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Convergent evolution in *Ophrys kotschyi* (Orchidaceae) revisited: a study using nrITS and cpIGS sequences

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Convergence in the endangered European bee-orchid species *Ophrys kotschyi* was studied using a molecular phylogenetic approach. We sequenced the nuclear ribosomal internal transcribed spacer (nrITS) and the *Rrn*5–*Trn*R intron of the chloroplast DNA (cpIGS) to resolve conflicting interpretations of its relationships. Some authors include all morphologically similar Greek taxa in the study species, others believe that similarity results from convergent evolution driven by a shared pollinator. Parsimony-based network building and three approaches of phylogenetic tree reconstruction provided a basic insight into the phylogeny of the studied taxa, revealing that the inclusion of the various Greek taxa in *O. kotschyi* results in a polyphyletic species. This implies the consideration of the species as a narrow endemic to Cyprus, and corroborates the view that convergent evolution is responsible for apparent morphological similarity. Additionally, nrITS sequencing revealed additive polymorphic sites in the nrITS, which implies significant inter-specific gene flow.

Introduction

For evolutionary plant biologists, one of the most exciting and fascinating genera of Europe is probably the orchid genus *Ophrys*, which has undergone a rapid and presumably adaptive radiation that has produced remarkable floral variability. This radiation is commonly thought to reflect its striking pollination system, which occurs by sexual deceit (Schiestl *et al*. 1999), making these plants intriguing for both botanists and entomologists. In this system, the flowers are pollinated by naïve hymenoptera males, who were sexually stimulated, and thus deceived by the female-mimicking odour bouquet of the flowers. Most *Ophrys* species supposedly have a unique pollinator species, and sympatric populations often differ in the preferred pollinators (Paulus & Gack 1990a). This pollination system induces rapid species diversification (Cozzolino & Widmer 2005): the plants are strongly isolated through prezygotic reproductive barriers by having a specific pollinator, but following isolation new taxa can emerge from shift to another specific pollinator (Schiestl & Ayasse 2002).

The above scenario, although leaving open the question of how the descendant is perfectly adapted to the new pollinator, is to our knowledge the best explanation of why the sexually deceptive genus *Ophrys* has radiated into more

than 260 supposed species (Delforge 2006). Although that number is undoubtedly inflated by inappropriate recognition of variants at species level (Pridgeon *et al*. 2001), it highlights the spectacular biodiversity of the genus.

The above interpretation of the genus' diversity, e.g. that the diversity of described species is connected to highly specific but unstable pollination (Paulus 2006), has been repeatedly challenged by recent works (Schiestl 2005, Pedersen & Faurholdt 2007, Devey *et al*. 2008, Bateman *et al*. 2011) because of the lack of genetic isolation among the supposed species in the genus. In fact, several works (Soliva & Widmer 2003, Gulyás *et al*. 2005, Devey *et al*. 2008, Pellegrino *et al*. 2008) showed significant gene flow between *Ophrys* "species", albeit within morphologically definable groups, i.e. between closely related taxa. The lack of evidence for genetic isolation of the > 250 currently described species led Pedersen and Faurholdt (2007) to define species much more widely than Delforge (2006). On the other hand, those authors neglected molecular phylogenetic information, and so developed their system without referring to the phylogenetic background provided by Bateman *et al*. (2003).

More recently, Devey *et al*. (2008, 2009) provided the deepest insight so far into the phylogeny of these plants by sequencing the nrITS and cpDNA *trn*D–*trn*T IGS, and by generating AFLP data from 85 putative species. They found nrITS to be the most valuable source of information on phylogenetic tree reconstruction in *Ophrys*. The phylogenetic tree presented had reliable support on the "spine" of the tree, but the "tips" (i.e. relationship between the currently defined species) remained unresolved; also, many morphologically similar species were segregated among clades. This finding was interpreted as evidence of a high level of hybridisation, and of the limitations of a morphological species-concept that is presently widely applied in the genus — a concept that, in effect, neglects the problem of morphological convergence, i.e. convergence in flower morphology toward similar pollinators.

The recognition of convergence could be crucial for distinguishing between morphologically similar, but genetically isolated species (Avise 2004). One species likely to be influenced by the phenomenon of convergence is *Ophrys kotschyi* (Soó 1926). The taxonomic treatment and systematic position of this species remain controversial. Though many monographers classified the taxon differently, they all considered it to be distinct from others. This view was changed by Sundermann (1975), who combined *O. cretica* in *O. kotschyi* as subsp. *cretica*, thus defining the taxon in a broader sense with a wider distribution (Fig. 1). Later, Pedersen and Faurholdt (2002) also included another taxon, *O. ariadnae*, in *O. kotschyi* as subsp. *ariadnae* (Fig. 1). Although these attempts to define the species *O. kotschyi* with subspecies were not adopted by later, more comprehensive works, the latest *Ophrys* monograph (Pedersen & Faurholdt 2007) presented *O. kotschyi* as a species with subspecies *cretica* and *ariadnae*, thus recognising a large distributional area (Fig. 1) and total population size for this species.

It seems that the above authors disregarded the study of Gölz and Reinhard (1985) which, based on floral morphometrics, proved the statistical distinctness of *O. kotschyi* from species of the *O. reinholdii* group, to where the other relevant taxa (*O. cretica* and *O. ariadnae*) belong (Delforge 2006, Devey *et al*. 2008). Gölz and Reinhard (1985) invoked convergence driven by the same pollinator to interpret the striking morphological similarity of *O. kotschyi* and the other taxa. Indeed, all taxa currently included in *O. kotschyi* are pollinated by bees of the genus *Melecta* (Paulus & Gack 1990b), whereas the other presumed relatives of *O. kotschyi* from the *O. umbilicata* group are pollinated by bees of the genus *Eucera* (Paulus & Gack 1990a).

A clear picture on the taxonomic state of *O. kotschyi* is especially important, because it is one of the four *Ophrys* species currently listed in the annexes of European Union's Habitats Directive (92/43/EEC). In fact, the experts on the species (Baumann & Künkele 1994, Kreutz 2004) reported that fragmented, very small populations occur in Cyprus. Although the species can be found on the island frequently (R. M. Bateman in litt. and our pers. obs.), only a few individuals occur at each site, so the perceived threat to the species can be justified.

A better understanding of the taxonomic status and species delimitation can be crucial to plan an adequate conservation strategy of

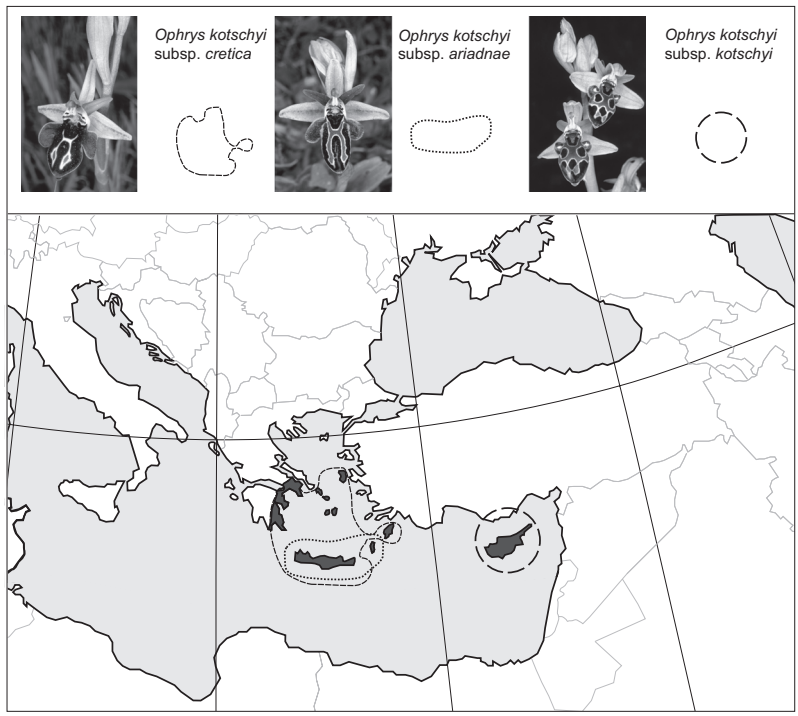

Fig. 1. Appearance and distribution range of *Ophrys kotschyi* and its subspecies as defined by Pedersen and Faurholdt (2007). Original photographs and drawing by the authors.

endangered species (Mace 2004), and is especially important in groups such as European orchids, where "taxonomic inflation" (Isaac *et al*. 2004) is caused by geopolitical bias in systematics (Pillon & Chase 2007). Our present paper focuses on one outcome of our molecular survey of the genus *Ophrys*, namely the implications for taxonomy and conservation consequences concerning the endangered *Ophrys kotschyi*. nrITS is one of the most widely applied markers in plant molecular systematics (Álvarez & Wendel 2003), and also seems to be the most powerful tool in the molecular systematics of *Ophrys* (Devey *et al*. 2008). Here, we apply the sequencing of the ribosomal ITS of the nucleus and the *Rrn*5–*Trn*R intron of the chloroplast DNA (hereafter also referred to as cpIGS) to examine the question whether or not the recent taxonomic viewpoint of Pedersen and Faurholdt (2007) on *O. kotschyi* is supported by the DNA sequences. In other words, do DNA data corroborate the hypothesis of Gölz and Reinhard (1985) drawn from floral morphometrics on the convergent evolution of *O. kotschyi* to other bee orchids assigned to the *O. reinholdii* group?

Material and methods

Plant material

Field-collected leaf-pieces from populations of *O. kotschyi* and all its presumed relatives, plus an additional population of *O. apifera* as outgroup (Table 1) were sampled. Although the work of Delforge (2006) is more comprehensive, its taxonomic treatment of the genus was repeatedly criticised (Pridgeon *et al*. 2001, Pedersen & Faurholdt 2002, Devey *et al*. 2008, Devey *et al*. 2009), therefore the nomenclature and thus the taxonomic treatment of the latest *Ophrys* monograph by Pedersen and Faurholdt (2007) is applied here.

Procedures of DNA work

Total genomic DNA was extracted from the ethanol-stored leaf material of 1–3 individuals per population after total desiccation. Approximately 1–30 mg of dried leaves were thoroughly ground in liquid nitrogen then resuspended in

lysis buffer (2% CTAB, 20 mM EDTA pH 8, 100 mM Tris-HCl pH 9 and 1.4 mM NaCl). After incubation at 65 °C for 60 minutes, the samples were centrifuged at 20 000 *g* for 10 min, than the supernatant was extracted with an equal volume of chloroform and centrifuged for 15 min at 20 000 *g*. The extraction procedure was repeated twice. The DNA was precipitated with two volumes of 96% ethanol and stored at –20 °C for 1 h. DNA was pelleted by centrifugation at 14 000 rpm for 30 min. The pellet was washed twice with 70% ethanol, dried and redissolved in 40 μl 0.1 M Tris (pH 7.5).

The nrITS of 1–3 individuals from each population was amplified by the newly devised (Gulyás *et al*. 2005) angiosperm-specific ITS1A primer and the universal primer ITS4 (White *et al*. 1990), and applied in polymerase chain reaction (PCR) to specifically amplify the plant nrITS. The PCR reaction mixture contained 0.1 volume 10× Taq buffer with $(NH_4)_2SO_4$ (Fermentas), 200 μM each of dNTPs (Fermentas), 2 mM $MgCl_2$, 0.2 μM of each primers, 1.25 U Taq DNA polymerase (Fermentas) and approximately 5 ng μl^{-1} genomic DNA extract. The amplifications were performed on a GeneAmp PCR System 2400 (Perkin Elmer Corp.), programmed for a denaturation step at 94 °C for 4.30 min, followed by 33 cycles of denaturation for 30 s at 94 °C, annealing for 30 s at 51 °C and extension for 30 s at 72 °C, the extension time being increased by one second in every cycle; the thermal cycling was ended by a final extension for 7 min at 72 °C. The quality and quantity of the PCR products were evaluated by loading it on a 1% agarose gel stained with ethidium bromide.

For direct sequencing, the PCR products were purified with Montage PCR Centrifugal Device (Millipore) using the protocol provided by the manufacturer. Abi Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems) was used for cycle sequencing and electrophoresis was carried out using commercially available service (Biomi Kft., Hungary). The same DNA extracts were used for the amplification of the *Rrn*5–*Trn*R intron of the cpDNA with the primers of Chung and Staub (2003). For the delimitation of the latter region, the complete chloroplast genome of maize (acc. no. NC_001666) was used. We selected this cpDNA region because we found it to be informative in our previous screening (results not shown) for variable regions in genus *Ophrys*. We found the PCR conditions applied in the amplification of nrITS to be effective in the case of the cpIGS; thus, we used the procedure detailed above for this DNA region too. The direct sequencing procedure was also carried out at a commercially available service.

Alignment

The plant-specific nrITS sequences of 1 to 3 individuals per population (average 2.36) and

Table 1. Location data of the analysed *Ophrys* taxa. Nomenclature follows Pedersen and Faurholdt (2007).

No.	Species	Subsp.	Location	Acronym	Sample size	Accession numbers (nrITS/cpIGS)
1	*kotschyi*	*ariadnae*	Crete: Spili	ariSpi	2	AM980101-2/FM945304-5
2	*kotschyi*	*cretica*	Rhodes: Kattavia	creKat	3	AM980103-5/FM945306-8
3	*kotschyi*	*cretica*	Crete: Makrigialos	creMak	3	AM980106-8/FM945309-11
4	*kotschyi*	*cretica*	Crete: Rethimno	creRet	3	AM980109-11/FM945312-14
5	*kotschyi*	*kotschyi*	Cyprus: Akrotiri	kotAkr	3	AM980112-14/FM945315-7
6	*oestrifera*	*oestrifera*	Ukraine: Nikita	oesNik	2	AM980115-16/FM945318-19
7	*reinholdii*	*reinholdii*	Rhodes: Nectaros	reiNec	3	AM980117-9/FM945320-2
8	*reinholdii*	*reinholdii*	Greece: Pigi	reiPig	2	AM980120-1/FM945323-4
9	*umbilicata*	*umbilicata*	Cyprus: Kato Drys	umbKat	1	AM980122/FM945325
10	*umbilicata*	*umbilicata*	Anatolia: Kizilkir	umbKiz	2	AM980123-4/FM945326-7
11	*apifera*	*apifera*	Hungary: Balatonszőlős	apiBal	2	AM980999-100/FM945302-3

the same number of the cpIGS were aligned with MEGA v. 4.0 (Tamura *et al*. 2007) using default settings. The nrITS region possesses additive polymorphic sites (APS), i.e. double peaks at certain places in the sequences that result from the presence of different paralogs and may refer to recent hybridisation or introgression of lineages (Gulyás *et al*. 2005, Devey *et al*. 2008). We carefully checked the electropherograms of the direct sequences with the program Chromas Lite 2.01 (Technelysium Pty), and APSs were coded with IUPAC symbols in terms of two nucleotides occurring together at the electropherogram rather than indication of ambiguous reading. As suspected, the nrITS had relatively few polymorphic sites, thus the nuclear and chloroplast data were combined to generate a dataset with more polymorphic sites.

Haplotype network building

To demonstrate the phylogenetic relationship between the accessions of *O. kotschyi* and its presumed relatives, we applied the methods of haplotype network building and phylogenetic tree reconstruction. The haplotype genealogy was estimated with the software TCS v. 1.21 (Clement *et al*. 2000), using a lowered (94%) connection limit to be able to present the relationship between the distantly related haplotype of the outgroup and the haplotypes of the ingroup. All sites were weighted equally and the gap was treated as 5th state during the procedure.

Phylogenetic tree reconstruction

Three different approaches were used to reconstruct the phylogeny of the accessions: the Neighbor-Joining (NJ) and Maximum-Parsimony (MP) methods were implemented in MEGA, and a Bayesian tree was constructed using MrBayes v. 3.1 (Ronquist & Huelsenbeck 2003). The Kimura 2P (Kimura 1980) model of sequence evolution was defined as model for the evolution of sequences at the NJ and Bayesian phylogeny reconstruction. This model was chosen not just because the usage of a simpler model is more satisfactory between closely related sequences (Nei & Kumar 2000), but it was also recommended as the best-fit model by Modeltest v. 3.7 (Posada & Crandall 1998). All sites were weighted equally, the single gap at the 58th site was treated as 5th state in the MP search, and "pairwise deletion" option was on during the NJ search. The statistical confidence in the inferred trees is demonstrated by bootstrap consensus trees inferred from 1000 replicates, while the Bayesian tree was drawn after running the program for one million generations, sampling every 10th generation, and discarding 25% as 'burn-in'.

Results

Alignment

The alignment of the nrITS sequences yielded a 625 bp-long matrix, in which 27 sites were polymorphic including the outgroup. For the ingroup, there were a total of 17 variable sites including 13 APSs. Of the variable sites four were parsimony informative (Table 2). It is also noticeable that APSs occurred at the same position in *O. kotschyi* subsp. *kotschyi* and *O. umbilicata* samples, whereas subsp. *ariadnae* and subsp. *cretica* shared APSs with *O. reinholdii* sequences (Table 2). Moreover, APSs were found within almost all accessions in the ingroup. The 128 bp long *Rrn*5–*Trn*R intron of the cpDNA possessed only one polymorphic site at the 27th base, which consistently separated *O. kotschyi* subsp. *kotschyi* and *O. umbilicata* samples from the rest (Table 2). This added an extra parsimony-informative site to the dataset, which was used in the following analyses as a combined dataset of nrITS and cpIGS sequences.

Haplotype-network building

The network constructed by TCS showed the close relationship of the studied ingroup, and identified three discrete sequence groups within it. The subspecies of *O. kotschyi sensu* Pedersen and Faurholdt (2007) were grouped separately: subsp. *kotschyi* was included in the *O. umbili-*

Table 2. Variable sites of the nrITS and the cp *Rrn*5–*Trn*T IGS (abbreviated as 'cp') of the analysed accessions of *Ophrys* species. Identical bases are represented by dots, and the single deletion is marked by a hyphen. For sample acronyms *see* Table 1.

	nrITS																											cp
Sample											1	1	1	1	2	2	2	2	3	4	4	4	5	5	5	5	6	0
	1	1	1	1	2	5	6	7	9	9	0	1	3	7	1	1	1	1	8	0	1	8	0	4	4	7	1	2
	2	4	5	6	0	8	0	5	4	8	8	5	6	3	2	3	8	9	0	6	5	9	6	0	2	5	1	7
apiBal1	C	G	A	G	A	A	C	T	A	G	C	C	C	A	T	G	C	A	A	A	A	G	C	C	G	T	T	C
apiBal2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ariSpi1	.	.	.	.	G	–	T	C	.	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
ariSpi2	.	.	.	.	G	–	T	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
creKat1	.	.	.	.	G	–	Y	C	.	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
creKat2	.	.	.	.	G	–	Y	C	.	.	.	T	.	T	.	A	.	.	G	.	.	.	A	T	.	C	C	.
creKat3	.	.	.	.	G	–	Y	C	.	.	.	T	.	T	.	A	.	.	G	.	.	.	A	T	.	C	C	.
creMak1	.	.	.	.	G	–	Y	C	W	.	.	T	.	T	.	A	.	.	G	.	.	R	A	T	.	C	C	.
creMak2	.	.	.	.	G	–	Y	C	.	.	.	T	.	T	.	A	.	.	G	.	.	.	A	T	.	C	C	.
creMak3	.	.	.	.	G	–	Y	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
creRet1	.	.	.	.	G	–	Y	C	.	.	Y	T	S	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
creRet2	.	.	.	.	G	–	T	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
creRet3	.	.	.	.	G	–	Y	C	.	.	Y	T	S	T	.	A	.	.	G	.	.	.	A	T	.	C	C	.
kotAkr1	Y	K	R	R	G	–	.	C	.	.	.	.	.	T	.	A	.	.	G	.	.	.	A	T	A	C	C	A
kotAkr2	Y	K	R	R	G	–	.	C	.	.	.	.	.	T	.	A	.	.	G	.	.	.	A	T	A	C	C	A
kotAkr3	Y	K	R	R	G	–	.	C	.	.	.	.	.	T	.	A	.	.	G	.	.	.	A	T	A	C	C	A
oesNik1	.	.	.	.	G	–	.	C	.	T	.	.	.	T	.	A	.	.	G	W	.	.	A	T	.	C	C	.
oesNik2	.	.	.	.	G	–	.	C	.	T	.	.	.	T	.	A	.	.	G	.	.	.	A	T	.	C	C	.
reiNec1	.	.	.	.	G	–	T	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
reiNec2	.	.	.	.	G	–	Y	C	.	.	.	T	.	T	K	A	.	.	G	.	.	.	A	T	.	C	C	.
reiNec3	.	.	.	.	G	–	T	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
reiPig1	.	.	.	.	G	–	T	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
reiPig2	.	.	.	.	G	–	T	C	W	.	.	T	.	T	.	A	.	.	G	.	W	.	A	T	.	C	C	.
umbKat1	Y	K	R	R	G	–	.	C	.	.	.	.	.	T	.	A	.	.	G	.	.	.	A	T	A	C	C	A
umbKiz1	Y	K	R	R	G	–	.	C	.	.	.	.	.	T	.	A	M	W	G	.	.	.	A	T	A	C	C	A
umbKiz2	Y	K	R	R	G	–	.	C	.	.	.	.	.	T	.	A	.	.	G	.	.	.	A	T	A	C	C	A

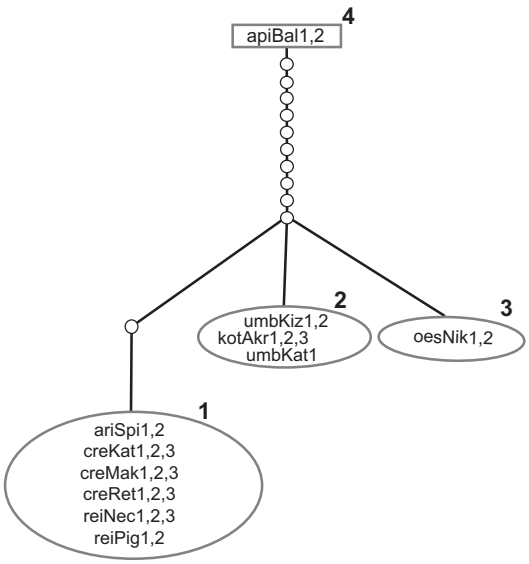

Fig. 2. Haplotype network of the combined nrITS and cpIGS sequences of the studied *Ophrys* individuals with their acronyms. The network was constructed with TCS v. 1.2 using a 94% connection limit. The open circles represent mutational steps between the groups. Arabic numbers directly next to the groups refer to clades *Reinholdii* (1), *Umbilicata* (2), *Oestrifera* (3), and the outgroup (4) as defined in this study. Note that the putative subspecies (abbreviated by "ari" and "cre") of *O. kotschyi* are included in group 1, whereas the nomenclatural type ("kot") is grouped in the Umbilicata clade (2).

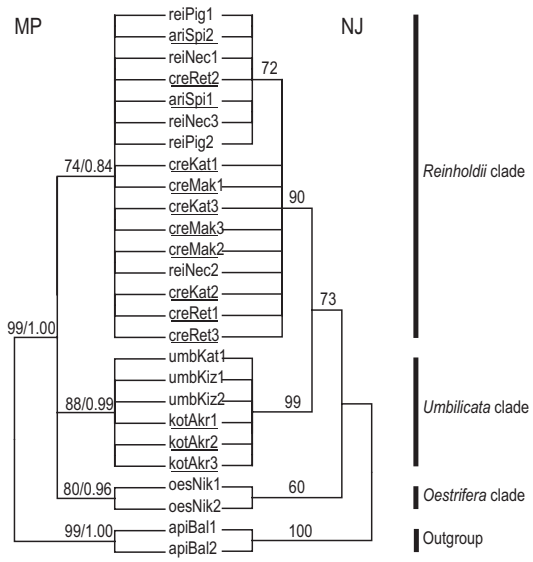

Fig. 3. Bootstrapped Majority Rule consensus phylogenetic trees of the studied *Ophrys* species using MP method (left-hand side) and NJ method (right-hand side). The trees were inferred after 1000 bootstrap replicates with bootstrap support above branches. Branches with < 50% bootstrap support are collapsed on both trees. The Bayesian Majority Rule tree had a topology identical with that of the left-hand tree; thus only posterior probabilities are shown from that analysis on the MP tree, following the bootstrap value by a slash. Clades defined in this study are presented on the right-hand side by a bar followed by the name of the clade. Accessions of *Ophrys kotschyi* (*sensu* Pedersen & Faurhold 2007) are underlined.

cata group and subsp. *ariadnae* and subsp. *cretica* were included in the *O. reinholdii* group (Fig. 2). This classification of samples suggests the polyphyletic nature of the species as defined by the above authors. The *O. oestrifera* group was closely related to the *O. umbilicata* group, and the outgroup, *O. apifera*, was ten mutations apart from the rest of the groups.

Phylogenetic tree-reconstruction

Three different phylogenetic tree reconstruction methods have yielded bootstrap consensus trees with low resolution at the tips of the tree, but the main branches received moderate or high bootstrap support. Regarding the statistically supported branches, the trees have largely congruent topologies (Fig. 3). The MP bootstrap consensus tree is based on 330 most-parsimonious trees (length: 21; CI: 1.0; RI: 1.0), and its topology was identical with the Bayesian Majority Rule tree (not shown), but with higher posterior probabilities on the main branches (indicated on the MP tree, Fig. 3). All three analyses identified three main clades within the ingroup: the "*reinholdii*-clade" (bootstrap NJ: 90%; bootstrap MP: 74%; Bayesian posterior probability: 0.84) including all *O. reinholdii*, *O. kotschyi* subsp. *ariadnae* and *O. kotschyi* subsp. *cretica* sequences; the "*umbilicata*-clade" (bootstrap NJ: 99%; bootstrap MP: 88%; Bayesian posterior probability: 0.99) with the accessions of *O. umbilicata* and *O. kotschyi* subsp. *kotschyi*; and the "*oestrifera*-clade" (bootstrap NJ: 60%; bootstrap MP: 80%; Bayesian posterior probability: 0.96) formed by the *O. oestrifera* samples. The NJ method provided a deeper insight into the

possible phylogeny of the samples, which may come from the usage of more variable sites in the tree-building than in the MP method (Nei & Kumar 2000). The latter found a moderately supported (72%) sub-branch within the "*reinholdii*-clade" including several accessions of *O. reinholdii*, all samples of *O. kotschyi* subsp. *ariadnae* and one accession of *O. kotschyi* subsp. *cretica*; and a moderately supported (73%) branch that divides the *reinholdii* clade and the *umbilicata* clade, and suggests a closer relationship of the two clades. However, the lack of these branches in the Bayesian tree suggests that we should regard them with caution, and not interpret them as robust branches.

Discussion

Taxonomic implications

The subspecies of *O. kotschyi* were placed into separate clades, clearly demonstrating the polyphyly of the species as defined by Pedersen and Faurholdt (2007). The tree topology also implies a closer relationship of the nomenclatural type (i.e. *O. kotschyi* subsp. *kotschyi sensu* Pedersen and Faurhold 2007) to *O. umbilicata*, whereas the other two subspecies are more closely related to *O. reinholdii*. In other words, the subspecies classified under *O. kotschyi* by Sundermann (1975) and Pedersen and Faurholdt (2002) are not connected to the nomenclatural type by their most recent common ancestor. Additionally, an alternative explanation exists for this situation, namely, the paraphyly of *O. reinholdii*, i.e. it could have evolved from *O. cretica*, and *O. umbilicata* from *O. kotschyi*, allowing *O. kotschyi* and *O. cretica* to potentially share a single common ancestor. In fact, the combination of *O. ariadnae* and *O. cretica* under *O. kotschyi* is polyphyletic, which can also be seen from the study of Devey *et al*. (2008). On their nrITS tree the clade with *O. umbilicata* forms as a cohesive group, whereas *O. cretica* is separated in another clade including *O. reinholdii*. Thus, the taxonomic treatment of *O. kotschyi*, *O. ariadnae* and *O. cretica* as conspecific by Pedersen and Faurholdt (2007) yields non-monophyletic taxa, and should therefore be rejected.

Convergent evolution

The above conclusion is supported, not only by the combined nrITS-cpIGS dataset, but also by the accurate morphological investigation of Gölz and Reinhard (1985). They invoked convergent evolution of floral traits to explain the morphological resemblance of *O. kotschyi* and other species of the *O. reinholdii* group. Indeed, the pollinators of the latter group are bees of the genus *Melecta*, and within the *O. umbilicata* group these pollinators are shared only by *O. kotschyi s. stricto*, whereas the remaining species within this group utilise bees of the genus *Eucera* as pollinators (Paulus & Gack 1990a). Selection to mimic the pollinator's females more accurately made *O. kotschyi* morphologically similar to the *O. reinholdii* group; however, the distinctness of the taxa is evidenced by its genome (exemplified here by nrITS) and the significantly different morphological flower traits, shown by Gölz and Reinhard (1985). These results indicate the limited value of apparent morphological similarity in the systematics of genera such as *Ophrys*, where the rapid isolation process is coupled with strong directional selection that arose after shifts in the preferred pollinator. These findings together corroborate the view of those taxonomists who regard *O. kotschyi* as monotypic species, a narrow endemic of the Isle of Cyprus, which should be placed in the *O. umbilicata* group. In fact, conspecificity of *O. kotschyi* and *O. umbilicata* is not out of the question if a broader species concept is applied.

Species concepts in the genus *Ophrys*

Although this paper deals only with a fraction of the species within the genus, a more generalised conclusion of its taxonomy and evolution may be drawn. On the one hand, there are frequent APSs in the nrITS sequences, which can be connected to inter-specific gene flow (Gulyás *et al*. 2005, Devey *et al*. 2008). Consequently, the temporal stability of strong reproductive barriers between species, maintained by highly specific pollinators, is challenged at this point, as is the narrow definition of species (Delforge 2006) that is clearly based on this hypothesis. This reasoning

also implies a smaller role for these reproductive barriers in the long-term evolution of the genus. This notwithstanding, we have to note that based on our current dataset we can not exclude shared ancestral polymorphism due to incomplete lineage sorting being responsible for the presence of paralogs in the nrITS in such a recently radiated genus as *Ophrys*. Therefore, the timing of the gene exchange (reticulation or hybridization) can not be readily assessed, however, the generally assumed high speed of concerted evolution (Baldwin *et al*. 1995, Elder & Turner 1995) may hint at a recent event. Whatever the case, the profound effect of pollinators on *Ophrys* speciation can still be questioned, since — in spite of a considerable morphological distinctiveness — the action of specific pollinators has failed to genetically distance *O. kotschyi* from its relatives (the *O. umbilicata* group). On the other hand, we also saw that a much broader species concept without strong phylogenetic basis can be misleading. In our case, the morphological similarity driven by convergence has led to the *bona fide* acceptance of a paraphyletic taxon. Thus, we should make detailed phylogenetic and population genetic investigations within the recently circumscribed clades (Devey *et al*. 2008, Devey *et al*. 2009) in *Ophrys* to gain a clearer picture of its taxonomy, and to unravel the evolutionary background of its variability.

Conservation issues concerning *Ophrys kotschyi*

Another important conclusion comes from adopting a narrower species concept for *O. kotschyi*, namely, its status as a Cypriot endemic. The inclusion in the latest monograph (Pedersen & Faurholdt 2007) of other, Aegean and southeastern Greek taxa into the species is neither supported by our study, nor by the morphometric study by Gölz and Reinhard (1985). The estimated total population size of *O. kotschyi s. stricto* is between 3000–5000 mature individuals (Baumann & Künkele 1994). This small number and the restricted distribution of the small subpopulations (Kreutz 2004) together imply that *O. kotschyi s. stricto* is highly endangered. Therefore, nature conservation efforts that aim to preserve this local endemic should focus on the declining populations on the island of Cyprus.

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