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Reproductive isolation in the Aegean *Ophrys omegaifera* complex (Orchidaceae)

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Abstract. The orchid genus *Ophrys* operates a system of sexual deception by which high specificity of pollination is attained. Reproductive isolation in *Ophrys* mainly rests upon prezygotic isolation mechanisms. The level of genetic separateness of *Ophrys* taxa with different pollinators is therefore likely determined by the fidelity of pollinators. The present study employs genetic fingerprinting to investigate this in the east Aegean *Ophrys omegaifera* s.l. complex, also including *O. dyris*, a west Mediterranean species of this complex. *Ophrys fleischmannii*, *O. basilissa*, and the west Mediterranean *O. dyris*, are found to be well-separated genetic entities whereas *O. omegaifera* s.str. and the putative hybrid taxon, *O. sitiaca*, are found to be genetically inseparable across their entire range of co-occurrence. This suggests that specific pollinators have high enough fidelity to act as effective isolating factors in east Aegean *O. omegaifera* s.l. as a whole, but that the situation in the species pair of *O. sitiaca* and *O. omegaifera* is likely to be more complex.

Key words: AFLP, *Ophrys omegaifera*, *Ophrys sitiaca*, *Ophrys basilissa*, *Ophrys fleischmannii*, *Ophrys dyris*, pollinators, reproductive isolation, sexually deceptive orchids.

The Mediterranean orchid genus *Ophrys* is remarkable for its pollination by sexual deception. *Ophrys* flowers mimic key traits used by female insects to elicit sexual behaviour in their male conspecifics (Kullenberg 1961, Paulus 2006, Paulus and Gack 1990b). Males attempt to copulate with orchid labella ('pseudocopulation') and by so doing receive pollinia or pollinate the flower. This exploitation of an insect's species-specific sexual signals ensures high pollination specificity in *Ophrys* (Grant 1994; Paulus 2006; Paulus and Gack 1990b).

Within *Ophrys*, sect. *Pseudophrys* Godfery is defined by those taxa attaching their pollinia to their pollinators' abdomens (Kullenberg 1961, Paulus and Gack 1994). This section comprises the *O. fusca* s.l., *O. lutea* s.l., and *O. omegaifera* s.l. complexes, which can be readily distinguished morphologically (Paulus 2001). DNA sequence data support the monophyly of sect. *Pseudophrys* but have so far not allowed finer distinctions to be made (Bateman et al. 2003, Bernardos et al. 2005, Soliva et al. 2001). Unlike other

members of the section which are mostly pollinated by *Andrena* (Hymenoptera: Andrenidae), taxa of the *O. omegaifera* complex are mainly pollinated by *Anthophora* bees (Hymenoptera: Anthophoridae) (Paulus and Gack 1981, 1990b). Due to its morphological intermediacy, *O. sitiaca* has been described as an intermediate between the *O. omegaifera* and *O. fusca* complexes (Paulus 1988). It may therefore be of hybrid origin or otherwise introgressed. Pollination of *O. sitiaca* by *Andrena nigroaenea* on Crete has been documented (Paulus and Gack 1990a).

The remarkable high specificity of pollinator attraction by *Ophrys* flowers rests upon mimicry of the insect sex pheromone, as was first elucidated in the case of *Andrena nigroaenea* and *O. sphegodes* (Schiestl et al. 1999, 2000). It has therefore been proposed that speciation in *Ophrys* may be linked with a change in floral odour and the associated attraction of a different specific pollinator (Paulus and Gack 1990b, Schiestl and Ayasse 2002). On the other hand, several *Ophrys* species utilising the same pollinator, such as *O. herae*, *O. fusca* and *O. sitiaca*, show strong convergence in pollinator-specific odour components (Stökl et al. 2005). In this case, interbreeding is prevented by different placement of pollinia on the insect or allopatry.

While specific pollination should allow for efficient prezygotic reproductive isolation (Paulus 2006, Paulus and Gack 1990b), there seems to be little postzygotic isolation (Cozzolino et al. 2004, Ehrendorfer 1980). In particular, only weak chromosomal differentiation has been observed in *Ophrys* (D'Emerico et al. 2005). Chromosome counts indicate that *Ophrys* taxa are generally diploids with $2n = 36$ chromosomes; the only tetraploid populations known in *Ophrys* so far are west Mediterranean representatives of the *O. fusca* s.l. and *O. omegaifera* s.l. groups (including *O. dyris* with $2n = 72$ and also $2n = 90$), for both of which no chromosome data are available from the eastern Mediterranean area (e.g. Bernardos et al. 2003, Greilhuber and Ehrendorfer 1975). Only diploid counts have been reported for *O. fusca* s.l. in Italy, where the *O. omegaifera* s.l. group is lacking (D'Emerico et al. 2005).

Since reproductive isolation in *Ophrys* mainly depends on prezygotic factors, the degree of genetic separateness of *Ophrys* taxa will depend on the fidelity of pollinators; occasional unspecific pollination events could potentially lead to a large number of seeds, potentially viable offspring and, dependent upon whether a suitable pollinator can be attracted, in considerable introgression or hybridisation (Arditti and Ghani 2000, Ehrendorfer 1980). We therefore ask whether *O. omegaifera* s.l. taxa with specific pollinators present in the same geographical area are genetically distinct groups, as one may expect under a biological species model and high pollinator specificity. *O. dyris* would be expected to be genetically separate in any event due to geography. Also, if *O. sitiaca* were of hybrid origin, we would expect its genome to contain neutral markers from either parent, i.e. most likely *O. omegaifera* and an *O. fusca* s.l. taxon.

We have used amplified fragment length polymorphism (AFLP) markers to address the question of reproductive isolation among *O. omegaifera* s.l. populations in the Aegean. The AFLP method (Vos et al. 1995) has been successfully employed in numerous recent studies to reveal genetic structure at a fine scale (e.g. Hedrén et al. 2001, Pfosser et al. 2006, Schönswetter et al. 2004, Trometsberger et al. 2003).

Materials and methods

Taxa. A summary of the main biological features of the taxa investigated in this study is presented in Table 1 and representative flowers depicted in Fig. 1 (see also Alibertis et al. 1990, Delforge 2005, Kretzschmar et al. 2002, Paulus 1988, Paulus and Gack 1990a). Taxonomic criteria and keys of Paulus (1998, 2001) were followed. West Mediterranean plant material was from *O. dyris* (and not *O. algarvensis*; Tyteca et al. 2003).

Table 1. Summary of some key biological data for the studied taxa, compiled from Alibertis et al. (1990), Paulus (1988), Paulus and Gack (1990b), and Paulus (unpublished), lip length values quoted referring to fresh material. Taxa are sorted according to flowering time for a typical lowland population

***Ophrys* taxon**	**Phenology**	**Pollinator (observations)**	**Lip length (mm) ± SD**	**Distribution**
	JanFebMarAprMay			
East Mediterranean taxa				
O. sitiaca Paulus, C. Alibertis & H. Alibertis		*Andrena nigroaenea* (Crete: >50; Samos: 2)	15.7 ± 0.82	E Crete and E Aegean
O. basilissa C. Alibertis & H. Alibertis & H. R. Reinhard		*Anthophora subterranea*[1] (Crete: >50; Kos: c. 30)	23.8 ± 3.26	Crete - Kos
O. fleischmannii Hayek		*Anthophora sicheli* (Crete: >30)	15.2 ± 0.96	Crete
O. omegaifera Fleischmann		*Anthophora dalmatica*[2] (>100)[3]	18.5 ± 1.26	Aegean
West Mediterranean taxa				
O. dyris Maire[4]		*Anthophora atroalba* (S Spain: >50)	16.3 ± 0.96	N Africa - NE Spain

[1] Previously known as *A. nigrocincta*
[2] Previously known as *A. atroalba* agamoides
[3] Data from Crete, Karpathos, Rhodes and Naxos. In addition, Anthophora nigriceps has been observed on early-flowering populations on Rhodes (10 observations) and Samos (10 observations)
[4] Data representative for populations from S Spain

Fig. 1. Representative photographs of members of the *O. omegaifera* s.l. complex. **A** *O. omegaifera* (28 March 2006, Gournia, Crete); **B** *O. sitiaca* (13 March 2005, Thripti, Crete); **C** *O. basilissa* (18 March 2005, Vasiliki, Crete); **D** *O. fleischmannii* (29 April 2004, Thripti, Crete); **E** *O. dyris* (8 April 2004, Sierra de Mijas, S Spain). All photographs by HFP

Plant material and DNA extraction. Plant leaves and/or flowers were collected in the field and dried in silica gel (Table 2 and Fig. 2). Wherever possible, individual plant photographs were taken and representative vouchers deposited in the herbaria at Vienna University (WU), Austria, and the herbarium of the Balkan Botanic Garden in Kroussia, Greece.

DNA was extracted from silica-dried plant material using DNeasy Plant Mini kit (Qiagen) and the manufacturer's protocol. DNA was eluted in 200 μL TE (10 mM Tris, 1 mM EDTA, pH 8) and its concentration estimated on agarose gel or by fluorimetry using a DyNA Quant 200 Fluorometer (Amersham) and the Hoechst 33258 dye binding assay following the manufacturer's protocol.

Table 2. Plant material used in this study, where n is the number of sampled individuals per population and the date is given in DD.MM.YYYY format

Island	Code	Place	n	Date	Collector	Population number
BAS - *O. basilissa* (n = 17)						
Crete	ANT	Antiskari	5	24.02.2004	P. M. Schlüter	171
Crete	APE	Aghia Pelaghia	1	21.02.2004	P. M. Schlüter	165
Crete	FES	Phaistos	2	20.02.2004	P. M. Schlüter	163
Crete	GRN	Gournia, Kreta	3	29.03.2003	H. F. Paulus	101
Kos	ASK	Asklepion	2	27.02.2002	H. F. Paulus	66
Samos	EKL	Klima	4	21.02.2004	H. F. Paulus	174
FLE - *O. fleischmannii* (n = 9)						
Crete	THR	Thripti	5	29.03.2003	H. F. Paulus	102
Crete	THR	Thripti	4	29.04.2004	H. F. Paulus	228
OMG - *O. omegaifera* (n = 25)						
Rhodes	ATT	Attaviros	5	24.04.2003	P. M. Schlüter	140
Rhodes	PEL	Profitis Elias, peak	2	22.04.2003	P. M. Schlüter	129
Rhodes	PEW	Profitis Elias, W side	3	22.04.2003	P. M. Schlüter	125
Samos	EKL	Klima	9	26.02.2004	H. F. Paulus	188
Crete	THR	Thripti	1	25.03.2002	H. F. Paulus	37
Crete	ZAR	Zaros	5	30.03.2003	H. F. Paulus	107
SIT - *O. sitiaca* (n = 24)						
Samos	EPK	Palaeokastro	4	21.02.2004	H. F. Paulus	173
Samos	ESB	Sideras bay	1	22.02.2004	H. F. Paulus	175
Samos	SWK	Kiriaki	3	24.02.2004	H. F. Paulus	185
Crete	JOU	Jouchtas	5	22.02.2004	P. M. Schlüter	169
Crete	THR	Thripti	8	29.03.2003	H. F. Paulus	99
Kos	ASK	Asklepion	3	25.02.2002	H. F. Paulus	64
DYR - *O. dyris* (n = 7)						
S Spain	ALH	Alhaurin	4	08.04.2004	H. F. Paulus	202
S Spain	SDM	Sierra de Mijas	2	10.04.2004	H. F. Paulus	201
Mallorca	MCA	S'Aranjassa	1	05.03.2003	J. Stökl	240

Fluorescent amplified fragment length polymorphism (AFLP) reactions. AFLPs followed the procedure of Vos et al. (1995), with modifications. Restriction and ligation of 100–300 ng genomic DNA were carried out in a single reaction in 11μL, using Fermentas T4 DNA ligase buffer. Reactions contained 40 mM Tris-HCl (pH 7.8 at 25°C), 10 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM adenosine triphosphate (ATP), 50 mM NaCl, 0.05μg μL^{-1} bovine serum albumin (BSA), 4.5 μM double-stranded (ds)-*Mse*I adapter, 0.45 μM ds-*Eco*RI-adapter, prepared in nuclease-free sterile water (Fluka), with 5 u *Mse*I (New England Biolabs), 5 u *Eco*RI (Fermentas) and 1 u T4 DNA ligase (Fermentas). Temperature was controlled in a thermocycler, using 3 h at 37°C, 30 min at 17°C and 10 min at 70°C. Reactions were checked on agarose gel, and 6 μL diluted with 94 μL $TE_{0.1}$ (20 mM Tris-HCl, 0.1 mM EDTA). Preselective and selective amplifications were carried out in 10 μL, using 2 μL diluted template, 0.2 u RedTaq DNA polymerase (Sigma), 1x RedTaq DNA polymerase buffer (i.e. reaction buffered in 10 mM Tris-HCl, pH 8.3, 1.25 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin), 0.22 mM each dNTP (Fermentas), and either 278 nM of each preselective primer (*Eco*RI-A and *Mse*I-CT) or 270 nM *Mse*I-selective and 54 nM *Eco*RI-selective primers. *Eco*RI-Selective primers (biomers.net and PE Applied Biosystems) were 5'-fluorophore labelled;

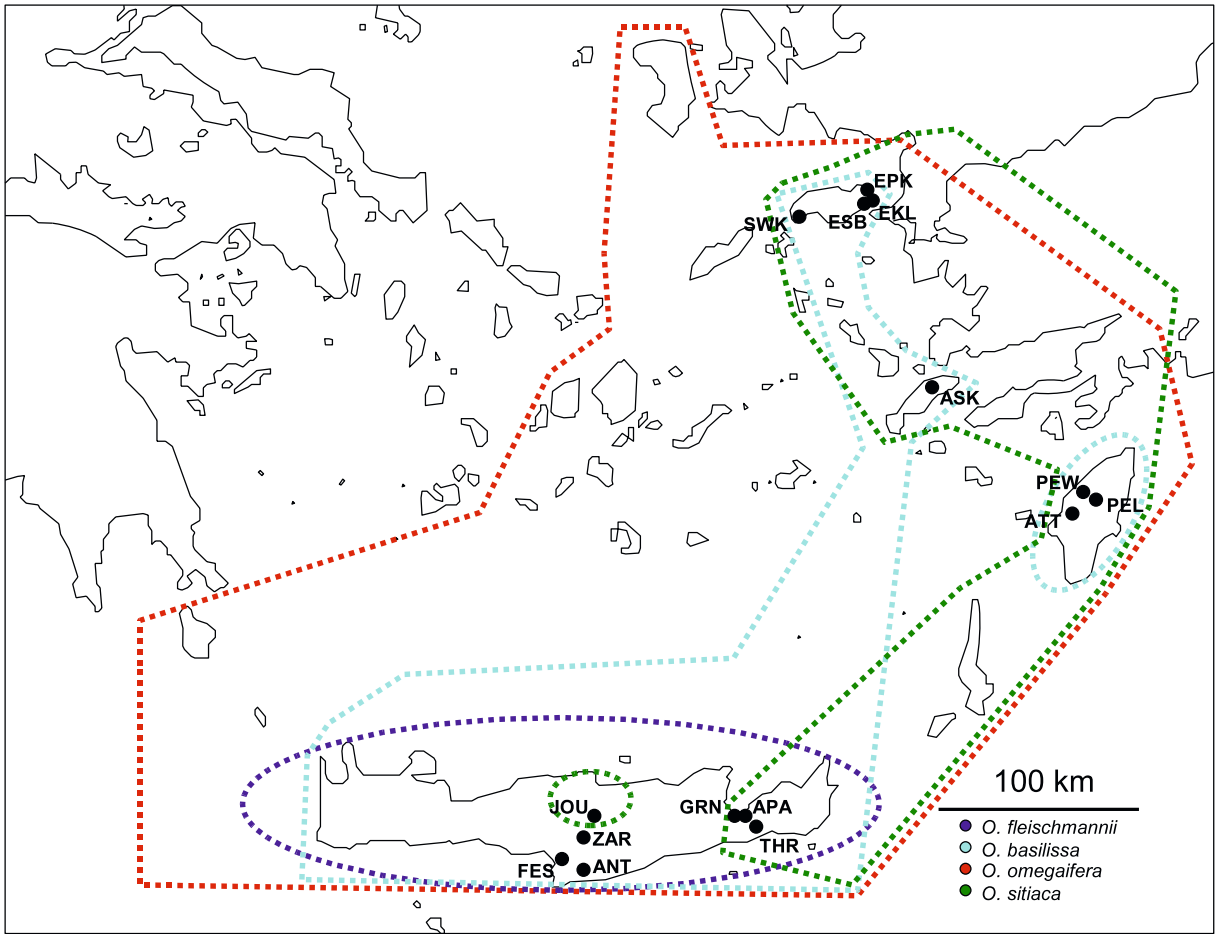


Fig. 2. Map of the Aegean indicating the *O. omegaifera* s.l. populations sampled from this area. Population codes as presented in Table 2. Known distribution areas of Aegean *O. omegaifera* s.l. taxa are indicated

the 6 combinations used were *Mse*I-CTCG with *Eco*RI-ACT (6-FAM), -ATC (HEX), -ACC (NED), and *Mse*I-CTAG with *Eco*RI-ACT (6-FAM), -AGG (HEX), -AGC (NED). All PCRs were performed on a T-CY thermocycler (Creacon Technologies), with a ramp time of 0.9°C s^{-1}. The preselective PCR was programmed as 1x (2 min at 72°C), 20x (1 s at 94°C, 30 s at 56°C, 2 min at 72°C), 1x (30 min 60°C); and the selective PCR as 1x (2 min at 94, 30 s at 65, 2 min at 72°C), 8x (1 s at 94, 30 s at T_A = 64°C, decreasing T_A by 1°C per cycle, 2 min at 72°C); 23x (1 s at 94°C, 30 s at 56°C, 2 min at 72°C), 1x (30 min at 60°C). Reactions were checked on agarose gel and preselective PCRs diluted 1:20 in $TE_{0.1}$ before use as template.

Selective PCRs were mixed in a dye ratio of 25% 6-FAM, 32% HEX and 43% NED, and 2.1 µL of this mix combined with 1.3 µL loading buffer containing 0.15 µL Genescan-500-ROX size standard (PE Applied Biosystems) and 0.3 µL loading dye in deionised formamide. Amplification products were separated on a denaturing 4% polyacrylamide (29:1 acrylamide:N,N'-methylene-bisacrylamide)-TBE gel containing 5.5 M urea and run on an ABI Prism 377 DNA sequencer (Perkin Elmer). Negative controls were performed substituting template with water for restriction/ligation, preselective and selective amplification; reference DNAs with known AFLP band profile were used as positive controls.

Scoring and data analysis. Raw data were aligned with the internal size standard using GeneScan 3.1.2 (PE Applied Biosystems). AFLP bands were scored twice as band presence ('*1*') or absence ('*0*'), explicitly coding ambiguous bands as '*?*' (i.e. missing data), using Genographer software (Benham et al. 1999) version 1.6.0 as a visual aid.

Both scorings were first analysed separately and second as a combined data set. AFLP error rates were estimated as suggested by Bonin et al. (2004); the mean genotyping error rate among controls and the mean error rate among scorings (of the same fragments) were estimated applying (a) strict and

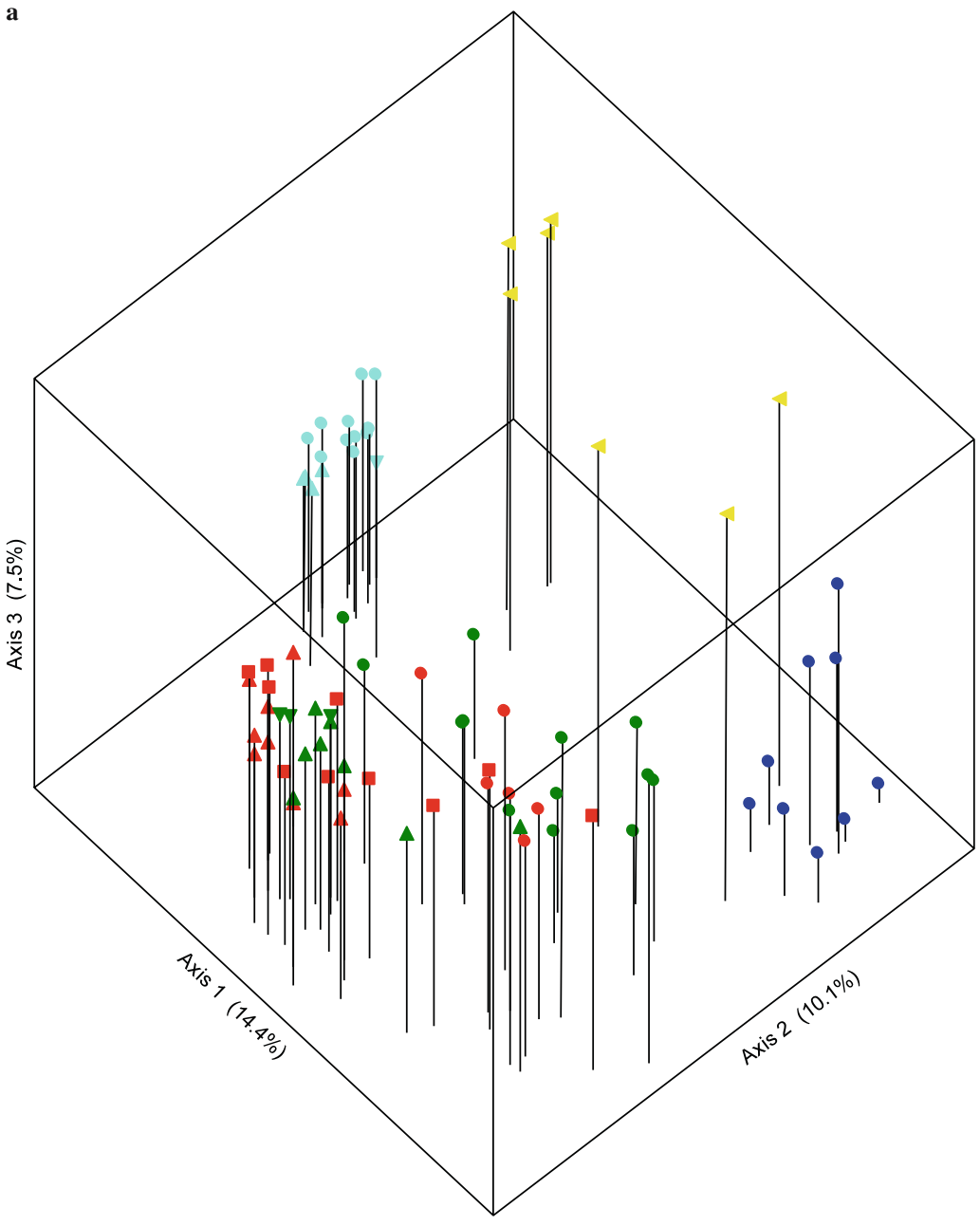


Fig. 3a. Principal coordinate analysis. **a** PCoA showing individuals from the entire *O. omegaifera* complex. Symbol colour represents taxa; yellow: *O. dyris*; cyan: *O. basilissa*; blue: *O. fleischmannii*; green: *O. sitiaca*; red: *O. omegaifera*. Symbol shapes represent sampling regions; ●: Crete; ■: Rhodes; ▲: Samos ▼: Kos; ◀: West Mediterranean. **b** PCoA of the *O. omegaifera*/*O. sitiaca* cluster only, where green symbols represent *O. sitiaca* individuals and red symbols *O. omegaifera*. Symbols: ●: Thripti, Crete; ■: Jouchtas, Crete; ▲: Zaros, Crete; ▼: Samos; ▶: Kos; ◀: Profitis Elias, Rhodes; +: Attaviros, Rhodes. The black line separates the northern and southern groups, as determined by BAPS

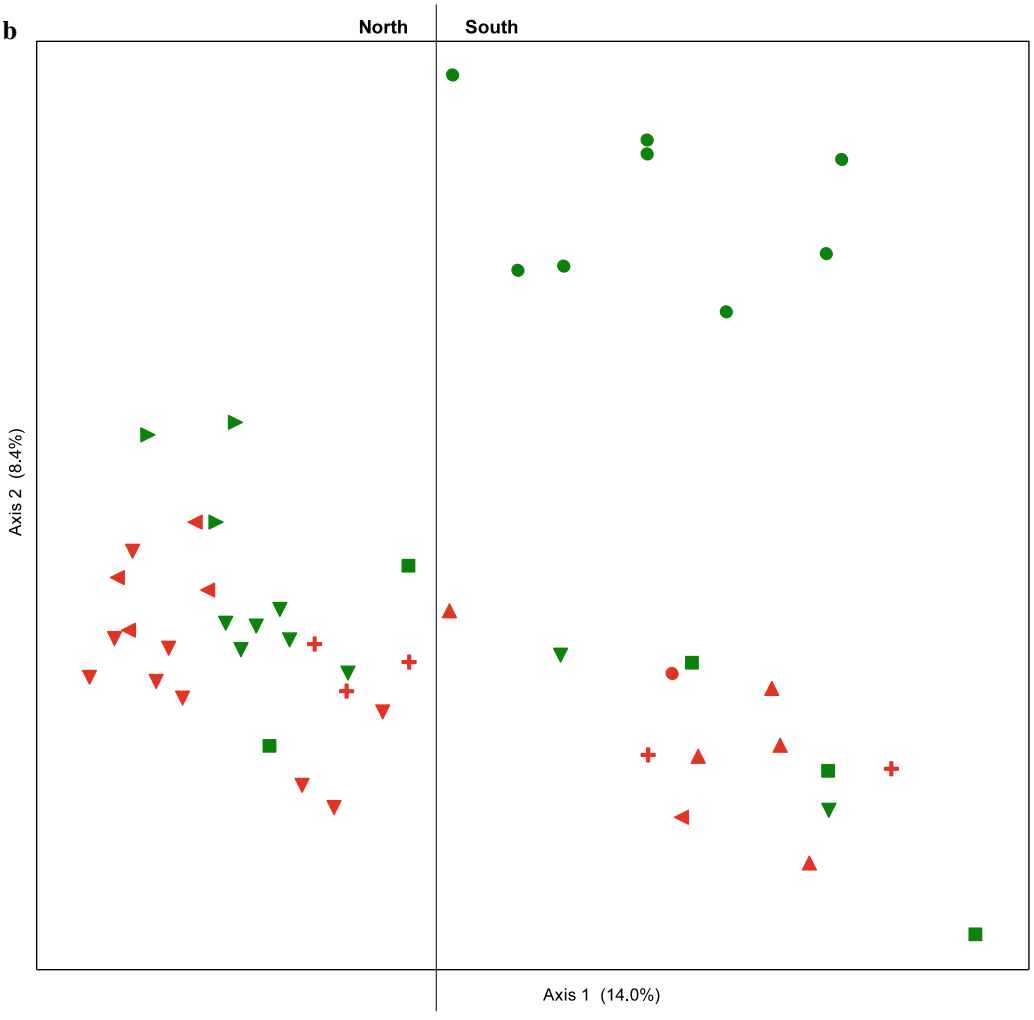
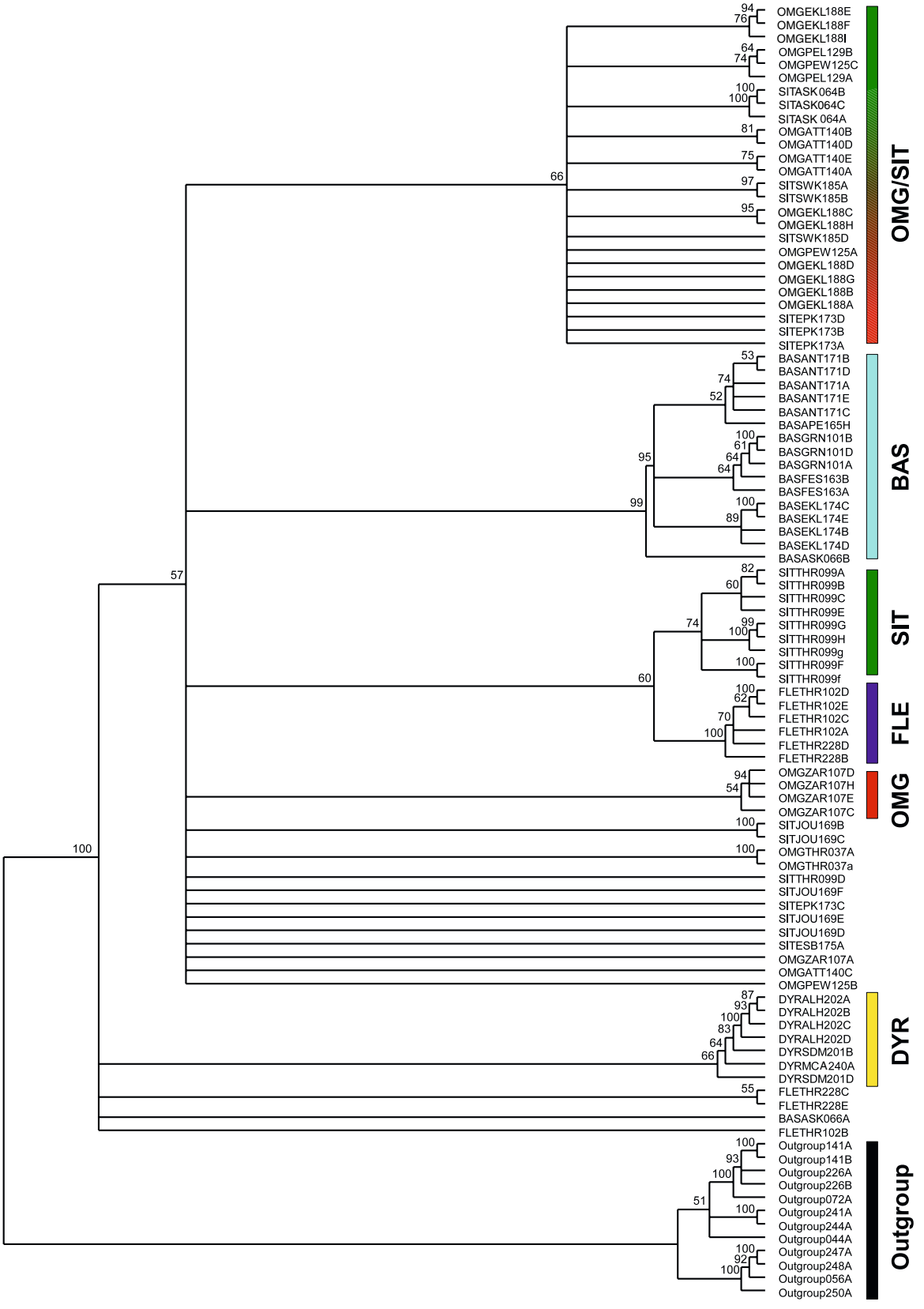


Fig. 3b. (Continued)

(b) relaxed criteria. Strict error rates treated *1/?* and *0/?* band combinations as errors, whereas these combinations were not treated as erroneous under relaxed criteria. All data matrices are available from PMS.

Initially, UPGMA and NJ dendrograms were constructed from the data matrix using Jaccard's similarity measure (Jaccard 1908) and Nei and Li's distance (1979, with r = 6) in FAMD 1.03 (Schlüter and Harris 2006) and PAUP* 4.0β10 (Swofford 2002) and the effect of missing data points investigated using FAMD. Bootstrap analyses (1000 pseudo-replicates) were performed likewise. Principal coordinate analyses were performed using SynTax 2000 (Podani 2001) and the R-package v.4d9 (Casgrain and Legendre 2004). Population structure was further investigated using BAPS 3.2 (Corander et al. 2003) and Structure 2.0

Fig. 4. Majority rule consensus tree from a bootstrap analysis (1000 pseudo-replicates) of UPGMA dendrograms based upon Jaccard's similarity measure. Numbers above branches indicate bootstrap support. Taxa are highlighted using the three letter species code given in Table 2. Labels for plant individuals include species and locality codes, and population number as stated in Table 2, plus a letter identifying an individual within a population


OMGEKL188E
OMGEKL188F
OMGEKL188I
OMGPEL129B
OMGPEW125C
OMGPEL129A
SITASK064B
SITASK064C
SITASK 064A
OMGATT140B
OMGATT140D
OMGATT140E
OMGATT140A
SITSWK185A
SITSWK185B
OMGEKL188C
OMGEKL188H
SITSWK185D
OMGPEW125A
OMGEKL188D
OMGEKL188G
OMGEKL188B
OMGEKL188A
SITEPK173D
SITEPK173B
SITEPK173A
OMG/SIT
BASANT171B
BASANT171D
BASANT171A
BASANT171E
BASANT171C
BASAPE165H
BASGRN101B
BASGRN101D
BASGRN101A
BASFES163B
BASFES163A
BASEKL174C
BASEKL174E
BASEKL174B
BASEKL174D
BASASK066B
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SITTHR099A
SITTHR099B
SITTHR099C
SITTHR099E
SITTHR099G
SITTHR099H
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FLETHR102D
FLETHR102E
FLETHR102C
FLETHR102A
FLETHR228D
FLETHR228B
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OMGZAR107D
OMGZAR107H
OMGZAR107E
OMGZAR107C
OMG
SITJOU169B
SITJOU169C
OMGTHR037A
OMGTHR037a
SITTHR099D
SITJOU169F
SITEPK173C
SITJOU169E
SITJOU169D
SITESB175A
OMGZAR107A
OMGATT140C
OMGPEW125B
DYRALH202A
DYRALH202B
DYRALH202C
DYRALH202D
DYRSDM201B
DYRMCA240A
DYRSDM201D
DYR
FLETHR228C
FLETHR228E
BASASK066A
FLETHR102B
Outgroup141A
Outgroup141B
Outgroup226A
Outgroup226B
Outgroup072A
Outgroup241A
Outgroup244A
Outgroup044A
Outgroup247A
Outgroup248A
Outgroup056A
Outgroup250A
Outgroup

(Pritchard et al. 2000) software. For Structure, AFLP profiles were treated as haplotypes, using the admixture model. For BAPS, data were treated as diploid with the second allele at every locus missing and both, individual and group-based clustering performed, followed by admixture analysis. Splitstree 4.2 (Huson and Bryant 2006) was used for construction of neighbour net and split decomposition networks on population samples. Maximum-likelihood estimates for hybrid indices were calculated for *O. sitiaca* samples using hindex 1.42 (Buerkle 2005).

Results

Eighty-two individuals (excluding duplicates) were scored and 723 markers obtained from the 6 primer combinations, with 2.85% of all data points coded as missing data. One individual had to be removed from the data matrix due to data quality issues. The mean genotyping error rate between controls (see Bonin et al. 2004) was 5.11% (relaxed) - 7.57% (strict) and the error among scorings 4.47% (relaxed) - 7.88% (strict).

Principal coordinate analysis (PCoA) revealed four distinguishable clusters, corresponding to *O. basilissa*, *O. dyris*, *O. fleischmannii* and a mixture of *O. sitiaca* and *O. omegaifera* (Fig. 3a). Population structure, as identified using BAPS, corresponded exactly to these four groups, identifying limited admixture among all groups. Dendrograms (Fig. 4) confirmed this overall picture, bootstrap analysis supporting the ingroup as well as the groups discernible in the PCoA. In addition, *O. sitiaca* from Mount Thripti and *O. omegaifera* from Zaros in Crete had moderate and weak support, respectively. However, the positions of several individuals (mostly *O. sitiaca* and *O. omegaifera*) were unresolved and there was hardly any support for relationships among groups. Network approaches did not yield any additional insights.

Within *O. omegaifera* and *O. sitiaca*, BAPS identified two putative populations, one composed mostly of individuals from the North of the sampled area (i.e. Samos, Kos, and Rhodes) and the second consisting mostly of individuals from Crete. Both groups contained individuals of both *O. omegaifera* and *O. sitiaca*. Both PCoA (Fig. 3b) and Structure revealed three groups, one corresponding to those outlined above, but decomposing the group from Crete into two clusters, one of them identical to the Thripti population of *O. sitiaca* (see also Fig. 4) and containing no *O. omegaifera* individuals.

Maximum likelihood estimates for hybrid indices were calculated for *Ophrys sitiaca*, testing for hybridity between *O. omegaifera* and a number of the *O. fusca* s.l. taxa present in the Aegean: *O. leucadica* Renz, *O. creberrima* Paulus, *O. creticola* Paulus and *O. mesaritica* Paulus, C. Alibertis & A. Alibertis (data from Schlüter et al. unpublished). Mean hybrid indices for *O. sitiaca* based on sample localities (Fig. 5) show values greater than 0.79 in all cases, where *0* would indicate identity with the putative *O. fusca* s.l. parent and *1* identity with *O. omegaifera*. The lowest indices for *O. sitiaca* on Crete were for putative parents *O. creberrima* (0.790) and *O. mesaritica* (0.793).

Discussion

Based on AFLP data, the five analysed taxa of the *Ophrys omegaifera* complex would seem to fall into four genetically distinct groups. The genetic separateness of *O. dyris* is certainly not surprising, since the distribution of this taxon is disjunct from the other, Aegean, taxa. On the contrary, the patterns observed in the Aegean are highly interesting, because *O. sitiaca*, which from the perspective of morphology and pollination biology would have been presumed the most divergent taxon, is genetically least well defined and, in fact, indistinguishable from *O. omegaifera*.

O. omegaifera, *O. basilissa* and *O. fleischmannii* are differentiated in terms of phenology; each is pollinated by different species of *Anthophora* bees, which vary in size and coloration (Paulus 2006, Paulus and Gack 1994). A correlation between pollinator body size and lip size has been documented in

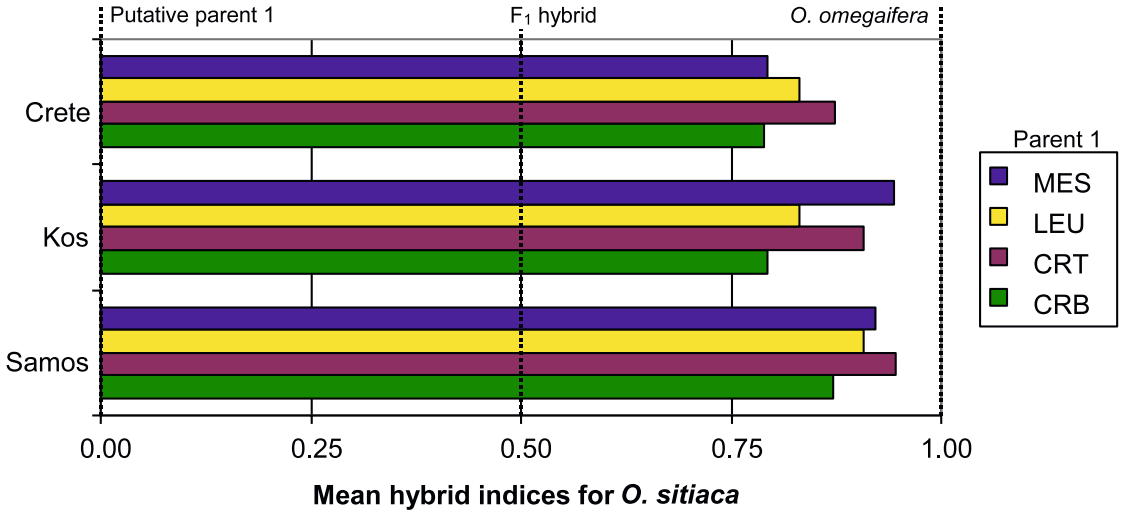

Fig. 5. Mean maximum likelihood estimates for hybrid indices for geographic groups of *O. sitiaca* individuals, under the hypothesis that *O. omegaifera* s.str. is one of the parental taxa. Four *O. fusca* s.l. taxa were each tested as the other putative parental taxon, where MES (blue): *O. mesaritica*; LEU (yellow): *O. leucadica*; CRT (purple): *O. creticola*; CRB (green): *O. creberrima*. One would expect tested *O. sitiaca* groups to score 0.5 if they were perfect F_1 hybrids, to score 1 if they were in fact *O. omegaifera* individuals, and 0 if they were identical to the other putative parental taxon tested

Ophrys and is also evident in this group (Gölz et al. 1996). These data suggest that the aforementioned taxa should be reproductively isolated, which is in agreement with the AFLP data presented here. Indeed, bootstrap support for *O. basilissa* and *O. fleischmannii* is high, and *O. basilissa* samples form a single cluster irrespective of sample origin. Furthermore, model-based clustering supports the strict partitioning of individuals into groups according to taxonomic expectations. Interestingly, this finding seemingly contrasts with the situation in *O. sphegodes* s.l. and *O. arachnitiformis* s.l., where there was no genetic separation among sympatric populations of different *Ophrys* taxa (Mant et al. 2005, Soliva and Widmer 2003). While we have not sampled sympatric populations extensively, *O. basilissa*, *O. fleischmannii* and *O. omegaifera* strongly overlap in their distributions and occur in sympatry in many localities. Although our data are not directly comparable to previous microsatellite studies (Mant et al. 2005, Soliva and Widmer 2003), we would argue that there is certainly stronger genetic differentiation in the case of *O. basilissa*, *O. omegaifera* and *O. fleischmannii* than observed in the case of Italian *O. sphegodes* s.l. and *O. arachnitiformis* s.l. This could indicate the groups in the *O. omegaifera* complex are comparatively older, that there is less gene flow across species boundaries, or that reproductive isolation is stronger in *O. omegaifera* s.l., whether due to different pollinator behaviour or otherwise.

Ophrys sitiaca has long been speculated to be of hybrid origin because of its morphological intermediacy between *O. fusca* s.l. and *O. omegaifera* (Alibertis et al. 1990, Paulus 1988). For example, Alibertis et al. (1990) suggested *O. mesaritica* (their "small, early-flowering *O. iricolor*") as a putative parent based on morphometric data. However, this could be misleading since the allopatric *O. mesaritica* is likely pollinated by *Andrena nigroaenea* (Schlüter et al., unpubl. data) and morphometry may merely reflect convergence of floral characters due to pollinator-driven selection. Alternative putative parents from the *O. fusca* complex may be *O. leucadica*, which is widespread in the Aegean but missing from Crete, or either *O. creberrima* or *O. creticola*, both endemic to

Crete. Also the *A. nigroaenea*-pollinated *O. lupercalis* from the west Mediterranean and northwest Aegean is a plausible second parent for *O. sitiaca*, these two taxa being strictly allopatric. *O. lupercalis*, however, was unavailable for analysis. Hybrid indices indicate that none of the sampled *O. sitiaca* were of likely F_1 origin and certainly much more similar to *O. omegaifera* than to any putative *O. fusca* s.l. parent. The most plausible putative parents tested were *O. creberrima* and *O. mesaritica* for *O. sitiaca* on Crete or *O. leucadica* for the populations on Kos (where neither *O. creberrima* nor *O. mesaritica* occur). While this does not exclude a hybrid origin involving any of the tested *O. fusca* s.l. taxa, it would at least suggest an advanced generation hybrid or introgressed population backcrossed with O. *omegaifera* rather than an *O. fusca* s.l. Alternatively, *O. sitiaca* may not be of hybrid origin despite its morphology and pollination, or the *O. fusca* s.l. parent of *O. sitiaca* could be a taxon not present in our sample, such as *O. lupercalis*.

Genetic data revealed a strong mixing of *O. sitiaca* and *O. omegaifera* on all sampled islands, much like that observed in Italian *Ophrys* populations (Mant et al. 2005). Limited structure in this *O. omegaifera*/*O. sitiaca* mix can be discerned as a tendency for samples from the southern island of Crete and the northern islands of Samos, Kos and Rhodes to cluster. The genetic mixing of *O. sitiaca* and *O. omegaifera* could be interpreted as (i) recent divergence or (ii) ongoing gene flow among these two taxa. While the observed biological divergence of *O. sitiaca* and *O. omegaifera* could be very recent and this divergence not yet reflected in neutral AFLP markers, the distribution of both taxa in the east Aegean does not suggest an origin more recent than that of *O. basilissa* or *O. fleischmannii*, both of which are genetically distinct from *O. omegaifera*. If there is ongoing gene flow between *O. sitiaca* and *O. omegaifera*, it is not clear why it is occurring in exactly this species pair and in the same way on different islands. This would suggest an as yet unidentified factor, specific to the *O. sitiaca*/*O. omegaifera* species pair that acts similarly in different geographic areas.

One explanation for the genetic pattern found in *O. omegaifera* and *O. sitiaca* would therefore be the existence of an alternative pollinator, different from *Anthophora atroalba* or *Andrena nigroaenea*, that is specifically attracted to these two *Ophrys* taxa, but none of the other *O. omegaifera* s.l. taxa, and that mediates gene flow in the same way from Crete to Samos. However, no field observations have yet suggested the existence of such an entity, and the same hypothetical pollinator would be expected to also mediate gene flow among *O. omegaifera* and other *Andrena nigroaenea*-attracting *Ophrys* taxa, such as *O. mesaritica* (which is not supported by AFLP data; Schlüter et al. unpublished). Nonetheless, the pollinator record of *O. omegaifera* and *O. sitiaca* may be incomplete, pollination of *O. sitiaca* being so far mostly undocumented outside of Crete. Moreover, hitherto little explored early-flowering populations of *O. omegaifera* on Rhodes and Samos are attractive to *Anthophora nigriceps* (Paulus, unpubl. data). If present in our sample, this may fit the genetic pattern observed in *O. omegaifera*, although this would not by itself explain a similar pattern in *O. sitiaca*. Clearly, there must be factors that render the situation of *O. sitiaca* and *O. omegaifera* more complicated than that of the other species in the Aegean *O. omegaifera* s.l. complex.

Taken together, our findings suggest that reproductive isolation is strong enough to keep specifically pollinated groups within *O. omegaifera* s.l. separate, with the notable exception of *O. sitiaca* and *O. omegaifera* where the situation may be more complex.

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