attica	Anavissos	5	26-03-2004	M. Fiedler	209
EPK	Samos	Paleokastro	7	21-02-2004	HFP	172
KEF	Kos	Kefalos	3	01-03-2002	HFP	067
KER	Attica	Keratea	2	26-03-2004	M. Fiedler	210
SWO	Samos	Ormos	8	24-02-2004	HFP	184
<i>O. parvula</i> Paulus (PRV), n = 7						
AGG	Rhodes	Agios Georgios	3	22-04-2003	PMS	131
APV	Rhodes	Agios Pavlos	2	23-04-2003	PMS	135
KAT	Rhodes	Katavia	2	28-03-2004	M. Fiedler	215
<i>O. persephona</i> Paulus (PRS), n = 9						
ARX	Rhodes	Archipolis	4	20-04-2003	PMS	118
DIM	Rhodes	Dimylia	3	20-04-2003	PMS	119
LER	Rhodes	Laerma	2	25-04-2003	PMS	142

were calculated in Arlequin 3.0 (Excoffier *et al.*, 2005) and FAMD based on Euclidean and average Jaccard distances. Chord and  $\Phi_{ST}$  population distances were subjected to UPGMA analysis in FAMD with 1000 bootstrap replicates. Geographic distances among sampling localities were based on the great circle distance via the haversine formula (Sinnott, 1984). A generalized linear model (GLM) with Gaussian error distribution was used to model genetic (pairwise chord and  $\Phi_{ST}$ ) population distance with the explanatory variables geographic distance (log-transformed), shared pollinator (categorical variable), and an interaction term among the two. This analysis was performed in R 2.11.0 (R Development Core Team, 2010).

Analysis of molecular variance (AMOVA) using three levels of hierarchy (taxon, island/region and population) was performed on the AFLP data from *O. cinereophila* and *O. leucadica* using Arlequin 3.0, both across all loci and on a locus-by-locus basis. Similarly, AMOVA using two levels of hierarchy (taxon and population) was calculated for all taxa, and for population groups within *O. leucadica*. Shannon's diversity index (calculated as  $H_{Sh} = -\sum p_i \log_2 p_i$  where  $p_i$  is the frequency of band presence in a locus) and its variance were estimated by bootstrapping using 10 000 pseudo-replicates in FAMD (Schlüter and Harris, 2006), sampling seven randomly chosen individuals per species and iteration, and randomly replacing missing data by 50 % band absences and 50 % band presences. Significance tests for Shannon's index were carried out as suggested previously (Hutcheson, 1970; Magurran, 1988).

#### DNA sequences

After screening several loci (Schlüter *et al.*, 2007a), sequences from the low-copy nuclear gene *LFY* were obtained from members of *Ophrys* sect. *Pseudophrys* to complement the AFLP analysis. Sampling included mostly Aegean taxa of the section, with several accessions of *O. cinereophila* and *O. leucadica* (Supplementary Data Table S2). Several samples (*O. attaviria* 117A; *O. bilunulata* 198A; *O. cinereophila* 114A, 130D; *O. leucadica* 67A, 172A, 209B; *O. persephona* 119B) were present in both AFLP and sequence data sets, and further samples represented different plant individuals from the same populations (*O. cinereophila* 25A; *O. parvula* 131C) or nearby populations (*O. leucadica* 333A). The nuclear gene *LFY* is probably a single-copy gene in *Ophrys* (Montieri *et al.*, 2004; Schlüter *et al.*, 2007a). The 5' fragment of *LFY* was amplified, sequenced and alleles compiled as described previously (Schlüter *et al.*, 2007a). The sequenced region covers exon 1, intron 1 and part of exon 2 of *LFY* and is approx. 3 kb in length, of which the intron constitutes two-thirds.

#### Sequence analysis

The *LFY* sequences were added to the alignment of Schlüter *et al.* (2007a) and manually aligned using BioEdit 7.0.1 (Hall, 1999). Since *LFY* sequences were amplified from a presumably nuclear locus, and as such are expected to undergo recombination, recombination among sequences was tested using the

program RDP3 with its default parameters (Martin *et al.*, 2010), either with all sequences in the alignment or with automatic masking of sequences. Three data sets were compiled: (a) all observed sequences; (b) only non-recombined sequences; and (c) all sequences, splitting each recombined sequence into two sequences at the inferred recombination breakpoints and treating the remaining nucleotides as missing data. The phylogenetic relationships among sequences were inferred using MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003), as detailed in the Supplementary Methods available online.

Observed heterozygosity ( $H_O$ ) for each species was calculated as the number of *LFY* heterozygotes divided by the total number of individuals sampled for that species. Expected heterozygosity was not calculated because our data do not allow us to estimate allele frequency.

## RESULTS

### AFLP results

Amplified fragment length polymorphism bands were scored twice for 97 individuals. After removal of bands that occurred only in single individuals, two scorings of the same 655 AFLP markers were available. Since initial analysis suggested congruent results, these two scorings were combined into a single data matrix containing 2.83 % missing data. The mean genotyping error between controls and error rate among scorings (Bonin *et al.*, 2004) were 5.11 % (relaxed) to 7.57 % (strict) and 4.47 % (relaxed) to 7.88 % (strict), respectively.

Principal coordinate analysis of pairwise distances (Fig. 2A), Bayesian clustering and Structure 2.3.1 analyses (both in Fig. 2B) were largely concordant, suggesting seven genetic groups in our data corresponding to the investigated taxa, with the exception of *O. leucadica* which appeared as two groups. One group contained the two western populations from Attica and one the eastern populations from Samos and Kos. Hereafter, these two groups are referred to as eastern (E) or western (W) groups of *O. leucadica*. Two individuals (139A and 184F) were considered to be outliers and excluded from all population-based analyses. Structure analysis suggested the *O. parvula* genome to be admixed (Fig. 2B). At the optimal value of  $K = 6$ , *O. parvula* is inferred to have genomic contributions of *O. attaviria* and *O. leucadica* group E; only the contribution of *O. leucadica* E is inferred at  $K > 6$ .

Clustering at the population level using the chord distance supported the above analyses, with *O. parvula* inferred as sister group to the eastern *O. leucadica* populations (Supplementary Data Fig. S1, available online). A GLM revealed that both geographic distance and shared pollinators significantly explain genetic distances (Supplementary Data Table S3), genetic distances being smaller among population pairs with the same pollinator (Supplementary Data Fig. S2). In most GLM analyses, shared pollinators were a more significant factor than geography.

The number of private AFLP bands was highest for *O. leucadica* (Supplementary Data Table S4). Likewise, Shannon's diversity index (Fig. 3; Supplementary Data Table S4) was highest for *O. leucadica*, being significantly greater than that of any other sampled taxon (all  $P < 0.01$ ). *Ophrys*

*cinereophila* displayed the second highest Shannon's index (significantly greater than that of the remaining Aegean taxa; all  $P < 0.01$ ). Analysis of molecular variance-derived  $\Phi_{ST}$  values for the taxa studied (Supplementary Data Table S4) generally suggested low intra-taxon differentiation, although this was not the case for *O. leucadica*. Nested AMOVA for *O. leucadica* and *O. cinereophila* (Supplementary Data Table S5), which were both sampled from different geographic regions, showed that both taxa harbour a similar amount of genotypic variation within geographic groups. However, differentiation among geographic groups was much stronger in *O. leucadica*. Inclusion of *O. bilunulata* as an additional group within *O. leucadica* did not alter the above findings (Supplementary Data Tables S4, S5).

### Sequence results

A total of 87 *LFY* alleles from 66 *Ophrys* individuals were analysed, of which ten sequences were putatively recombined (Supplementary Data Table S6). For no individual could more than two alleles be found, which is consistent with the hypothesis that the studied taxa are diploids. The same two alleles were found in both *O. sphegodes* 392A and *O. archipelagi* 393A. *LFY* gene genealogies obtained with different treatments of recombined sequences were largely concordant (Fig. 4, Supplementary Data Figs S3, S4). Three groups of sequences were identified from *Ophrys* sect. *Pseudophrys* taxa (Fig. 4, Supplementary Data Table S7): group A contained *O. fusca s.l.* endemics from Crete such as *O. cretica* or *O. creberrima*; group B contained, for example, *O. omegaifera s.l.* and *O. iricolor s.l.*; and group C contained, for example, *O. bilunulata* and *O. lutea s.l.* The allele from *O. parvula* was placed in group A, whereas *O. attaviria* and *O. persephonae* alleles were in group B. Only alleles from *O. leucadica*, *O. cinereophila* and *O. thriptiensis* (endemic to Crete) were found in more than one sequence group. Recombination among *O. fusca s.l.* sequences was found only among sequence groups A and B (Fig. 4, Supplementary Data Table S6). No geographic pattern was discernible for *O. cinereophila* and *O. leucadica LFY* alleles. *Ophrys cinereophila* from Crete had alleles from groups A and B; *O. leucadica* from western localities also used for AFLP (and population KRP nearby) had alleles in groups A and B, while those from eastern localities had alleles in groups A and C, the C alleles being very similar to alleles from *O. bilunulata*. Observed heterozygosity was highest for *O. thriptiensis*, *O. leucadica* and *O. cinereophila* (Supplementary Data Table S7).

## DISCUSSION

### Genetic structure is shaped by pollinators and geography

Pollinators of *Ophrys* are thought to be highly specific and would be expected to act as isolating factors (Paulus and Gack, 1990), although the effectiveness of pollinators in maintaining species boundaries and preventing hybridization or gene flow among *Ophrys* taxa has been questioned by some authors (e.g. Devey *et al.*, 2008). Phylogenetic studies of *Ophrys* show limited divergence among taxa in nuclear ribosomal ITS (internal transcribed spacer) and chloroplast DNA

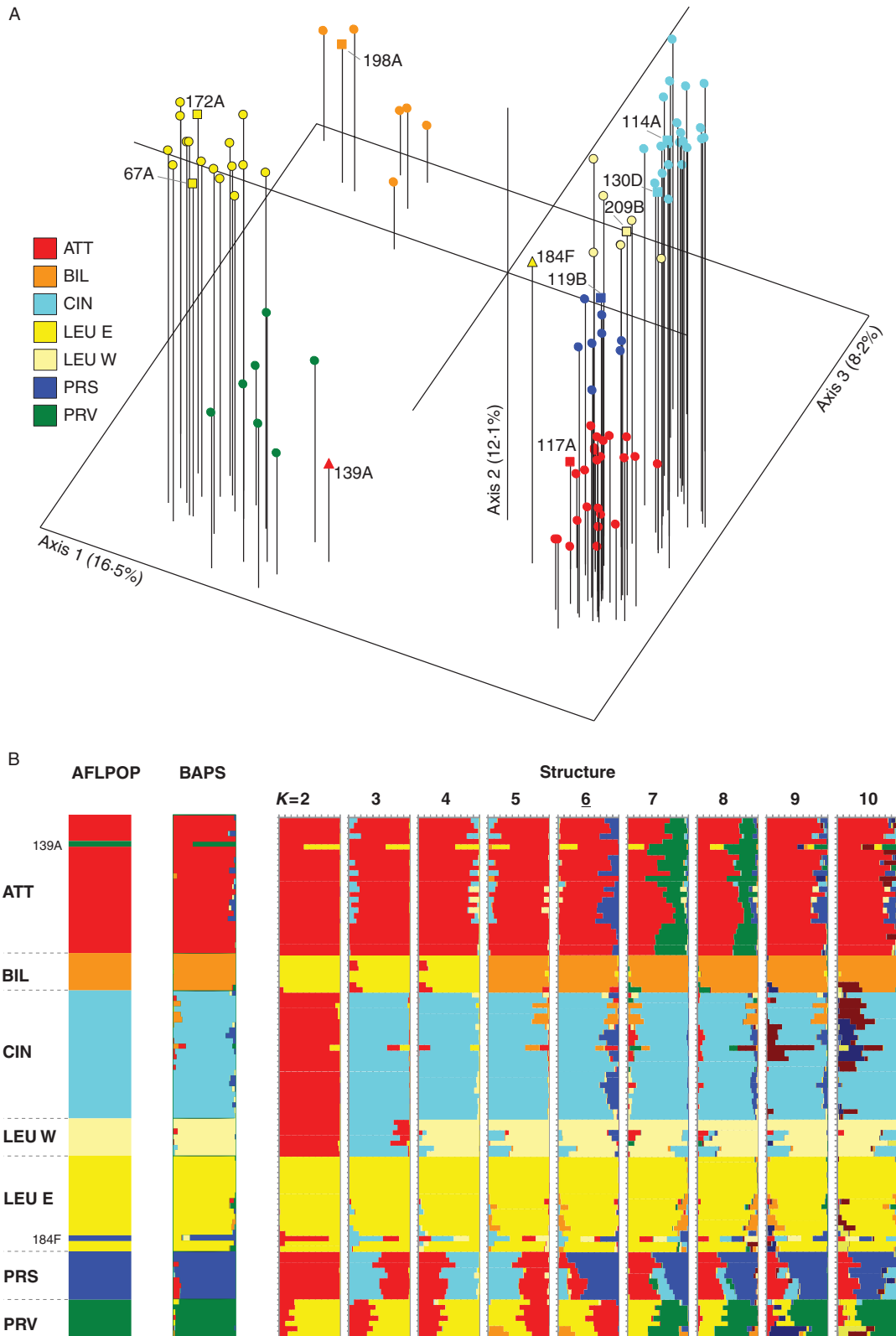


FIG. 2. Results from individual-based analyses of AFLP data. (A) PCoA plot based on an average Jaccard's coefficient after 100 random draws from the interval of possible values (Schlüter and Harris, 2006). Points of different colour represent individuals of different taxa, as indicated in the figure. In the case of *O. leucadica*, two shades of yellow are used to differentiate the two geographical groups of individuals from the east and west of the sampled area, denoted by the letters E and W, respectively. Circles represent samples only present in the AFLP data sets, whereas squares represent samples present in AFLP and sequence data sets (annotated with the sample number). Triangles represent the two individuals treated as outliers. (B) Left to right, results from AFLPOP, BAPS and Structure ( $K = 2-10$ ) analyses, the underlined  $K$  value (6) indicating the most likely value following Evanno *et al.* (2005). Species codes are as shown in Table 1.

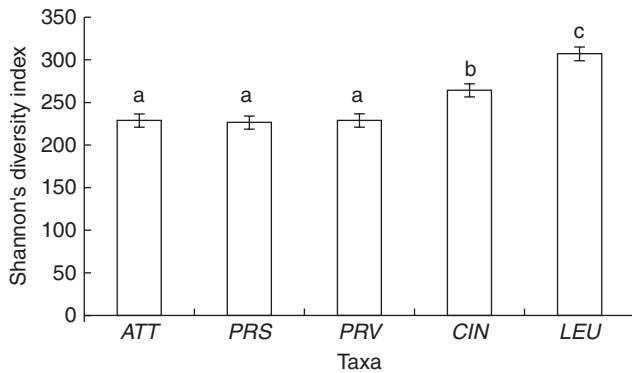


FIG. 3. Shannon's diversity index, a measure of genetic diversity, for the Aegean taxa studied, randomly sampling seven individuals per taxon. The abbreviations for taxa are given in Table 1. Error bars indicate  $\pm$  s.d. Different letters indicate values that are significantly different from each other ( $P < 0.01$ ).

(e.g. Soliva *et al.*, 2001; Bateman *et al.*, 2003; Devey *et al.*, 2008), which can be taken as evidence for fast divergence and radiation of taxa, or as evidence for gene flow and hybridization (or both). Also, previous population studies using molecular markers have yielded conflicting data, some suggesting a weak or absent differentiation among taxa with different pollinators (e.g. Soliva and Widmer, 2003; Mant *et al.*, 2005b; Schlüter *et al.*, 2007b), whereas in other studies genetic groups delimited by different pollinators were evident (e.g. Grünanger *et al.*, 1998; Caporali *et al.*, 2001; Schlüter *et al.*, 2007b; Gögler *et al.*, 2009; Stökl *et al.*, 2009). In this study, we found that apart from *O. leucadica*/*O. bilunulata*, all investigated *Ophrys* taxa were identified as cohesive genetic groups in our AFLP analysis. Since these orchid taxa differ in their specific pollinators (different species of the genus *Andrena*; Supplementary Data Table S1), this supports the role of pollinators in maintaining species boundaries among the taxa analysed. However, the correlation of geographic and genetic population distances in our data set implies that isolation by distance (i.e. drift) cannot be rejected. Genetic drift has recently been highlighted as an important factor in orchid population biology (Tremblay *et al.*, 2005) and could in principle explain the patterns of population structure in presumably neutral AFLP markers that were observed in this study. However, large effective population sizes have been estimated for *Ophrys* (Soliva and Widmer, 2003; Mant *et al.*, 2005b), which should mitigate genetic drift. Nonetheless, it is evident that pollinators also have a strong and significant effect on population structure (Supplementary Data Fig. S2, Table S3). In fact, in many analyses, pollinators are more strongly associated with population structure than is geography (Supplementary Data Table S3). Taken together, this implies that the observed population structure in our study taxa is largely (but not entirely) shaped by pollinators.

#### Genetic structure in restricted and widespread taxa

The three taxa sampled only from Rhodes, *O. attaviria*, *O. parvula* and *O. persephoniae*, all formed separate groups in AFLP data, which was expected because of their different

specific pollinators (Fig. 2; Supplementary Data Table S1). None of these taxa displayed any obvious geographic population structure. In Structure analyses, *O. parvula* showed signs of a mixed genomic composition, which may reflect either ancestral polymorphism or a hybrid origin. A hybrid origin, however, seems unlikely, because this species is genetically separate from the other species with which it shares alleles. Moreover, morphological assessments are not suggestive of hybridity, the flowers (and pollinator) of *O. parvula* being considerably smaller than those of both putative parents, *O. leucadica* and *O. attaviria* (Paulus, 2001a; Paulus and Schlüter, 2007).

Strong genetic structure was observed in *O. leucadica*, but not in *O. cinereophila*. This was evident in PCoA (Fig. 2), and in the higher  $\Phi_{ST}$  value for *O. leucadica* (Supplementary Data Tables S4, S5). The Aegean samples of *O. leucadica* were present in two geographic groups, one from the west Aegean (Attica) and one from the east Aegean (Samos and Kos). If *O. bilunulata* is regarded as conspecific with *O. leucadica* (Paulus, 2001b; Paulus and Salkowski, 2007) then the sampled *O. bilunulata* population would represent one further geographic group in *O. leucadica*. In contrast, *O. cinereophila* did not display a similar amount of genetic structure, even though the sample from the Aegean covered a similar geographic range to that from *O. leucadica*. Although in *O. leucadica*, differentiation among geographic groups was higher than in *O. cinereophila* (where samples from Rhodes and Crete were included), both taxa displayed a similar amount of genotypic variation within groups (Supplementary Data Table S5). There are currently no data suggesting a difference in the ecology of *O. leucadica* (and *O. bilunulata*) and *O. cinereophila*, apart from the specialization for different species of pollinating bees. This in turn cannot explain the differences in geographic structure in the two *Ophrys* species, although the apparent absence of the *O. cinereophila*'s pollinator, *Andrena cinereophila*, from the west Mediterranean (Supplementary Data Table S1) (Gusenleitner and Schwarz, 2002) probably explains the absence of this *Ophrys* taxon from that area.

#### Higher genetic diversity in widespread taxa

The taxa that are common in the Aegean, *O. leucadica* and *O. cinereophila*, displayed significantly greater genetic diversity than the taxa sampled from Rhodes alone (Fig. 3; Supplementary Data Tables S4, S7). This would suggest that the more widespread taxa harbour greater genetic diversity than the more restricted ones. In particular, the highest genetic diversity was found in *O. leucadica*, which is more widely distributed than any of the other sampled taxa, including *O. cinereophila*. This may be expected because more widespread taxa are likely to consist of a higher number of individuals and populations, and therefore have a higher chance for mutations to accumulate. However, while higher genetic diversity (but not population differentiation) is often found in widespread plant species as compared with rare congeners, this is not always the case and cannot be taken as a general trend (Gitzendanner and Soltis, 2000).





### Shared ancestral polymorphism

The distinct genetic groups identified from AFLP data contrast markedly with the pattern found among *LFY* alleles, several plant individuals or taxa containing alleles from different sequence groups (Fig. 4; Supplementary Data Table S7). *LFY* was the only marker chosen from a large number of candidates that had enough sequence variation to be informative even within closely related *Ophrys* species (Schlüter et al., 2007a). However, the pattern of allelic variation found at this locus implies that phylogenetic reconstructions based on *LFY* sequences may be regarded as gene genealogies representing the evolutionary history at this locus rather than a phylogeny that reflects organismal history. This is further complicated by the inferred presence of recombination at this locus. The observed allelic patterns, in particular the fact that sometimes two alleles found within the same individual were more strongly divergent from each other than from alleles restricted to other species, could be explained by extensive hybridization in the study group or by ancestral polymorphism shared among species (i.e. the retention of allelic diversity that was present prior to speciation in the descendant species). In sexually deceptive systems, genic speciation processes are likely (Schlüter and Schiestl, 2008; Schiestl and Schlüter, 2009; Schlüter et al., 2011), and divergent selection on very few loci may separate incipient species despite ongoing gene flow at other loci in the genome. Initially, species divergence will only be detectable at the few loci under selection. As a consequence, concordant genetic differentiation at multiple neutral loci (like AFLP) will only be detectable later in the process of divergence (see Harrison, 1991). Conversely, an AFLP profile in which species are inseparable (e.g. *O. omegaifera* and *O. sitiaca*) (Schlüter et al., 2007b) is consistent with both incomplete divergence and genetic mixing due to hybridization. The fact that the taxa studied here are separable using AFLP implies that the evolutionary history across the entire genome is not obscured by extensive hybridization and thus favours retention of ancestral polymorphism as an explanation for the allelic variation observed at the *LFY* locus. Moreover, hybridization can be rejected as a plausible explanation for the close relationship of *O. bilunulata* and *O. leucadica* alleles (sequence group C), because the respective populations are separated by 2800 km. Therefore, we conclude that incomplete lineage sorting due to retention of ancestral polymorphism is largely responsible for the conflicting patterns among AFLP and *LFY* data.

### *Ophrys leucadica* as a progenitor species

The genetic patterns observed in this study are congruent with a scenario in which the widespread *O. leucadica* represents the oldest of the sampled taxa from the *O. fusca* s.l. group and has acted as a progenitor species for more restricted taxa in the Aegean. Whereas the geographic groups found within *O. leucadica* may represent evolutionarily independent

lineages (cryptic species) that are convergent in their morphology, phenology and pollinator attraction, there is currently no biological evidence that would strengthen this view, and a greater age of *O. leucadica* seems more plausible given the available data. The hypothesis of a greater age of *O. leucadica* – and therefore a higher likelihood of having acted as a progenitor species – compared with other members of the Aegean *O. fusca* group is in agreement with (a) the greater distribution of this species (assuming equal rates of dispersal, habitat and pollinator availability); (b) its greater genetic differentiation among populations; (c) its greater genetic diversity; and (d) the finding of ancestral polymorphism in this species. First, while evolution in *Ophrys* may occur on a short time scale, and highly restricted taxa, such as *O. parvula*, may arise relatively quickly, it is obvious that colonization of the entire Mediterranean basin by *O. leucadica* (if it has the same origin as *O. bilunulata*) or at least the Aegean (if it does not) would require more time. Secondly, *O. leucadica*, being older than the other widespread species, *O. cinereophila*, would explain why geographic groups in *O. leucadica* have ‘drifted apart’ and show genetic differentiation, whilst such structure is absent from *O. cinereophila*. Thirdly, genetic diversity may likewise be the result of accumulation of mutations over a longer time scale. Conversely, populations (or parts thereof) that diverged from a progenitor species due to selection by a novel pollinator are expected to have reduced genetic diversity because of founder effects. This is consistent with the lower genetic diversities of *O. attaviria*, *O. parvula* and *O. persephoniae*, their lower numbers of private AFLP bands, and the observed pattern of *LFY* alleles. Among these taxa, *O. parvula* is inferred to have genomic similarities with *O. leucadica* in Structure analyses, which may reflect an ancestral genomic contribution from the progenitor species.

Although *O. cinereophila* is relatively widespread in the Aegean and is polymorphic for *LFY* alleles, it does not show genetic differentiation among geographic regions. By the same reasoning as above, *O. cinereophila* would be expected to be younger than *O. leucadica* and less likely to have acted as a progenitor for other *O. fusca* s.l. taxa in the Aegean. However, another potential example of a progenitor–derivative species pair may be *O. iricolor* and *O. mesaritica* (also from section *Pseudophrys*), although, in that case, AFLP profiles cannot yet separate the two taxa despite evidence for a pollinator shift and likely strong floral isolation among these species (Schlüter et al., 2009). Therefore, it seems possible that pollinator-mediated progenitor–derivative speciation is common in the genus *Ophrys*.

### Progenitor–derivative speciation in sexually deceptive orchids

In his recent review, Crawford (2010) points out that, apart from cases of ecogeographic and mating system differences,

FIG. 4. Relationships among *LFY* alleles from *Ophrys* sect. *Pseudophrys* (and some outgroup individuals) as determined by Bayesian inference phylogeny reconstruction, with brackets indicating alleles (a1 or a2) sampled from the same individual. Plant individuals and accession numbers are listed in Supplementary Data Table S2 (available online). Branches are labelled with Bayesian posterior probabilities (where >0.5) and underlined sequences represent putatively recombined alleles (see Supplementary Data Table S6, available online). Sequence groups A, B and C referred to in the text are indicated by the respective letters. \*, partial sequence; \*\*, both alleles were found in *O. sphagodes* 392A and *O. archipelagi* 393A; \*\*\*, merged outgroup sequence (see Supplementary Data Table S2).

data on pre-mating barriers to gene flow among progenitor–derivative species pairs are largely lacking, identifying only two such cases in his literature survey: first, *Camassia angusta* (Agavaceae) may be a recent derivative of *C. scilloides*; these species are crossable and a difference in flowering time has been suggested as a reproductive barrier (Ranker and Schnabel, 1986). Secondly, in addition to post-mating barriers, pollinator behaviour has been suggested to act as a partial pre-mating barrier between *Mimulus guttatus* (Phrymaceae) and its derivative *M. nudatus* (Macnair and Gardner, 1998). Hence, *Ophrys* orchids may represent one of the few known cases where specific pollinators are both the main reproductive barrier among progenitor and derivative species, and potentially also the drivers of the speciation process.

Pollinator-mediated progenitor–derivative speciation may, however, be more widespread. For instance, as in *Ophrys*, highly specific pollinators provide the main reproductive barrier among Australian sexually deceptive orchids of the genus *Chiloglottis*, making pollinator-driven speciation due to scent changes likely (Bower and Brown, 2009; Peakall et al., 2010; Ayasse et al., 2011; Gaskett, 2011). This process may have a simple genic basis, allowing for sympatric divergence with gene flow (Mant et al., 2005a; Peakall et al., 2010). Thus, the occurrence of progenitor–derivative species patterns in *Chiloglottis* would not be surprising. More generally, one might expect that progenitor–derivative species patterns may be a common result for genic ecological speciation processes, in which strong pre-mating isolation allows the swift establishment of barriers to gene flow in sympatry.

### Conclusions

The taxa studied here from the *O. fusca* s.l. group, each with a different pollinator, represent genetically distinct units, pollinators (besides geography) significantly affecting population structure. This supports the role of pollinators in maintaining reproductive isolation among the studied taxa. Secondly, the widespread Aegean taxa *O. leucadica* and *O. cinereophila* have higher genetic diversities than the restricted taxa found on Rhodes, and the comparison of AFLP and sequence data suggests the retention of ancestral polymorphism. Furthermore, *O. leucadica* (whether including *O. bilunulata* or not) shows a strong geographic population structure, which contrasts with *O. cinereophila*. The genetic pattern is consistent with the scenario that *O. leucadica* is a progenitor species for restricted or endemic *Ophrys fusca* s.l. taxa in the Aegean region, in particular *O. parvula*. Genic ecological speciation processes may be expected to result in patterns of progenitor–derivative speciation. This study illustrates that such patterns are indeed detectable in sexually deceptive orchids.

### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxfordjournals.org](http://www.aob.oxfordjournals.org) and consist of the following. Supplementary methods: details of the phylogenetic sequence analysis. Table S1: summary of the study species and their pollinators. Table S2: plant samples used for sequence analysis. Table S3: generalized linear models of genetic distance, geographic distance and pollinators. Table S4: diversity and differentiation

statistics from AFLP data. Table S5: results from nested AMOVA analyses of AFLP data. Table S6: summary of *LFY* recombination analysis. Table S7: summary statistics for *LFY* sequence data. Figure S1: dendrogram of inter-population relationships from AFLP data. Figure S2: analysis of genetic vs. geographic distance between populations. Figure S3: relationships among all non-recombined *LFY* alleles. Figure S4: relationships among all *LFY* alleles and partial alleles.

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