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Friends or Relatives? Phylogenetics and Species Delimitation in the Controversial European Orchid Genus *Ophrys*

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• *Background and Aims* Highly variable, yet possibly convergent, morphology and lack of sequence variation have severely hindered production of a robust phylogenetic framework for the genus *Ophrys*. The aim of this study is to produce this framework as a basis for more rigorous species delimitation and conservation recommendations.
• *Methods* Nuclear and plastid DNA sequencing and amplified fragment length polymorphism (AFLP) were performed on 85 accessions of *Ophrys*, spanning the full range of species aggregates currently recognized. Data were analysed using a combination of parsimony and Bayesian tree-building techniques and by principal coordinates analysis.
• *Key Results* Complementary phylogenetic analyses and ordinations using nuclear, plastid and AFLP datasets identify ten genetically distinct groups (six robust) within the genus that may in turn be grouped into three sections (treated as subgenera by some authors). Additionally, genetic evidence is provided for a close relationship between the *O. tenthredinifera*, *O. bombyliflora* and *O. speculum* groups. The combination of these analytical techniques provides new insights into *Ophrys* systematics, notably recognition of the novel *O. umbilicata* group.
• *Conclusions* Heterogeneous copies of the nuclear ITS region show that some putative *Ophrys* species arose through hybridization rather than divergent speciation. The supposedly highly specific pseudocopulatory pollination syndrome of *Ophrys* is demonstrably 'leaky', suggesting that the genus has been substantially over-divided at the species level.

Key words: AFLP, DNA sequencing, hybridization, introgression, *Ophrys*, pseudocopulation, species delimitation, systematics.

INTRODUCTION

The genus *Ophrys* will be familiar to many botanists, conservationists and orchid enthusiasts, having become widely recognized as a model system for exploring floral evolution. Both molecularly and morphologically distinct (Bateman *et al.*, 2003), the genus occurs within a clade of genera delimited by a chromosome number of 2*n*, 36 (records of tetraploids in *Ophrys* are rare: Bernardos *et al.*, 2003). Like *Serapias*, but unlike most other related genera, *Ophrys* has a relatively narrow distribution that encompasses much of Europe but does not extend east of Asia Minor.

The genus is most remarkable for the complex morphology of the flower in general, and of the insect-like labellum in particular (Fig. 1). This is thought to have evolved primarily through pollinator mimicry. Pollination occurs by sexual deception through a process termed pseudocopulation; the flower (the mimic) imitates a female of one or more pollinator species (the model) in order to attract males of the same species (the operator). Pollinators are usually sexually inexperienced male bees of Andrenidae, Anthophoridae, Megachilidae, Apidae and Colletidae, wasps of Sphecidae and Scolidae and, in two cases, beetles of Scarabidae (Paulus, 1997). Males of the pollinator species are attracted to the flowers through a combination of olfactory, visual and tactile stimuli. Several active compounds in the scent trigger behavioural responses in the pollinator when present in specific ratios (Schiestl *et al.*, 1999). The visual signals are based on shape and chromatic variation in the flower (including a highly reflective region termed the speculum), whereas the main tactile component is variation in pilosity across the labellum. Once attracted, males attempt to mate with the flower, and pollinia are transferred to the pollinator through the adhesion of viscid discs. Further attempts to mate with other *Ophrys* flowers result in the transfer of pollinia, thereby effecting cross-pollination.

This remarkable pollination syndrome may help explain the wide range of often subtle morphological differentiation evident among putative species of *Ophrys*. Centuries of predominantly morphological study have resulted in monographs of the genus that recognize from as few as 16 species plus 34 subspecies (Sundermann, 1980) or 19 species plus 46 subspecies (Faurholdt and Pedersen, 207), through 150 species forming 29 complexes (Devillers and Devillers-Terschuren, 1994), to as many as 252 species forming 32 complexes (Delforge, 201). Species-rich classifications have become dominant in recent years, reflecting in part the increasing influence of a species concept in *Ophrys* systematics that even subtly distinct morphological

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FIG. 1. (A–J) Single representative species of each of the ten clades of *Ophrys* delimited in the ITS tree, presented in alphabetical order (i.e. image A represents clade A) and at a constant magnification (original images were slides taken at 1:1 scale; thus, the long axis of each image represents 35 mm): (A) *O. insectifera*, Hampshire, UK; (B) *O. tenthredinifera*, Sicily; (C) *O. speculum*, Sicily; (D) *O. bombyliflora*, Sicily; (E) *O. bilunulata*, Crete; (F) *O. apifera*, Kent, UK; (G) *O. sphegodes*, Dorset, UK; (H) *O. apulica*, Gargano, Italy; (I) *O. heldreichii*, Crete; (J) *O. attica*, Peloponnese. (K–L) Two examples from Crete of natural hybrids between highly divergent clades within *Ophrys*: (K) *O. bombyliflora* (Group D) × *O. cretensis* (Group G); (L) *O. iricolor* (Group E) × *O. spruneri grigoriana* (Group G). All photographs by R. M. Bateman.

variants each have a specific, dedicated pollinator. It is further assumed that this supposed precise and intimate relationship forms a reliable pre-zygotic mating barrier around each putative species (Delforge, 205). However, other lines of evidence have challenged these finely drawn species boundaries. For example, breeding experiments performed by S. Malmgren (pers. comm., 2006) regularly recovered multiple morphological 'species' from single selfed *Ophrys* flowers. Also, Soliva and Widmer (2003) clearly demonstrated recent and apparently

ubiquitous introgression among at least some members of the *O. sphegodes s.l.* group, while Gulyás *et al.* (2005) reported similar gene flow among certain members of the *O. fuciflora s.l.* group.

The higher-level classification of *Ophrys* also reflects inferences about pollination biology. The genus was previously divided into two sections (treated as subgenera by some authors) based on suites of morphological characters later inferred to dictate the position of the pollinator during pseudocopulation. A 'head-up' (cephalic) position, with the abdomen pressed against the labellum, characterized section *Ophrys (*often referred to as section *Euophrys*). In contrast, a 'head-down' (abdominal) position, in which pollinia are removed via the abdomen, characterized section *Pseudophrys*. However, field observations, in the form of close-up video footage (F. Schiestl, pers. comm., 2005), clearly show that the widely accepted and allegedly mutually exclusive concepts of abdominal and cephalic pseudocopulation are apocryphal, an observation reinforced by the natural occurrence of 'wide crosses' between members of the two supposed sections (e.g. Bateman and Devey, 2006; Fig. 1K, L).

Controversies over species delimitation in the genus are not simply theoretical debates between evolutionary biologists. Being both numerous and charismatic, *Ophrys* species figure prominently in local and national conservation strategies across Europe, and thus attract a great deal of conservation attention. Once a species has been named it must then be considered seriously by conservationists, potentially diverting limited resources from arguably more deserving cases. It is therefore highly desirable that each of these species be distinct and stable, rather than a subjective and poorly tested taxonomic entity.

Here, a suite of DNA-based techniques, spanning the boundary between systematics and population genetics, is applied in order to clarify species delimitations. Multiple accessions have been obtained from many of the species groups recognized by Delforge (2005), and are subjected to DNA-based analyses. First, phylogenetic trees have been generated from sequence data obtained from fast-evolving regions of both the nuclear and plastid genomes. Secondly, a set of multi-locus markers were generated and scored for each accession using the AFLP technique (Vos *et al.*, 1995) and subjected to multivariate ordination. The AFLP technique uses restriction enzymes to cut genomic DNA. Complementary double-stranded adaptors are ligated to the ends of the restricted fragments. A subset of these restriction fragments is then further amplified using a pair of primers complementary to the adaptor and restriction site fragments. Fragments are visualized on denaturing polyacrylamide gels where polymorphisms are apparent as presence or absence of peaks. AFLP markers sample restriction endonuclease sites widely across the nuclear genome by selective amplification (Remington *et al.*, 1999). AFLP markers were initially developed for population and species delimitation studies (Mueller and Wolfenbarger, 1999), but they have also been used to resolve relationships among closely related species (e.g. Richardson *et al.*, 2003).

Then the possibilities of integrating these techniques are reviewed, along with appropriate morphological analyses, into a metapopulation-based approach to species delimitation. The objectives include:

(a) circumscribing minimum resolvable genetically distinct entities within the genus;
(b) determining the degree of morphological convergence evident within the genus;
(c) inferring whether some putative *Ophrys* species may have arisen through hybridization rather than through divergent speciation;
(d) assessing the likelihood of gene flow between genetically distinct entities, both sympatric and allopatric.

MATERIALS AND METHODS

Generating sequences

Accessions of *Ophrys* species and outgroup taxa used in this study are listed in Appendix 1. Genomic DNA was extracted from both fresh and silica-dried material. Extractions followed the 2× CTAB protocol (Doyle and Doyle, 1987), but used a $CsCl_2$/ethidium bromide density gradient (1·55 g mL^{-1}) for purification (Creeth and Denborough, 1970).

Amplification of the *trnH–psbA* intergenic spacer was carried out in 50 μL reactions, containing 45 μL of 2·5 mM Mg PCR master mix (Abgene Ltd, Epsom, UK), 1·5 μL bovine serum albumin (0·04 %), 0·6 μl H_20, 60 ng of each primer, *trnH* F (Tate and Simpson, 203) and *psbA* R (Sang *et al.*, 1997) with approx. 40 ng DNA template. The PCR profile was as follows: initial denaturation of 94 °C for 2 min, followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 3 min, followed by a final extension of 7 min at 72 °C.

Amplification of the *trnD–trnT* intergenic spacer was carried out in 50-μL reactions, containing 45 μL Abgene PCR mastermix (2·5 mM Mg), 1·5 μl bovine serum albumin (0·04 %), 0·8 μL H_20, 50 ng of each primer *trnD* F and *trnT* R (Demesure *et al.*, 1995) and approx. 40 ng DNA template. The PCR profile was as follows: initial denaturation of 94 °C for 2 min, followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 3 min, followed by a final extension of 7 min at 72 °C.

Amplification of internal transcribed spacers 1 and 2 (ITS) and the 5·8S gene was carried out in 50-μL reactions, containing 45 μL PCR Abgene mastermix (1·5 mM Mg), 1·5 μL bovine serum albumin (0·04 %), 60 ng H_20, 0·6 μL of each ITS primer, 17SE and 25SE (Sun *et al.*, 1994) and approx. 40 ng DNA template. The PCR profile was as follows: initial denaturation of 94 °C for 2 min, followed by 28 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 3 min, followed by a final extension of 7 min at 72 °C.

Any ITS trace files showing evidence of introgression in the form of heterogeneous ITS copies were cloned into a vector (pGem-T Easy Vector, Cat. No. A1360; Promega Ltd, Madison, WI, USA) to isolate single sequences.

The ITS region was then re-amplified from the transformed bacterial colonies using the M13 primers contained in the kit and a small portion of the colony as the DNA template. Cloning was undertaken for the following taxa: *O. aegirtica*, *O. apifera*, *O. apulica*, *O. araneola*, *O. bombyliflora*, *O. bornmuelleri*, *O. bremifera*, *O. dyris*, *O. garganica*, *O. lacaitae*, *O. mammosa*, *O. murbeckii*, *O. pallida*, *O. phillipei* and *O. rhodia.* Ten ITS clones were obtained for each of the above taxa, though only the example of each clone type with the best sequence was included.

All PCR products were purified using DNA purification columns according to the manufacturers' protocols (QIAquick; Qiagen Ltd, Crawley, UK.). Dideoxy cycle sequencing was then performed using the chain termination method and ABI Prism Big Dye version 3·1 reaction kit, following the manufacturers' protocols (Applied Biosystems Inc., Warrington, UK). The products were run on an ABI 3700 Genetic Analyser, also according to the manufacturers' protocols. Sequence editing and assembly of contigs were performed using Sequence Navigator and AutoAssembler software programs (ABI). All sequences were aligned by eye, following the guidelines of Kelchner (2000).

For the AFLP analysis, a primer trial was conducted using 14 primer combinations to identify pairs of selective primers that would be appropriate to the study. The standard primer *Mse*1–AGG and a modified (by a fourth selective base) *Eco*RI–CTAT primer (both 5 mM) were used, following the manufacturers' instructions (MWG Biotech Ltd. UK), to produce AFLP profiles for all *Ophrys* species, since this combination yielded a suitable number of bands and variation among loci. The addition of an extra base to reduce the number of peaks produced was described by Vos *et al*. (1995), Fay and Krauss (2003) and Fay *et al.* (2005) for use in cases where the genome of the plant in question is substantially larger than those of plants for which the AFLP kits are optimized.

Data analysis strategy

The ITS data were analysed using the Fitch parsimony model (equal weight, unordered (Fitch, 1971) and a bootstrapping (Felsenstein, 1985) approach using the software program PAUP 4·0b2A (Swofford, 2001). One thousand replicates were performed using the sub-tree pruning and re-grafting (SPR) algorithm, with MulTrees on but holding only five trees at each step, to reduce the time spent in swapping on large numbers of potentially suboptimal trees. Support for branches was evaluated by bootstrapping using 1000 random addition replicates with simple sequence addition, SPR swapping and holding five trees at each stage.

A Bayesian analysis was also conducted using Mr Bayes version 2·01 (Ronquist and Huelsenbeck, 2003) for the ITS matrix. Prior to the Bayesian analysis, a model of DNA evolution was obtained using the Model Test software version 3·0 (Posada and Crandall, 1998) following the authors' protocols. Model Test implements three different model selection frameworks: hierarchical likelihood ratio tests, Akaike information criterion, and Bayesian information criterion, and the model selected was HKY85. For the analysis itself, 5 000 000 cycles were performed, sampling one tree every 20 generations. A graph of generation number versus log likelihood values was plotted and any trees preceding the plateau phase (burn in) were discarded. A 50 % majority rule consensus was constructed in PAUP 4·0b2A from the remainder of the trees with the 'include compatible grouping' and 'show frequency of all observed bipartitions' options selected. To test whether taxon order could affect tree topology and nodal support, five replicates of 500 000 cycles with randomized taxon order were also performed, sampling a tree every 40 generations and comparing the results. No incongruence was detected between these topologies and those resulting from the 5 000 000-generation analysis.

Analyses of the plastid datasets, both individually and in combination, were carried out using a maximum parsimony approach incorporating a Fitch parsimony model (equal weight, unordered) and a bootstrapping approach using the software program PAUP 4·0b2A. Analysis settings followed those detailed for the ITS analysis.

AFLP analysis was performed using primers selected for use across the genus. Fragment data were analysed using Genescan (version 2·02) and Genotyper (version 1·1) analysis software (ABI). The AFLP traces were carefully compared by eye to ensure homology of bands. Markers with evidence of 'false negative' peaks (small, unscorable peaks in a size range where other samples have larger, scorable peaks) were discarded from all samples. This screening strategy prevented the potential introduction of artefacts into the data due to uneven amplification among samples. Bands ranging in size from 50 bp to 500 bp were scored as present or absent. Principal co-ordinates analysis (PCoA), using the Jaccard similarity coefficient (Jaccard, 1908) to exclude shared zeros, was performed using the program 'R' Version 4·0 (Casgrain and Legendre, 2001).

RESULTS

DNA sequencing

Figure 2 shows one of 6287 equally most-parsimonious trees obtained with maximum parsimony. The trees produced by both parsimony and Bayesian inferences were congruent with respect to the groups recovered. The analyses were undertaken with outgroups included but, in order to facilitate the display of the resulting tree as a phylogram, these taxa were reduced to a single aggregate terminal in the tree presented.

The analyses confirm that *Ophrys* is monophyletic [posterior probability (pp) 0·98, bootstrap percentage (bp) 97], as shown in previous studies (Cozzolino *et al.*, 2001; Bateman *et al.*, 2003). Outgroups are placed in the following order: *Neotinea maculata*, *Orchis italica*, *Steveniella satyrioides* as successive sister clades to a combined ((*Anacamptis laxiflora* + *Serapias lingua*) *Ophrys*) clade. *Ophrys* can be divided into ten clades. These clades (labelled A–J in Fig. 2) are, with one exception, subsets of section *Ophrys*, namely the groups corresponding to *O. insectifera* (A), *O. tenthredinifera* (B), *O. speculum* (C), *O. bombyliflora* (D), *O. apifera* (F), *O. sphegodes s.l.* (G), *O. fuciflora s.l.* (H), *O. scolopax s.l.* (I) and *O. umbilicata*

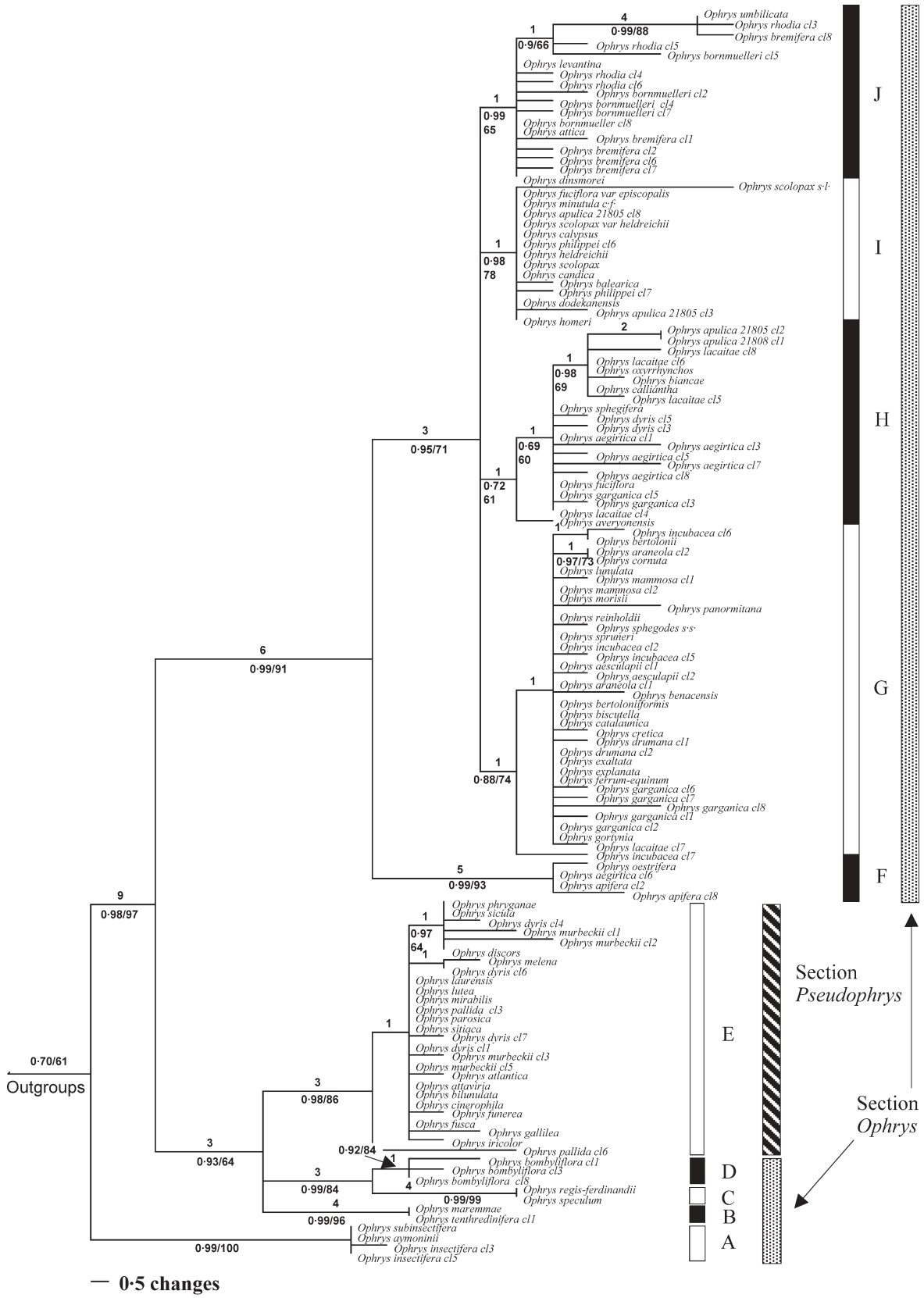

FIG. 2. One of 6287 equally parsimonious trees for the transcribed spacers 1 and 2 (ITS) and the 5·8S gene dataset. Numbers above lines represent branch lengths. Of the numbers below the branches, the first is the Bayesian posterior probability (pp) and the second the bootstrap percentage (bp). Values $<50\,\%$ not shown.

(J). Section *Pseudophrys* (E) forms a monophyletic group nested within a paraphyletic section *Ophrys* (*sensu* Delforge).

The *O. insectifera* clade (A) is strongly supported (pp 0·99, bp 100) and is placed with weak support as sister to the rest of *Ophrys* (pp 0·70, bp 61). Within the clade containing groups A–E, the *O. tenthredinifera* group (B) is strongly supported as a monophyletic entity with a posterior probability of 0·99 (bp 96). The *O. speculum* group (C) is strongly supported (pp 0·99, bp 99) and is sister to the morphologically contrasting *O. bombyliflora* group (D) (pp 0·99, bp 84). Group E (Section *Pseudophrys*) is well supported with a posterior probability of 0·98 (bp 86). Although the precise relationships between group B, groups (C and D) and group E remain uncertain, they form a clade in all the most parsimonious trees. This clade receives a posterior probability of 0·93 (bp 64) indicating that these groups are more closely related to each other than to any other *Ophrys* species sampled.

Cloning of the ITS amplification products from *O. dyris* produced heterogeneous ITS copies, and the respective placements of these copies challenge the monophyly of section *Pseudophrys*. Three copy types were, as expected, placed in Group E (i.e. section *Pseudophrys*), but the other two were located in the *O. fuciflora* clade (H), part of section *Ophrys*.

There is strong support (pp 0·99, bp 91) for a clade containing several of the groups assigned to section *Ophrys* by Delforge (groups F, G, H, I and J), but this excludes other groups traditionally assigned to section *Ophrys*, specifically the *O. insectifera*, *O. speculum*, *O. bombyliflora* and *O. tenthredinifera* groups. Within this clade the *O. apifera* group (F) (pp 0·99, bp 93) is strongly supported as sister to a clade containing the *O. sphegodes s.l.*, *O. fuciflora s.l.*, *O. scolopax s.l.* and *O. umbilicata s.l.* groups (G, H, I and J, respectively) with a posterior probability of 0·95 (bp 71). In addition to *O. apifera*, this group contains *O. oestrifera*, which is treated by Delforge and many others as a synonym of *O. apifera*. Additionally, this group contains one ITS copy type from *O. aegirtica*.

There is good support for an *O. sphegodes* clade (G) with a posterior probability of 0·88 (bp 74), although relationships within the group are poorly resolved. Some taxa previously identified as members of this group also appear in other clades due to their possession of heterogeneous ITS copies. For example, *O. garganica* has ITS copies that are also placed in the *O. fuciflora s.l.* clade, suggesting either this species is of relatively recent hybrid origin or introgression has occurred in the genealogy of the sampled specimen.

There is weak support (pp 0·72, bp 61) for the *O. fuciflora s.l.* clade (H), which also contains taxa with ITS copies appearing elsewhere in section *Ophrys.* As previously stated, *O. aegirtica* has one copy type that is strongly supported as belonging to the *O. apifera* clade. Some copy types from *O. dyris* are also located in the *O. fuciflora s.l.* clade. In addition, *O. lacaitae* possesses an ITS copy type that is placed in the *O. sphegodes* clade (G), and *O. apulica* has ITS copies that appear in the *O. scolopax* clade (I).

Clade I contains taxa that generally correspond to the *O. scolopax s.l.* group and is well supported (pp 0·98, bp 78). Clade J (pp 0·99, bp 65) consists of taxa that had previously been placed in the *O. bornmuelleri*, *O. scolopax* and *O. umbilicata* groups *sensu* Delforge; consequently the groups corresponding to *O. bornmuelleri* and *O. scolopax* appear paraphyletic, as members of both of these groups are distributed between more than one clade.

No well-supported examples of incongruence were found between phylogenetic reconstructions produced by maximum parsimony and Bayesian analysis.

Figure 3 shows one of the 2053 most-parsimonious cladograms for plastid data; specifically, the combined *trnH–psbA* intergenic spacer and *trnD–trnT* intergenic spacer data matrices. The topology is largely congruent with that of the nuclear ITS tree described from Figure 2. The main differences are the position of the *O. insectifera* group, which in this analysis is placed as sister to all other *Ophrys*, and the placement of the *O. umbilicata* group rather than the *O. apifera* group as sister to the non-*Pseudophrys* clade. Both of these incongruencies have little bootstrap support in the plastid tree.

AFLP

With 79 accessions spanning 74 putative species, the AFLP dataset contains fewer taxa than the sequencing analysis, due to the greater sensitivity to DNA quality of AFLP in comparison to DNA sequencing. All ingroup taxa (listed in Appendix 1) were originally included in the AFLP study, but some AFLP traces from the analysis were subsequently discarded due to weak signal strength.

The primer combination selected resulted in the generation of 165 AFLP markers, each accession possessing between 22 and 41 peaks. The sample of *O. dyris* had the most peaks (41), the next highest having only 35. This may reflect hybrid additivity, as this accession is the only sample that fell outside sections *Ophrys* and *Pseudophrys* as depicted in the PCoA. The PCoA analysis derived from AFLP data (Fig. 4A) shows partitioning of *Ophrys* species into four discrete clusters that correspond to amalgamations of groups recovered in the ITS analysis. Letters in parentheses refer to clades recovered in the ITS analysis. Clusters correspond to the *O. insectifera* group (A), the *O. tenthredinifera* group (B–D), section *Pseudophrys* (E) and the *O. apifera* group (F–J). The *O. apifera* cluster itself contains two groups: the *O. apifera* group (F) and the *O. fuciflora* group (G–J). Subgroup *fuciflora* contains two clusters that correspond to the *O. umbilicata* aggregate (J) and an aggregate that is a combination of subgroup *O. sphegodes s.l.*, subgroup *O. fuciflora s.l.* and subgroup *O. scolopax s.l.* (G, H and I, respectively). Details of taxa contained within each of the above-mentioned clusters are given in Appendix 2.

Ophrys dyris, of the *O. omegaifera* group, is placed between the section *Pseudophrys* cluster and the cluster representing the larger subset of section *Ophrys*. It is apparent from the combination of sequencing and AFLP analysis that either this particular accession represents an intersectional hybrid individual or the species *per se* is of

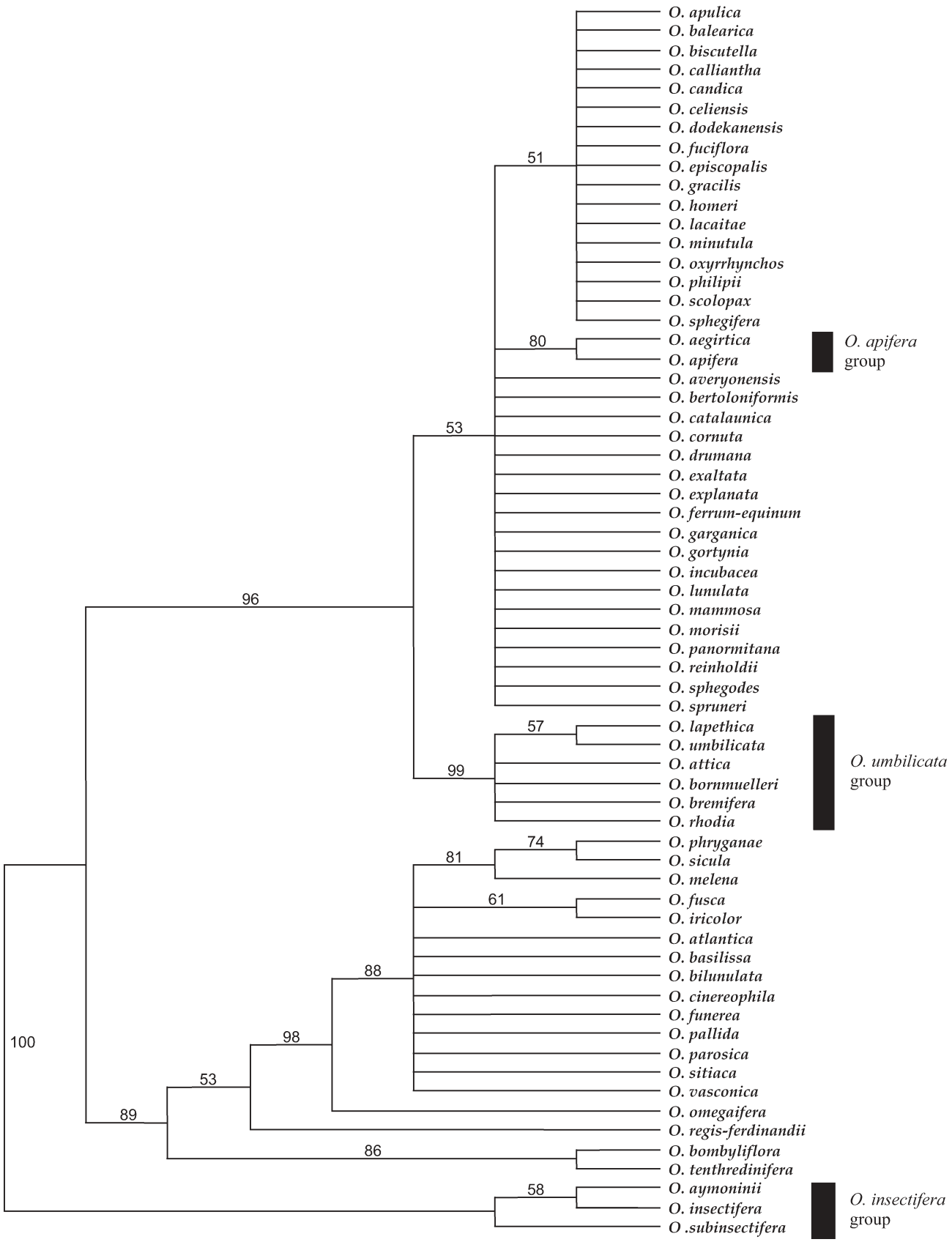

FIG. 3. One of the 2053 most parsimonious trees for the combined *trnH–psbA* intergenic spacer and *trnD–trnT* intergenic spacer data matrices. Numbers represent the bootstrap percentage (bp).

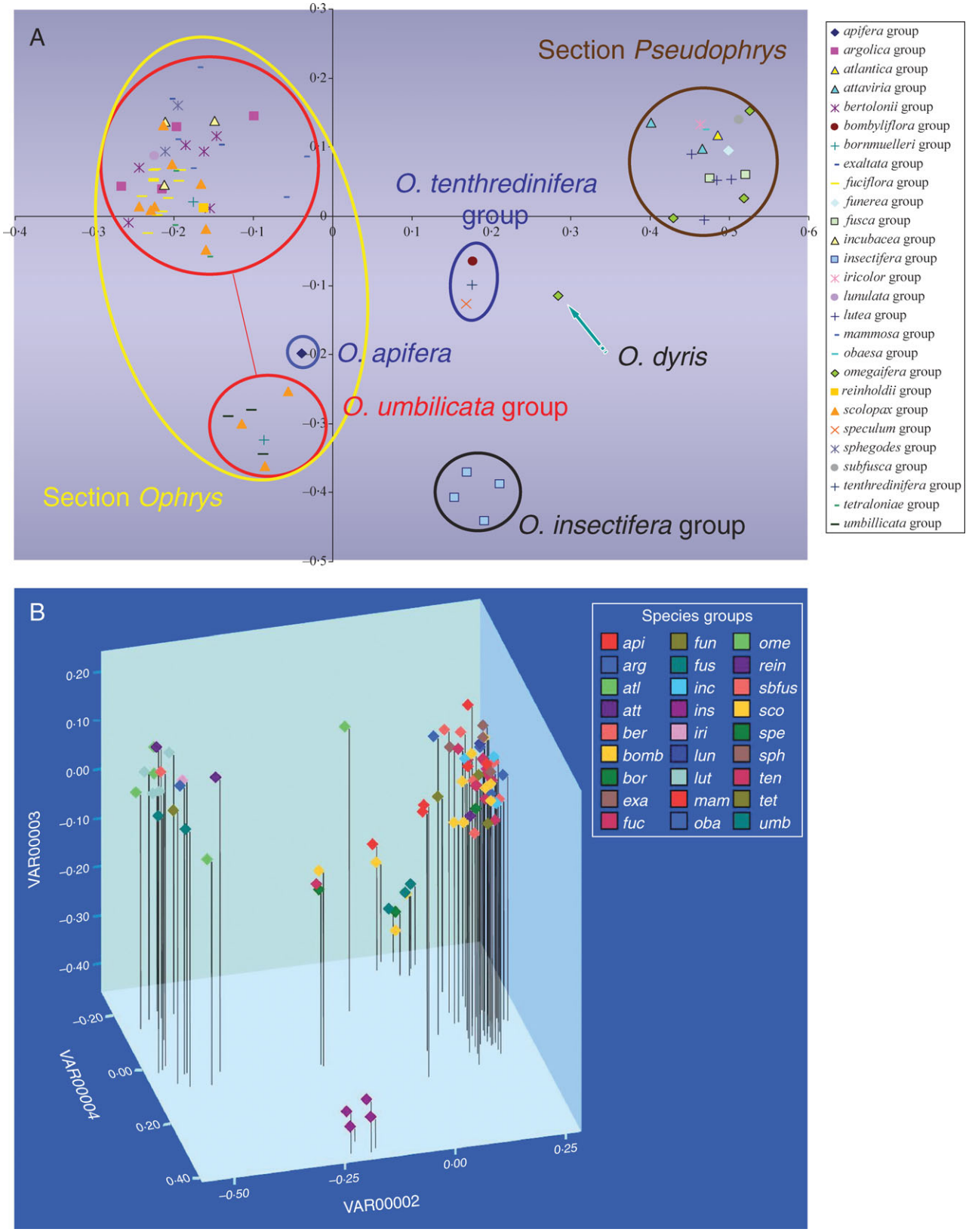

FIG. 4. (A) PCoA plot of AFLP results. (B) Three-dimensional PCoA plot of AFLP results. Species groups: *api*, *apifera*; *arg*, *argolica*; *atl*, *atlantica*; *att*, *attaviria*; *ber*, *bertolonii*; *bomb*, *bombyliflora*; *bor*, *bornmuelleri*; *exa*, *exaltata*; *fuc*, *fuciflora*; *fun*, *funerea*; *fus, fusca;* *inc*, *incubacea*; *ins*, *insectifera*; *iri*, *iricolor*; *lun*, *lunulata*; *lut*, *lutea*; *mam*, *mammosa*; *oba*, *obaesa*; *ome*, *omegaifera*; *rein*, *reinholdii*; *sbfus*, *subfusca*; *sco*, *scolopax*; *spe*, *speculum*; *sph*, *sphegodes*; *ten*, *tenthredinifera*; *tet*, *tetraloniae*; *umb*, *umbilicata*.

TABLE 1. *PCoA summary statistics*

Axis	Eigenvalues	% of variance	Cumulative (%)
1	6·104	24·8	28·4
2	1·907	7·9	32·6
3	1·414	5·9	38·5

hybrid origin. Since only one accession labelled *O. dyris* was included in this particular analysis, it is impossible at present to determine which of these scenarios is correct. *Ophrys aegirtica*, which showed evidence of hybridization with *O. apifera* in the ITS analysis, is positioned on the periphery of the larger section *Ophrys* cluster and, when viewed in the three-dimensional PCoA space (Fig. 4B), it is the closest member of this cluster to *O. apifera*.

The groups corresponding to *O. bornmuelleri* and *O. scolopax* (*sensu* Delforge) are split between different clusters in the PCoA. Some members are found in the *O. umbilicata* cluster, whereas others are positioned in the larger section *Ophrys* subset Table 1.

DISCUSSION

Species concepts

One of the most crucial decisions to be made when dealing with the systematics of any group, but in particular a group of closely related taxa like *Ophrys* distributed in a biodiversity hotspot such as the Mediterranean, is selecting the appropriate species concept and species delimitation method. Since all subsequent descriptions and implications depend on these initial assumptions, these need to be specified for any investigation of this genus.

Many of the previous attempts to classify *Ophrys* species have adopted a loosely defined and intuitive approach to taxonomy, based largely on field observations while incorporating analysis of some micro-morphological characters (e.g. Devillers and Devillers-Terschuren, 1994; Delforge, 2001, 2005). In effect, the problems posed by homoplasy are ignored. These are especially important in a pseudocopulatory pollination scenario, in which sympatric taxa are supposedly reliant on differential pollinator attraction and pseudocopulatory positioning for reproductive success. Convergence in morphology is likely if plants from separate lineages share pollinators. It was due to the problems associated with potential homoplasy that the phenetic species concept (at least, when applied to only morphological characters) was discarded as inappropriate for this study.

The data presented in this study and elsewhere (e.g. Soliva and Widmer, 2003) clearly show evidence of introgression in many of the taxa sampled. Since intersectional hybrids are evident (Bournérias and Prat, 2005; Bateman and Devey, 2006), adoption of the biological species concept (Mayr, 1963), which recognizes actually or potentially inbreeding individuals as members of the same species, is impractical. Nevertheless, some consideration of isolation through pollinator behaviour, as inferred by patterns of introgression, seems desirable for species delimitation in this group. In older species groups, where sufficient time has passed for fixed genetic variation to accumulate to a level detectable by conventional sequencing, the adoption of a pattern-based approach is relatively straightforward. However, within a genus such as *Ophrys*, where there is an absence of complete differentiation and lineage sorting due to the suspected recentness of the radiation and the likelihood of continuing introgression, an approach incorporating a combination of the two concepts is more appropriate. Specifically, for this study a fundamentally phylogenetic pattern cladistic approachadopted, but it still incorporated a mechanistic component in which putative species are viewed as participants in an ongoing evolutionary process, rather than the fixed end-products of the process. It is this ongoing introgression, both within and between members of different species groups, which gives rise to the 'fuzzy margins' of the species clusters described here.

We believe that this combined 'pattern–process' model is a pragmatic compromise that best reflects the morphology and evolutionary biology of the group. This approach is broadly congruent with that of Sundermann (1980) and Bateman (2001), but differs radically from that of Devillers and Devillers-Terschuren (1994) and Delforge (1995 *et seq.*), in which many minor variants and morphological oddities are categorized as full species.

With this in mind, two interpretations are possible for the patterns of variation shown in *Ophrys*: an incipient speciation scenario and a reticulate evolution scenario.

(1) Incipient speciation, in which several species clusters are recognized. The self-imposed criteria for the species name chosen to represent these clusters are prioritized as follows: (*a*) they must fall wholly within one of the lettered groups described in the Results section, (*b*) they must be geographically widespread, (*c*) they must be central to a cluster of genetically distinct entities, and (*d*) they must be central to a morphologically distinct suite of characters. These species clusters are equivalent to the clades recovered in the ITS and AFLP analyses and it is assumed that they actually attract a specific spectrum of pollinators. In addition to these species, there also exist reproductively suboptimal species, which are still sufficiently successful in terms of pollinator attraction to maintain populations and reproduce to a level where it is not uncommon to find their offspring. This is demonstrated by the presence of heterogeneous peaks in ITS electropherograms for some accessions prior to cloning. This variation demonstrates evidence of relatively recent gene flow, where insufficient time has passed for complete copy conversion. The contribution of each progenitor lineage can be estimated as the ratio of copy types that would be proportional to the ratio of peak heights (Y. Pillon, pers comm, 2005). These species may be present due to the existence of odour-bouquet variability within populations produced by negative frequency-dependent selection in response to pollinator learning (Moya and Ackerman, 1993), which would lead to mistakes by pollinators and hence to introgression between species.

In terms of lowered fitness through lowered reproductive viability, members of Orchidaceae pose an additional problem. Due to the abundance of dust-like seed produced by *Ophrys* (typically 5000–10 000 per fertilized capsule),

even a dramatically reduced fruiting success can result in hundreds or thousands of viable seeds. Thus, infrequent aberrant pollination events can permit the dispersal of the offspring of these slightly suboptimal 'species' and thereby induce 'fuzzy margins' to species clusters. This situation may occur because the group is too young for divergence to have taken place and for reliable genetic or behavioural barriers to reproduction to have become established. There may, in addition, be an anthropogenic component to *Ophrys* speciation, as many species favour 'man-made' (anthropogenic) and 'man-maintained' (anthropostatic) environments such as grazed but unimproved scrubland and grassland. In this case, there may not have been any time in the past when reproductive isolation was complete (such as glacial refugia). Phenotypic variation in floral morphology, and probably more importantly pheromone mimicry among sympatric individuals, would attract different suites of pollinators and lead to the formation of putative 'prospecies' by the erection of initially weak reproductive barriers. Different 'species' may coalesce if they share pollinators and introgress to a level where no pure individuals of the parent species remain (hybrid speciation). An example of speciation based on geographical vicariance could be the *O. umbilicata* group (as presently circumscribed), in which the members are located exclusively in the eastern Mediterranean.

(2) Reticulate evolution by the mechanism of incipient genetic homogenization. At some point in the past, there was sufficient isolation of *Ophrys* populations, perhaps in glacial refugia, to lead to speciation. Due to the subsequent increase in areas habitable by *Ophrys*, reflecting post-glacial recolonization and/or anthropogenic effects on European biogeography, the ranges now overlap, allowing increased levels of hybridization and introgression between species that still lack effective mechanisms of reproductive isolation. If this scenario is valid, homogenization of the genomes of formerly distinct *Ophrys* species is likely to increase through time, potentially as a result of anthropogenic disturbance and possibly climate change.

The two scenarios outlined above need not be mutually exclusive. However, it is important to determine how much gene exchange between diverging populations or groups of genetically distinct entities is possible without arresting, or even reversing, the divergence. Introgression among populations tends to make gene pools progressively more similar. Nevertheless, populations or meta-populations may continue to diverge despite some introgression, if it is subordinate to divergent selection or stochastic processes such as genetic drift. Net genetic convergence or divergence, at least in sympatry, will be determined by the balance of these opposing forces. Exactly how much isolation is required (be it contrasting geographical distributions, habitat preferences or pollinators) to permit prospecies formation and divergence in sympatry depends upon the intensity of the differentiating selection. It seems probable that, within *Ophrys*, the average level of genetic divergence slightly exceeds that of genetic reticulation. There may be a breakout threshold within *Ophrys* in terms of levels of genetic differentiation, which has been exceeded by each of the groups A–J in Fig. 2. This threshold would represent a point at which phenotypic or genotypic differences between groups or populations have a negative effect on the likelihood of successful reproduction given the available pollinators in those locations. Once this threshold has been passed and additional divergence takes place, lineages may undergo an evolutionarily positive feedback loop as fixed changes accrue more rapidly due to increased isolation. It is interesting to speculate, though almost impossible to determine, how many times this threshold has been approached, only for introgression to eradicate the divergence.

Phylogeny reconstruction

Variation observed within the sampled plastid loci (Fig. 2) among *Ophrys* species was, as expected, low. A total of 2211 nucleotide characters was sequenced from two non-coding regions. Analysing data for each plastid locus independently provided insufficient potentially parsimony-informative characters for well-resolved phylogenetic reconstructions. Even when the regions were combined, few of the groups were resolved with high levels of bootstrap support. Combining the plastid datasets into a single matrix provided increased resolution, but nevertheless only 4 % of characters proved potentially parsimony-informative. Improved resolution was obtained from the ITS analysis, in which 21 % of characters proved potentially parsimony-informative. Even then, clade resolution was concentrated along the spine of the tree, with little or no resolution near the tips. High consistency and retention indices indicated that most of the variable sites were congruent. Low bootstrap support was expected for many clades, as re-sampling within a matrix is likely to remove groups defined by only one or two character states. No hard incongruence was found among the different partitions. The *O. insectifera* group proved the most labile, albeit with only weak bootstrap support for any particular position. The *O. apifera* group was also somewhat labile, typically appearing as sister to the remainder of section *Ophrys* but occasionally as sister to the *O. umbilicata* group.

Unfortunately, due to the rarity and conservation protection offered to many *Ophrys* species, in some cases it was only possible to collect very small amounts of plant tissue to avoid excessive damage to the plant. This lack of material and therefore the limited amount of extracted DNA available meant that it was not possible to amplify all the DNA-based markers for all of the sampled taxa. It was the combination of this limitation and the presence of polymorphic ITS sequences that prevented production of a combined plastid and nuclear DNA phylogenetic reconstruction.

These results demonstrate that, within a potentially actively evolving group such as *Ophrys*, species-level trees can be difficult to reconstruct when based on DNA sequence data alone. Sequences from loci routinely exploited for phylogenetic purposes may not contain sufficient signal to make confident statements about relationships, even when the group has been well sampled for a large number of nucleotides in rapidly evolving regions of

the various genomes. In an attempt to overcome this limitation, AFLP techniques were incorporated. The use of AFLP data for tree-building purposes can present problems due to the assumption of homology of all co-migrating fragments and the difficulties associated with assigning homology to alleles of differing sizes. However, this approach may be justified as the use of modern automated genetic analysers greatly increases the accuracy of fragment size measurement, and therefore resolution of the technique with regard to the co-migrating fragments. More pragmatically, within this project at least, results are consistent with those produced by the sequence analyses, and this mirrors the congruence between datasets described by other authors using the same combination of techniques (e.g. Richardson *et al.*, 2003; Goldman *et al.*, 2004; Koopman, 2005).

As putative species within groups in the genus *Ophrys* are so closely related, it is almost impossible to rule out chimaeric recombination within those groups, though even if it had taken place the groups are so similar in terms of ITS sequences that any conclusions reached would not be affected. Sequences were visually inspected for evidence of chimeras; however, wherever heterogeneous sequences indicating hybridization or introgression between groups (notably *O. dyris*) were found, cloned sequences fell in either one or the other putative parental groups and not in intermediate positions in the phylogenetic analysis, suggesting chimaeric recombination had not taken place. There is the possibility that chimeric sequences could be generated from combining fragments of ITS copy types inherited from different species groups but had these sequences been amplified, the analyses of nuclear and plastid sequencing and those of AFLP data would have displayed incongruencies that were not evident.

The best-supported estimate of relationships appeared to be from combined analysis of AFLP and sequence data: the retention index remained high and, although the consistency index was lowered, it remained sufficiently high for continued statistical confidence in tree topology. Additionally, analysis of AFLP, plastid DNA and nuclear DNA all independently recovered the same groups, including the novel, geographically constrained *O. umbilicata* group, indicating that the combined data provide relatively stable and reliable estimates of relationships at the level of species groups.

The results produced in this molecular study show some incongruence with those of previous studies based solely on morphological characters. For example, we refute the suggestion of Devillers & Devillers-Terschuren (1994) that section *Ophrys* is best divided into three assemblages organized around the *O. insectifera*, *O. speculum* and *O. bombyliflora–fuciflora–sphegodes* groups. Their study also used shape of the labellum, details of the stigmatic cavity and organization of labellum pilosity as morphological synapomorphies to suggest that *O. speculum* is sister to the rest of the section, but the present molecular data strongly reject this inference.

Analysis of the *O. aegirtica* sample used in this study demonstrates conclusively that introgression between members of different groups has taken place. As this accession was collected from the type locality of the species, we can be confident that no misidentification has taken place. Because the *O. aegirtica* site was within 200 m of a population of *O. apifera s.s.*, we propose that *O. aegirtica* is of recent hybrid origin and should be considered a nothospecies.

The Bayesian posterior probabilities and levels of bootstrap support for some nodes within the phylogram (Fig. 2), particularly those that would assign sister relationships between the larger, better differentiated groups, are relatively low. Only once posterior probabilities exceed $>0{\cdot}9$ and bootstrap percentages exceed 85 % can we be reasonably confident of support for a particular tree topology. Hence, we are confident that these species groups are meaningful entities, but the relationships among them must still be considered cryptic. Although the fine detail observed within phylograms has only moderate support and collapses into several large polytomies, the fact that the taxa are sufficiently closely related to allow reliable scoring of AFLP bands across the entire genus provides an indication of the closeness of the relationships among these species.

We believe the congruence between the ITS and AFLP datasets, which respectively sample a very small section of the nuclear genome and many loci distributed across the genome, lend credibility to our conclusions. Taken together, these data refute claims of Paulus and Gack (1990), that the supposedly species-specific pseudocopulatory pollination syndrome of *Ophrys* provides reliable reproductive isolation among species.

Two explanations are feasible for the poorer differentiation within the *fuciflora–scolopax–sphegodes* clade using AFLP analysis relative to the ITS analysis. The first is evolutionary and the second technical. Firstly, in species of recent origin, few changes will have become fixed and even then only in a few functional genes directly involved in the speciation event, which form a small proportion of the whole genome sampled by AFLP. Less constrained regions of the genome, such as the ITS spacers, are not likely to have participated in the speciation event but will subsequently accumulate changes more rapidly than most other regions (Bateman, 1999). There may not have been sufficient time for the accumulation of changes in plastid regions (which in general accrue sequence changes more slowly) to track those occurring in more rapidly mutating regions. As AFLP techniques sample restriction sites from across the genome there will be evolutionary rate heterogeneity between the sites sampled. Consequently, phylogenetic patterns recovered by the quickly evolving markers may be swamped by the addition of regions that evolve more slowly.

The second, technical factor reflects the rationale behind AFLP primer selection. The initial focus of the present investigation was based on a need to obtain primers for use across the genus. Consequently, primers that could potentially have differentiated between closely related members of section *Ophrys* were not selected. Further studies (D. S. Devey *et al.*, unpubl. res.) will therefore be needed to address *Ophrys* systematics within the major species groups recognized here.

Taxonomic implications

Delimitation of the *O. scolopax s.l.* group is based mainly on the occurrence and size of the 'horns' on the lateral lobes of the labellum. The present data suggest that the *O. scolopax* group, as currently delimited (Figs 2 and 3), is polyphyletic; several lineages have independently converged on this visually distinct morphology. We assert that the presence of long 'horns' cannot be used as a synapomorphy delimiting the group.

Some sampled taxa show evidence of introgression, as ITS copy types appear in multiple, well-supported clades. It is therefore proposed that they be treated as taxa of hybrid origin and excluded from the aforementioned groupings. The taxa are as follows: *O. aegirtica*, *O. apulica*, *O. araneola*, *O. dyris*, *O. garganica*, *O. lacaitae*, *O. mammosa*, *O. murbeckii*, *O. pallida* and *O. phillipei.* Should these patterns be repeated in a wider range of accessions of these taxa, we suggest that for systematic and conservation purposes, they be treated as hybrids.

We attribute these patterns to introgression rather than any contamination of samples, as in many cases evidence of hybridization was expected from field observations of the taxa concerned. Additionally, careful examination of samples at every step of the extraction and PCR process, and robustness of data produced in other laboratory studies using the same extraction techniques indicates that the chances of cross-contamination are remote.

Several species groups (*sensu* Delforge, 2005) are polyphyletic with respect to ITS sequences, including the *O. bornmuelleri* group, the *O. tetraloniae* group, the *O. scolopax* group and the *O. tenthredinifera* group.

Biogeography

This study clearly demonstrates biogeographic partitioning within section *Ophrys*. Members of the *O. umbilicata* group (as redelimited using genetic evidence), which are genetically distinct and therefore presumably reproductively isolated from the remainder of the section, occur exclusively to the south-east of the Balkans. Within the group, *Ophrys umbilicata s.s.* has the widest reported distribution, stretching from Greece in the north-west to Israel and Jordan in the south and Asiatic Turkey in the east. As the accession used in this study was collected on Cyprus, any future study should analyse *O. umbilicata* from the northern and western extremes of its distribution. The distribution of the *O. umbilicata* group (as recircumscribed here) is centred on Cyprus, but two members are located in southern Greece. One species from section *Ophrys* (*O. levantina*) was sequenced from Cyprus and did not appear in this clade, thus ruling out the possibility that all the relevant species from this island are undergoing extensive introgression.

General conclusions

Despite the interesting results currently being produced by researchers in the field of pheromone-mediated pollinator attraction, until the odour bouquets of many more putative species (and many individuals sampled from a wide geographical spectrum within species) have been examined and overlaid onto DNA-based frameworks, it will be impossible to determine with any degree of certainty which component(s) of pollinator attraction selection is acting upon. In particular, pollinator data need to be obtained from the full range of target species and their relatives, ideally sampled throughout the flowering season. Only then can gene flow be estimated directly, rather than indirectly though genetic fingerprinting of the orchids themselves as reported here.

As a general approach to *Ophrys* systematics, we suggest caution with regard to segregation of the genus into trivial partitions. The partitions must be demonstrably real and should ideally be based on a combination of discrete characters: genetic, chemical and/or morphological. Such characters should be prioritized over arbitrary divisions of continuous morphological spectra that are hypothesized to coincide with species-specific pollinator relationships. This is particularly important, as the identification of many putative pollinators is itself contentious, and frequently based on largely anecdotal – and, most crucially, geographically restricted – evidence of pollinator visitation. Although, for both systematics and conservation purposes, it would be remiss to ignore a biologically significant species or species group, at the same time it is a severe hindrance to conservation bodies to have to factor into their plans poorly substantiated taxa or taxonomic groupings. This is particularly true with relation to supposed island endemics, which would receive a high conservation priority as they stand but would be considerably less significant if proved to be trivially distinct local morphological variants of the same species or comparatively transient hybrid swarms.

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APPENDIX 1

TABLE A1. *List of all* Ophrys *taxa, Genbank ID numbers and voucher information*

Species	Voucher	Source	ITS Genbank ID	*trnD–trnT* Genbank ID	*trnH–psbA* Genbank ID
Anacamptis laxiflora (Lam.) Bateman, Pridgeon and Chase	Bateman 4	Crete, marsh imm. W Frangocastello Castle, E Hora Sf.,12 April 1996	AM711747	AM711971	AM711707
Neotinea maculata (Desf.) Stearn	Bateman 35	1·2 km SE Karines, Karines–Spili road, Crete; 19 April 1996	AM711744	AM711970	AM711706

Continued

TABLE A1. *Continued*

Species	Voucher	Source	ITS Genbank ID	*trnD–trnT* Genbank ID	*trnH–psbA* Genbank ID
Ophrys aegirtica P.Delforge	Fay 555		AM711759 AM711760 AM711761 AM711762	AM711905	AM711641
Ophrys aesculapii Renz	Chase O- 901 K	Oncidiinae, Kew 1984-8117	AM711787 AM711788	N/A	N/A
Ophrys apifera Huds.	Chase 13839	England, Avon, off A432 between Bristol and Chipping Sodbury, Cuckoo Lane, 16 June 2002	AM711789 AM711790	AM711906	AM711642
Ophrys apulica	Bateman 705	Bosco di Pianelle, Martina Franca, Italy	AM711794 AM711795	AM711907	AM711643
Ophrys apulica (Danesch) O.Danesch & E.Danesch ex Gölz & H.R.Reinhard	Bateman 696	Massafra-Martina Franca, Italy	AM711791 AM711792 AM711793	N/A	N/A
Ophrys araneola Rchb.	Chase O- 701 K	Orchidinae, Kew 1983-8174	AM711796 AM711797	N/A	N/A
Ophrys atlantica Munby	Bateman 208	Alhaurin el Grande, Malaga, Spain	AM711798	AM711908	AM711644
Ophrys attaviria D.Rückbr., U.Rückbr., Wenker & S.Wenker	Bateman 209	Alhaurin el Grande, Malaga, Spain	AM711799	N/A	N/A
Ophrys attica Soó	Bateman 1158		AM711800	AM711909	AM711645
Ophrys aveyronensis (J.J.Wood) H.Baumann & Künkele	Bateman 1236		AM711801	AM711910	AM711646
Ophrys aymoninii (Breistr.) Buttler	Bateman 1235		AM711802	AM711911	AM711647
Ophrys balearica P.Delforge	Bateman 257	Cabo Gros, Alcudia, Mallorca	AM711803	AM711912	AM711648
Ophrys basilissa	DSD14		N/A	AM711913	AM711649
Ophrys benacensis (Reisigl) O.Danesch & E.Danesch	Bateman 571	Picnic site NW Gardola, N Lake Garda, NW Verona, Italy	AM711804	N/A	N/A
Ophrys bertoloniformis O.Danesch & E.Danesch			AM711806	AM711914	AM711650
Ophrys bertolonii Moretti	Devey DSD 60A	Sicily, Forest of Ficuzza April 2004	AM711805	N/A	N/A
Ophrys biancae Macch.	Bateman 527	4·1 km Pantalica-Ferla, SW Sortino, Syracusa, Sicily	AM711807	N/A	N/A
Ophrys bilunulata Risso	Chase 16363	Crete, SW Bridge, Kanevos-Kali Sikea rd, NW Spili, *c*. 14 April 1996	AM711808	AM711915	AM711651
Ophrys biscutella O.Danesch & E.Danesch	Bateman 653	Monte Sacro, Gargano, Italy	AM711809	AM711916	AM711652
Ophrys bombyliflora Link	Bateman 22	SW Bridge, Kanevos–Kali Sikea road, NW Spili, Crete; 14 April 1996	AM711810 AM711811	AM711917	AM711653
Ophrys bornmuelleri (*s.s.*) Schulze	Bateman 396	SE Cyprus	AM711766 AM711767 AM711768 AM711769 AM711770	AM711918	AM711654
Ophrys bremifera (*c.f.*) Stev. ex M.Bieb.	Bateman 297	Skopelos, Greece	AM711812 AM711813 AM711771 AM711772	AM711919	AM711655
Ophrys calliantha Bartolo & Pulv.	Devey DSD43	Sicily E Pantalica. April 2004	AM711815	AM711920	AM711656

Continued

TABLE A1. *Continued*

Species	Voucher	Source	ITS Genbank ID	*trnD–trnT* Genbank ID	*trnH–psbA* Genbank ID
Ophrys calypsus M.Hirth & H.Spaeth	Bateman 445	Agiasos, Lesvos	AM711816	N/A	N/A
Ophrys candica (E.Nelson ex Soó) H.Baumann & Künkele	R. Bateman 037	Crete 29a/96	AM711814	AM711921	AM711657
Ophrys catalaunica O.Danesch & E.Danesch.	Bateman 321	Barcelona, Spain.	AM711817	AM711922	AM711658
Ophrys celiensis	DSD11		N/A	N/A	AM711923
Ophrys cinerophila Paulus & C.Gack	Bateman 225	Tekke, Cyprus	AM711818	AM711924	AM711660
Ophrys cornuta Stev. ex M.Bieb.	Bateman 287	Kokkonicasto, Greece	AM711819	AM711925	AM711661
Ophrys cretica (Vierh.) Erich Nelson	Chase O- 709 K	Orchidinae, Kew 1983-5694	AM711820	N/A	N/A
Ophrys dinsmorei			AM711822	N/A	N/A
Ophrys discors Bianca	Cozzolino 1827		AM711821	N/A	N/A
Ophrys dodekanensis H.Kretzschmar & Kreutz	Bateman 1148		AM711823	AM711926	AM711662
Ophrys drumana (*c.f.*) P.Delforge	Civegiel *et al.* 502	France, nr Murs. similar to Delforge ed.1lower photo page 439.	AM711824 AM711825	AM711927	AM711663
Ophrys dyris Maire	Bateman 93	Faunia, Portugal	AM711749 AM711750 AM711751 AM711752 AM711753 AM711754	N/A	N/A
Ophrys episcopalis Poir.	Bateman 17		AM711826	AM711932	AM711668
Ophrys exaltata Ten.	Bateman 365	C sicily	AM711827	AM711928	AM711664
Ophrys explanata (Lojac.) P.Delforge	Devey DDA	Sortino, Sicily 2004	AM711828	AM711929	AM711665
Ophrys ferrum-equinum Desf.	Chase O- 707 K	Orchidinae, Kew 1984 2540	AM711829	AM711930	AM711666
Ophrys fuciflora (F.W.Schmidt) Moench	Devey DSD29	Sicily, E Pantalica 2004	AM711830	AM711931	AM711667
Ophrys funerea Viv.	Chase 16364	Crete, SW Bridge, Kanevos-Kali Sikea rd, NW Spili, *c*. 14 April 1996	AM711831	AM711933	AM711669
Ophrys fusca subsp. fusca Link	Chase O- 711 K	Orchidinae, Kew 1983-5696	AM711832	AM711934	AM711670
Ophrys galilaea H.Fleischm. & Bornm.	Cozzolino 865		AM711836	N/A	N/A
Ophrys garganica E.Nelson ex O.Danesch & E.Danesch	Bateman 671	Monte San Angelo, Gargano, Italy	AM711833 AM711834 AM711835 AM711764 AM711765	AM711935	AM711671
Ophrys gortynia (Baumann & Künkele) Paulus	Bateman 24	Prov.: Hillock imm. N Agia Triada ruins, Timbaki, Crete; 16 April 1996	AM711837	AM711936	AM711672
Ophrys gracilis			N/A	AM711937	AM711673
Ophrys heldreichii Schltr.	Bateman 13	Orchidinae	AM711734 AM711838	N/A	N/A
Ophrys homeri M.Hirth & H.Spaeth	Bateman 1104		AM711839	AM711938	AM711674
Ophrys incubacea Bianca ex Tod.	Devey DSD62A	Sicily, Forest of Ficuzza; coll. April 2004	AM711783 AM711784 AM711785	AM711939	AM711675
Ophrys insectifera L.	Fay 570	Italy,W Verona, E L Garda, Bizzano-St Zeno	AM711840	AM711940	AM711676
Ophrys iricolor Desf.	Bateman 33	Crete 24/96	AM711841	AM711941	AM711677
Ophrys lacaitae Lojac.	Devey DSD36	Sicily, E Pantalica 2004	AM711708 AM711709	AM711942	AM711678

Continued

TABLE A1. *Continued*

Species	Voucher	Source	ITS Genbank ID	*trnD–trnT* Genbank ID	*trnH–psbA* Genbank ID
			AM711842 AM711763 AM711777		
Ophrys lapethica				AM711943	AM711679
Ophrys laurensis Geniez & Melki	Cozzolino 1417		AM711710	N/A	N/A
Ophrys levantina Gölz & H.R.Reinhard	Bateman 230	Limasol, Cyprus	AM711711	N/A	N/A
Ophrys lunulata Parl.	Devey DSD1	Sicily, Pantalica Road. Limestone bank across gorge from Sortino 2004	AM711712	AM711944	AM711680
Ophrys lutea Biv.	Fay 543		AM711713	N/A	N/A
Ophrys mammosa Desf.	Bateman 12	Orchidinae	AM711714 AM711715	AM711945	AM711681
Ophrys maremmae O.Danesch & E.Danesch.	Cozzolino 974		AM711716	N/A	N/A
Ophrys melena (Renz) Paulus & Gack	Devey DSD66A	Sicily, Forest of Ficuzza; April 2004	AM711717	AM711946	AM711682
Ophrys minutula c.f. Gölz & H.R.Reinhard	Bateman 1107		AM711718	AM711947	AM711683
Ophrys mirabilis P.Geniez & F.Melki	Cozzolino 1408		AM711719	N/A	N/A
Ophrys morisii (Martelli) G.Keller & Soó	Bateman 1232		AM711720	AM711948	AM711684
Ophrys murbeckii H.Fleischm.	Cozzolino 1329		AM711773 AM711774 AM711775 AM711776	N/A	N/A
Ophrys oestrifera M.Bieb.	Bateman 682	Vieste-peschicchi, Gargano, Italy	AM711721	N/A	N/A
Ophrys omegaifera H.Fleischm.	Bateman 364	Purchased	N/A	AM711949	AM711685
Ophrys oxyrrhynchos Tod.	Devey DSD50	Sicily, Forest of Ficuzza; April 2004	AM711722	AM711950	AM711686
Ophrys pallida Raf.	Devey DSD59A	Sicily, Forest of Ficuzza; coll. April 2004	AM711723 AM711724 AM711725 AM711726	AM711951	AM711687
Ophrys panormitana (Tod.) Soó	Bateman 541	Piazza Amerina, Sicily	AM711727	AM711952	AM711688
Ophrys parosica P.Delforge	Bateman 1125		AM711728	AM711953	AM711689
Ophrys philippei Gren.	Bateman 1234		AM711779 AM711780	AM711954	AM711690
Ophrys phryganae J.Devillers-Terschuren & P.Devillers	Chase 16355	Crete, zig-zag above Gouverneto Gorge, Akrotiri, *c*. 11 April 1996	AM711729	AM711955	AM711691
Ophrys regis-ferdinandii (Renz) Buttler	Chase O- 905 K	Orchidinae, Kew 1984-2613	AM711730	AM711956	AM711692
Ophrys reinholdii Sprun. ex Boiss.	Bateman 1146		AM711731	AM711957	AM711693
Ophrys rhodia (H.Baumann & Künkele) P.Delforge	Bateman 229	Tekke, Cyprus	AM711756 AM711755 AM711757 AM711758	AM711958	AM711694
Ophrys scolopax Bory & Chaub.	Chase O- 703 K	Orchidinae, Kew 1984-2590	AM711732 AM711733	AM711959	AM711695
Ophrys sicula Tineo	Bateman 36	5·1 km E Spili, Spili–Gerakari road, Crete; 20 April 1996	AM711735	AM711960	AM711696
Ophrys sitiaca Paulus, C.Alibertis & A.Alibertis	Bateman 39	SW T-junction, Spili–Agia Galini/Melanbes, Crete; 21 April 1996	AM711736	AM711961	AM711697
Ophrys speculum Link			AM711737	N/A	N/A

Continued

TABLE A1. *Continued*

Species	Voucher	Source	ITS Genbank ID	*trnD–trnT* Genbank ID	*trnH–psbA* Genbank ID
Ophrys sphegifera Willd.	Bateman 211	Alhaurin el Grande, Malaga, Spain	AM711738	AM711962	AM711698
Ophrys sphegodes	DSD17		AM711739	AM711963	AM711699
Ophrys splendida	DSD21		N/A	AM711964	AM711700
Ophrys spruneri Nyman	Bateman 001	Crete 4/96	AM711740	AM711965	AM711701
Ophrys subinsectifera C.E.Hermos. & J.Sabando	Bateman 318	Navarra, Spain	AM711741	AM711966	AM711702
Ophrys tenthredinifera Willd.	Chase 15846	Source: RBG-Kew, LivColl.2002-745 [Provenance: Italy]	AM711743	AM711967	AM711703
Ophrys umbilicata Desf.	Bateman 397	SE Cyprus	AM711742	AM711968	AM711704
Ophrys vasconica	DSD19		N/A	AM711969	AM711705
Orchis italica	Cotrim, Pinto, Chase & Fay 456 A	Casais Robustos, nr Vila Moreira Alcanena, Portugal, 24 March 2001	AM711745	N/A	N/A
Serapias lingua L.	Bateman 8	Crete, marsh imm. W Frangocastello Castle, E Hora Sf.,12 April 1996	AM711748	N/A	N/A
Steveniella satyrioides Schlechter	Güner 12838	Turkey, A4 Bolu, 5 km W of Mengen, open scrub and meadows, 590 m a.s.l., 7 May 2000	AM711746	N/A	N/A

APPENDIX 2

TABLE A2. *The four clusters of* Ophrys *species as indicated by PCoA analysis derived from AFLP data (Fig. 4A)*

Ophrys species	PCoA cluster	*Ophrys* species	PCoA cluster
Ophrys aegirtica	Section *Ophrys* large subset (SOLS)	*Ophrys panormitana*	SOLS
Ophrys aesculapii	SOLS	*Ophrys philippeii*	SOLS
Ophrys apulica 2	SOLS	*Ophrys reinholdii*	SOLS
Ophrys apulica 5	SOLS	*Ophrys scolopax*	SOLS
Ophrys apulica 7	SOLS	*Ophrys scolopax*	SOLS
Ophrys apulica 8	SOLS	*Ophrys sphegifera*	SOLS
Ophrys araneola	SOLS	*Ophrys sphegodes*	SOLS
Ophrys argolica	SOLS	*Ophrys spruneri*	SOLS
Ophrys averyonensis	SOLS	*Ophrys atlantica*	Section *Pseudophrys*
Ophrys balearica	SOLS	*Ophrys attaviria*	Section *Pseudophrys*
Ophrys bertolonii	SOLS	*Ophrys basilissa*	Section *Pseudophrys*
Ophrys bertoloniiformis	SOLS	*Ophrys bilunulata*	Section *Pseudophrys*
Ophrys biscutella	SOLS	*Ophrys cinereophila*	Section *Pseudophrys*
Ophrys calliantha	SOLS	*Ophrys funerea*	Section *Pseudophrys*
Ophrys calypsus	SOLS	*Ophrys fusca* s.s.	Section *Pseudophrys*
Ophrys candica	SOLS	*Ophrys iricolor*	Section *Pseudophrys*
Ophrys catalaunica	SOLS	*Ophrys lutea*	Section *Pseudophrys*
Ophrys celiensis	SOLS	*Ophrys melena*	Section *Pseudophrys*
Ophrys cornuta	SOLS	*Ophrys pallida*	Section *Pseudophrys*
Ophrys dodecanensis	SOLS	*Ophrys parosica*	Section *Pseudophrys*
Ophrys drumana	SOLS	*Ophrys phryganae*	Section *Pseudophrys*
Ophrys episcopalis	SOLS	*Ophrys sicula*	Section *Pseudophrys*
Ophrys exaltata	SOLS	*Ophrys sitiaca*	Section *Pseudophrys*
Ophrys explanata	SOLS	*Ophrys vasconica*	Section *Pseudophrys*
Ophrys ferrum-equinum	SOLS	*Ophrys aymoninii*	*insectifera*
Ophrys fuciflora	SOLS	*Ophrys insectifera*	*insectifera*
Ophrys garganica	SOLS	*Ophrys insectifera*	*insectifera*
Ophrys gortynia	SOLS	*Ophrys subinsectifera*	*insectifera*
Ophrys gracilis	SOLS	*Ophrys bombyliflora*	*bombyliflora/speculum/tenthredinifera* (BST)
Ophrys homeri	SOLS	*Ophrys speculum*	BST
Ophrys heldreichii	SOLS	*Ophrys tenthredinifera*	BST

Continued

TABLE A2. *Continued*

Ophrys species	PCoA cluster	*Ophrys* species	PCoA cluster
Ophrys incubacea	SOLS	*Ophrys apifera*	*apifera*
Ophrys lacaitae	SOLS	*Ophrys oestrifera*	*apifera*
Ophrys lucis	SOLS	*Ophrys attica*	*umbilicata*
Ophrys lunulata	SOLS	*Ophrys bornmuelleri*	*umbilicata*
Ophrys mammosa	SOLS	*Ophrys bremifera*	*umbilicata*
Ophrys minutula	SOLS	*Ophrys lapethica*	*umbilicata*
Ophrys morisii	SOLS	*Ophrys rhodia*	*umbilicata*
Ophrys oxyrrhynchos	SOLS		