Journal of Heredity 2005:96(1):15–23
doi:10.1093/jhered/esi006
Advance Access publication December 23, 2004

Reproductive Versus Floral Isolation Among Morphologically Similar *Serapias* L. Species (Orchidaceae)

G. Pellegrino, A. Musacchio, M. E. Noce, A. M. Palermo, and A. Widmer

From the Dipartimento di Ecologia, Università della Calabria, Arcavacata di Rende, I-87036 Cosenza, Italy (Pellegrino, Musacchio, Noce, Palermo); and the Geobotanisches Institut, ETH Zürich, Zollikerstr. 107, CH-8008 Zürich, Switzerland (Widmer)

Address correspondence to G. Pellegrino at the address above, or e-mail: giuseppe.pellegrino@unical.it.

Abstract

Flowers of the Mediterranean orchid genus *Serapias* L. form small, dark tubes that vary among taxa in diameter and depth. Visiting insects use the floral tube as shelter and act as pollinators if they touch the sticky viscidium at the rear of the tube and remove the pollinarium. It has been assumed that floral tube size and shape limit access to the flowers and thus may act as a barrier to gene flow between different *Serapias* species. Here we investigated floral characters and nuclear microsatellite markers in populations belonging to three morphologically similar *Serapias* species to test whether these species show evidence for floral or reproductive isolation. We found strong overlap of floral traits between two species, suggesting that floral isolation is nonexistent between them. Microsatellite markers applied to the same populations were highly polymorphic and revealed clear genetic differentiation among all three species. These results suggest that reproductive isolation exists, despite the lack of floral isolation between two of the species. In contrast to morphological characters, diagnostic microsatellite alleles were found for all *Serapias* species. The microsatellite markers could thus provide a useful tool to identify *Serapias* species and further investigate evolutionary relationships in this fascinating orchid lineage.

A central tenet of evolutionary plant biology is that floral trait variation may induce differential visitation or constancy of pollinators, thus promoting isolation between distinct plant phenotypes, lineages and species, a vision that was originally formulated in the floral isolation concept of Grant (1949). Orchids often show highly specialized and species-specific plant–pollinator relationships and for this reason have been commonly indicated as the most obvious example of diversification due to floral or prezygotic isolation (Dressler 1993; Van der Pijl and Dodson 1966). However, there is now increasing evidence that generalized pollination systems are more widespread than previously thought. This insight has spurred new interest in classical topics like selection on floral traits, divergence of populations, and the nature of the reproductive barriers among the species (Johnson and Steiner 2000; Waser 1998). In this context, research on orchids species may be particularly interesting because the role of the postzygotic barrier has been typically ignored in this diverse plant family.

The Mediterranean region harbors a high diversity of orchid species that differ widely in floral morphology, mating, and pollination system. Among the most intriguing orchids are members of the genus *Serapias* L. Their flowers differ from those of other Euro-Asiatic orchids in the peculiar floral shape and pollination system. Sepals, lateral petals, and lobes of the basal portion of the central petal, the hypochile, together form a small, dark tube that varies in diameter and depth among taxa (Figure 1) (Baumann and Künkele 1989). The plants produce no nectar reward but instead attract insects that use the floral tube to rest or sleep (Dafni et al. 1981), as a draft-free hiding place under bad or rainy weather conditions (Gumprecht 1977), or for thermoregulation, because the temperature in the flower tube may exceed the ambient temperature by up to 3°C during the morning hours (Felicioli et al. 1998). Insects entering the floral tube encounter two guiding ridges at the base of the labellum.

Behind these ridges is a single viscidium, a removable, sticky plate to which the two pollinia are attached. While visiting the flowers, pollinators of appropriate size pick up and remove the pollinia. Pollination occurs when insects carrying pollinia visit another flower and deposit pollen on the stigma (Dafni et al. 1981). It has long been assumed that differences in size and shape of the floral tube control access to pollinia and thus act as a prezygotic reproductive barrier between

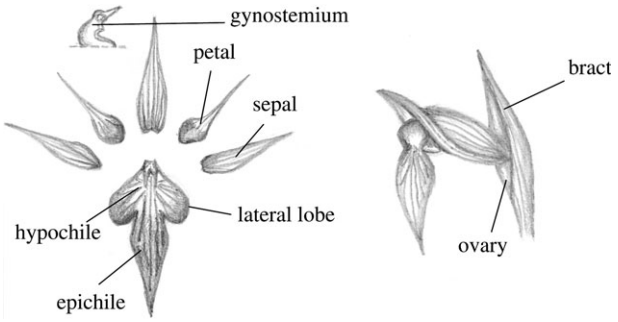

Figure 1. Drawing of a whole and partitioned flower of a *Serapias* species.

Serapias species. However, floral tube size varies within populations and species, and considerable overlap exists among species. It thus has been concluded that species boundaries are poorly defined in *Serapias* (Bateman et al. 2003, Pridgeon et al. 1997).

As in most other Mediterranean orchids, vegetative characters are similar across species and do not distinguish them. Consequently, the number of species recognised varies widely (e.g., 10 or 21 species according to Nelson 1968 and Delforge 1994, respectively). The present classification of *Serapias* species is essentially based on quantitative differences in floral traits and inflorescence size. The observed variation in these traits has often led to disagreement about the taxonomic rank and the corresponding nomenclature of several *Serapias* taxa (Gölz and Reinhard 1993; Grünanger 2001; Lorenz 2001).

Molecular systematic studies have provided little help to date with phylogenetic relationships among *Serapias* species. Sequence variation is weak or absent in the nuclear ribosomal internal transcribed spacer (nrITS) region (Bateman et al. 2003; Pridgeon et al. 1997), and in two noncoding chloroplast DNA regions (Widmer and Kocyan unpublished data). Also, a recent karyological analysis has found homogeneity among species with respect to heterochromatin distribution (D'Emerico et al. 2000). These data suggest that *Serapias* species are closely related and have diverged relatively recently.

Other orchid groups that are characterized by morphological and often molecular similarity, such as the genus *Dactylorhiza* Necker and *Epipactis* Zinn, have recently been analysed with AFLPs (Hedrén et al. 2001) and polymerase chain reaction (PCR)-RFLPs of cpDNA (Squirrel et al. 2002), reaching a more satisfying scenario of their taxonomic and evolutionary relationships. Well-characterized, codominant, and highly variable markers, such as microsatellites, might represent an even more adequate approach. To date, microsatellites have been characterized for a few European orchids, including *Ophrys* L. (Soliva et al. 2000), *Gymnadenia* Brown (Gustafsson and Thorén 2001), and *Serapias* (Pellegrino et al. 2001) and have been used successfully to estimate genetic population structure (Gustafsson 2000; Gustafsson and Sjögren-Gulve 2002), and to test for gene flow across species boundaries in *Ophrys* (Soliva and Widmer 2003).

In the present study we used nuclear microsatellite markers that were recently developed for *Serapias* (Pellegrino et al. 2001) to investigate populations of *Serapias politisii* Renz, *S. parviflora* Parl., and *S. vomeracea* (N.L. Burm.) ssp. *laxiflora* (Soò) Gölz & Reinhard (= *S. bergonii* Camus) from southern Italy and the Greek island Corfu. These three species are morphologically very similar and thus have caused considerable discordance with respect to their taxonomic rank and relationships (Baumann and Künkele 1989; Gölz and Reinhard 1993; Lorenz 2001).

We tested the potential of microsatellites or simple sequence repeat (SSRs) markers to evaluate genetic differentiation among the three selected taxa and to address taxonomic problems within *Serapias*. At the same time, we estimated the level of correspondence among genetic and phenetic markers (diagnostic floral traits), exploring whether these species show evidence for floral differentiation or reproductive isolation. Finally, by comparing populations from well-separated localities, we assessed also the importance of geography for the spatial genetic structure of these taxa.

Materials and Methods

Study Organisms

The genus *Serapias* is distributed throughout the Mediterranean region but has its center of diversity in southern Italy and on Greek islands (Baumann and Künkele 1989). The present classification of *Serapias* species is essentially based on quantitative (rather than qualitative) differences in floral traits and has remained controversial. Consequently, the number of species and subspecies recognized differs markedly among authors (Baumann and Künkele 1989; Delforge 1994; Gölz and Reinhard 1977; Nelson 1968). In the present study, we focus on three taxa with overlapping distribution areas (Figure 2) that are generally accepted and can be identified reliably (Grünanger 2001). Nonetheless, their strong resemblance and similar floral traits provide little information about their relationships (Delforge 1994; Grünanger 2001; Lorenz 2001).

S. parviflora was originally described from Sicily by Parlatore (1837) but is widely distributed in the Mediterranean region. Its taxonomic position has never been much debated. Two qualitative characters, friable yellow pollinia and the presence of a thickened ovary during blooming, are typical for *S. parviflora* (Grünanger 2001). *Serapias vomeracea* ssp. *laxiflora*, originally described as *S. bergonii* (Camus et al. 1908), is considered a subspecies of *S. vomeracea* (Gölz and Reinhard 1977), but its taxonomy is under debate (Gölz and Reinhard 1993; Grünanger 2001; Lorenz 2001). *S. vomeracea* ssp. *laxiflora* is common in the eastern Mediterranean region. Populations from southern Italy (Apulia and Sicily) are at the western limits of the species range (Delforge 1994). *S. politisii*, originally described from the Greek island Corfu (Renz 1928), has since been reported from many other Greek islands and from sites along the Ionian coast (Baumann and Künkele 1989; Kaptein den Boumeester and Willing 1988). Recently it has also been reported from southern Italy (Apulia, Salento) (Bianco et al. 1992; Delforge 1994; Grünanger 2001). *S. politisii* differs from *S. vomeracea* ssp.

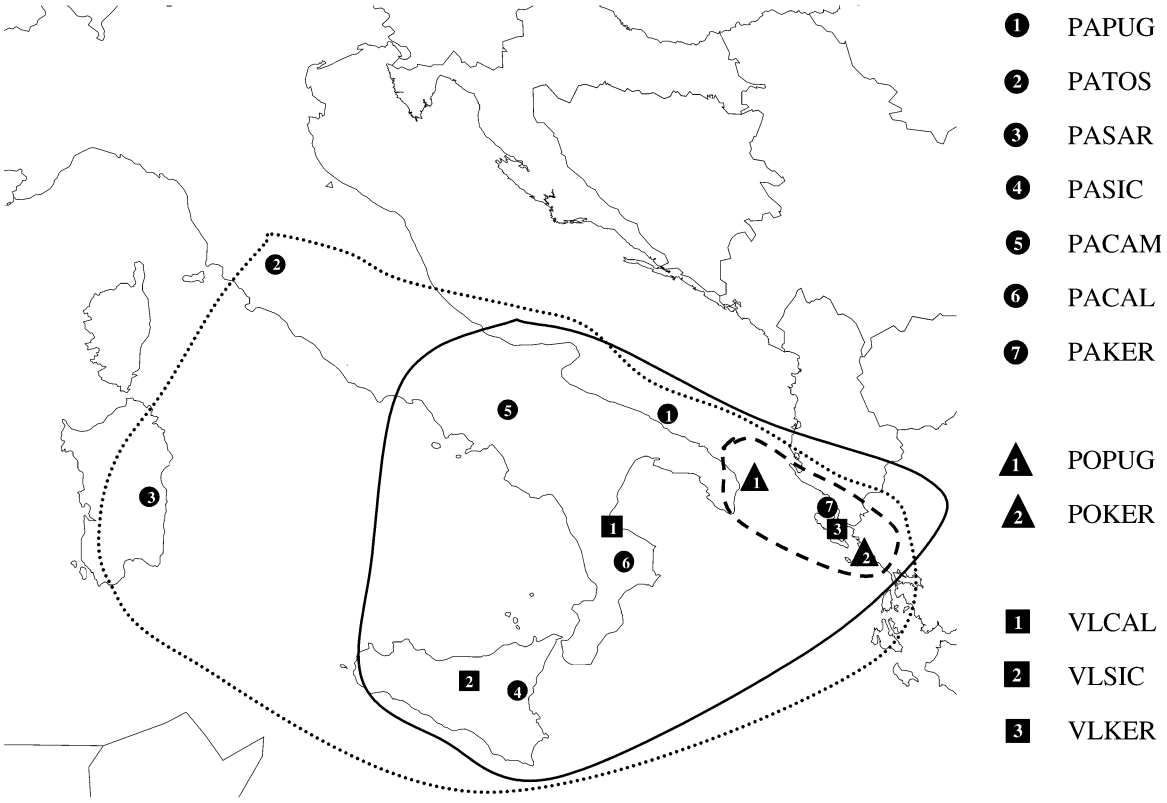

Figure 2. Map of Italian and western Greek territories with overlapping distribution areas of *S. parviflora* (dotted line; circles), *S. politisii* (dashed line; triangles) and *S. vomeracea* ssp. *laxiflora* (solid line, squares), and locations of the sampled populations. Abbreviations as in Table 1.

laxiflora in a few discriminating characters, namely, features of the petal base, the dark red-purple flowers, and the smaller epichile (10–13 × 3–5 mm) (Grünanger 2001). Close relationships between *S. parviflora* and *S. politisii* have been recently attested by karyological evidence. In fact, it has been shown that the two species produce identical banding patterns of their very similar karyotypes ($2n = 36$), whereas the karyotype of *S. vomeracea* ssp. *laxiflora* differs from that of the two other species (D'Emerico et al. 2000).

Sample Collections

Specimens of *S. politisii*, *S. parviflora*, and *S. vomeracea* ssp. *laxiflora* were collected during the flowering season from natural populations on Corfu and in central and southern Italy in spring 2000 (Table 1). We collected 30 individuals per species and population. Individuals were sampled at least 5 m apart and were otherwise collected randomly. Two, three, and seven populations of *S. politisii*, *S. vomeracea* ssp. *laxiflora*, and *S. parviflora*, respectively, were sampled. For each specimen, we stored freshly collected leaf material individually in silica gel for DNA extraction. In addition, the second and third flowers from the bottom of the inflorescence of each specimen were removed and preserved in ethanol for morphometric analysis.

Floral Traits

We measured 13 floral traits (Table 2, Figure 1) to the nearest mm on the second and third flowers from the bottom of the inflorescence of each specimen, and used the average values from these two flowers in statistical analyses. These same traits were used by Gölz and Reinhard (1993) in their morphological analysis of *Serapias* and are considered

Table 1. Origin of *Serapias politisii*, *S. parviflora*, and *S. vomeracea* ssp. *laxiflora* populations investigated in this study

Species	Population	Abbreviation
S. parviflora	Torre Canne (Apulia, Italy)	PAPUG
	Argentario (Tuscany, Italy)	PATOS
	Tuvixeddu (Sardinia, Italy)	PASAR
	Monreale (Sicily, Italy)	PASIC
	Sassano (Campania, Italy)	PACAM
	Marcellinara (Calabria, Italy)	PACAL
	Lefkimmi (Corfu, Greece)	PAKER
S. politisii	S. Cataldo (Apulia, Italy)	POPUG
	Perivoli (Corfu, Greece)	POKER
S. vomeracea ssp. *laxiflora*	Marcellinara (Calabria, Italy)	VLCAL
	Vittoria (Sicily, Italy)	VLSIC
	Liapades (Corfu, Greece)	VLKER

Table 2. Morphological characters analyzed in populations of *S. politisii*, *S. parviflora*, and *S. vomeracea* ssp. *laxiflora*, together with character coefficient for the first two principal components

No.	Character	Abbr.	PC1	PC2
1.	Length of bracts	LBR	0.231	−0.251
2.	Width of bracts	WBR	0.128	−0.217
3.	Length of ovary	LOV	0.602	−0.066
4.	Length of sepals	LSE	0.306	−0.211
5.	Width of sepals	WSE	0.598	−0.405
6.	Length of petals	LPE	−0.298	−0.142
7.	Width of petals	WPE	0.701	−0.268
8.	Length of labellum	LLA	−0.267	0.010
9.	Length of lateral lobes	LLL	−0.240	−0.155
10.	Length of epichile	LEP	0.643	−0.329
11.	Width of hypochile	WHY	−0.226	−0.209
12.	Width of base of epichile	WEB	−0.296	−0.384
13.	Maximum width of epichile	WEP	0.159	−0.397

diagnostic for species identification. Floral traits were measured to the nearest 1 mm using a ruler and were replicated on both collected flowers.

Molecular Methods

Total genomic DNA was extracted from approximately 0.5 g dried leaf material using the CTAB protocol (Doyle and Doyle 1990). We used five nuclear microsatellite loci previously developed for *S. vomeracea* (Pellegrino et al. 2001), and carried out PCR amplifications after Pellegrino et al. (2001). One of the PCR primers for each locus was 5′ labeled with fluorescent dye (FAM, TET). Labeled PCR products were run together with the internal size standard ROX-500 on an ABI 373A (Perkin Elmer, Biosystems). Individuals were genotyped using Genescan Analysis and Genotyper software (Perkin Elmer, Biosystems).

Data Analysis

Floral Traits

Ranges, means and standard deviations were estimated for each trait using DataDesk 6.1 software (Krzanowski 1997). Significant differences in structural traits at the species level were determined by analysis of variance (ANOVA), and pairwise contrasts were done with $\alpha = .05$. The data set was ordinated by principal components analysis (PCA) based on a correlation matrix by means of DataDesk 6.1 (Krzanowski 1997).

Molecular Data

We calculated population variability estimators such as allele frequencies, numbers of alleles per locus, mean allele numbers (Ao), and mean observed (Ho) and expected heterozygosities (He) across loci using POPGENE 3.2 (Yeh et al. 1997). *F* statistics were calculated using Genepop 3.2a (Raymond and Rousset 1995).

Based on allele size variations, a hierarchical analysis of molecular variance (AMOVA) among species and populations was performed using Arlequin 2.0 (Schneider et al. 2001). The computer program Gene Class, version 1.0.02, was used for assignment tests, which test how indicative an individual's multilocus genotype is of its population of origin (Cornuet et al. 1999). Gene Class employs a Bayesian approach, which derives from the sample population frequencies the probability density of population allele frequencies. Assignment tests are most typically applied at the intraspecific level, where they may be useful for describing ecogeographical diversity (Gustafsonn and Sjögren-Gulve 2002), but they can also be applied to interspecific data (Roques et al. 1999; Soliva and Widmer 2003). The allelic data set was ordinated by PCA using DataDesk 6.1 (Krzanowski 1997). We tested for isolation by distance using the Mantel test as implemented in Genepop 3.2a (Raymond and Rousset 1995).

Nei's (1972) genetic identities among populations were calculated using TFPGA 1.3 (Miller 1997), and a phenogram was constructed using the unweighted pair group method of Sneath and Sokal (1973) with arithmetic averages (UPGMA).

Results

Floral Traits

All floral traits, except width of petals (WPE), differed significantly among species (Table 3), but most traits did not differ significantly between *S. politisii* and *S. parviflora*. Exceptions were length of ovary ($F_{1,269} = 3.91$, $P < .01$), width of sepals ($F_{1,269} = 6.58$, $P < .001$), and width of petals ($F_{1,269} = 3.38$, $P < .01$). By contrast, the majority of traits differed significantly between *S. vomeracea* ssp. *laxiflora* and the two other taxa. Interestingly, the length of epichile (LEP) was significantly different from that of *S. parviflora* ($F_{1,299} = 7.43$, $P < .001$) and *S. politisii* ($F_{1,149} = 6.98$, $P < .001$).

Two instead of three groups were thus observed on the ordination diagram derived from PCA (Figure 3A). The first principal component accounted for 47.4% of the total variance, whereas the second component explained 12.2%. Examination of character coefficients (Table 2) revealed that some variables were strongly and positively correlated with PC1, including LOV, WSE, WPE, and LEP. Individuals of *S. politisii* and *S. parviflora* overlap almost entirely, whereas most *S. vomeracea* ssp. *laxiflora* individuals are well separated from the other two species. Only in a small area did two individuals of all three species overlap.

Molecular Variation

The nuclear microsatellite loci were found to be polymorphic in all populations examined, with the exception of locus SV01 that was fixed in two *S. vomeracea* ssp. *laxiflora* populations. Total numbers of alleles per locus ranged between 5 and 15. Mean allele numbers were 3.1 in *S. vomeracea* ssp. *laxiflora*, 3.5 in *S. parviflora*, and 5.2 in *S. politisii* (Table 4). Overall, 53 alleles were detected. Only 20% of all alleles were shared among the *Serapias* taxa, and allele frequencies differed within and among taxa (Table 4). *S. politisii* and *S. vomeracea* ssp. *laxiflora* exhibited more species-specific alleles than *S. parviflora* (Table 4). Observed heterozygosity was lowest in *S. vomeracea* ssp. *laxiflora*

Table 3. Mean, standard deviation, and *F*-ratio of morphological measurements for populations of *S. parviflora*, *Serapias politisii*, and *S. vomeracea* ssp. *laxiflora*.

Character	PAPUG	PATOS	PASAR	PASIC	PACAM	PACAL	PAKER	POPUG	POKER	VLCAL	VLSIC	VLKER	*F*-ratio
Length of bracts	31.50 ± 2.91	25.25 ± 1.90	30.92 ± 3.5	24.50 ± 2.97	29.72 ± 5.33	30.71 ± 5.77	28.48 ± 3.31	25.72 ± 5.73	28.00 ± 4.85	39.50 ± 2.90	32.03 ± 3.90	35.03 ± 3.47	7.03**
Width of bracts	9.10 ± 1.24	8.12 ± 0.64	8.85 ± 1.09	8.00 ± 1.06	8.86 ± 1.47	8.14 ± 1.15	8.22 ± 1.76	7.60 ± 1.52	8.00 ± 1.34	10.95 ± 0.99	9.30 ± 1.49	9.13 ± 1.36	7.58**
Length of ovary	10.67 ± 1.35	11.75 ± 2.31	15.07 ± 1.85	10.75 ± 1.66	13.36 ± 2.10	14.04 ± 2.92	15.26 ± 2.09	10.17 ± 1.68	10.10 ± 2.13	14.20 ± 2.86	11.80 ± 1.85	13.17 ± 1.86	2.42*
Length of sepals	16.03 ± 1.43	14.12 ± 0.83	15.28 ± 0.72	13.12 ± 0.64	16.80 ± 2.15	14.71 ± 1.23	14.70 ± 1.18	14.07 ± 1.89	15.14 ± 1.53	18.05 ± 1.13	17.13 ± 1.72	17.80 ± 1.34	7.98**
Width of sepals	3.63 ± 0.49	3.62 ± 0.51	3.92 ± 0.26	3.25 ± 0.46	3.43 ± 0.50	3.28 ± 0.46	3.74 ± 0.45	3.10 ± 0.40	3.07 ± 0.65	4.00 ± 0.59	3.60 ± 0.66	3.67 ± 0.65	5.50**
Length of petals	14.03 ± 1.45	12.25 ± 0.70	13.00 ± 0.55	11.50 ± 1.06	14.33 ± 2.26	12.90 ± 1.26	12.65 ± 1.61	12.47 ± 1.61	12.41 ± 1.74	15.90 ± 1.70	14.10 ± 1.15	14.53 ± 1.26	5.56**
Width of petals	3.50 ± 0.51	3.50 ± 0.53	3.28 ± 0.46	3.25 ± 0.46	3.40 ± 0.50	3.14 ± 0.35	3.30 ± 0.47	3.17 ± 0.46	3.14 ± 1.96	3.80 ± 0.64	3.10 ± 0.46	3.10 ± 0.51	0.98ns
Length of labellum	16.37 ± 2.30	13.75 ± 0.70	14.50 ± 1.34	12.00 ± 0.75	18.64 ± 2.74	15.00 ± 1.76	14.48 ± 1.44	14.47 ± 2.24	16.76 ± 2.25	24.40 ± 1.78	18.83 ± 1.84	19.73 ± 0.79	7.61**
Length of lateral lobes	5.03 ± 0.81	5.62 ± 0.51	5.82 ± 0.61	4.75 ± 0.46	5.13 ± 0.78	5.33 ± 0.57	5.35 ± 0.57	4.67 ± 0.61	5.10 ± 0.90	6.25 ± 0.98	5.56 ± 0.60	5.70 ± 0.56	4.61**
Length of epichile	9.83 ± 1.76	7.87 ± 0.35	8.28 ± 1.20	6.62 ± 0.51	12.14 ± 2.29	8.95 ± 1.11	8.39 ± 0.99	9.37 ± 2.11	10.38 ± 1.70	16.3 ± 1.45	13.43 ± 1.81	13.50 ± 0.90	12.16***
Width of hypochile	8.97 ± 1.33	9.00 ± 0.53	9.57 ± 0.51	8.62 ± 0.51	9.97 ± 1.03	9.28 ± 0.46	8.87 ± 0.81	8.40 ± 1.25	8.83 ± 0.80	12.00 ± 1.53	9.70 ± 0.83	10.07 ± 1.01	5.90**
Width of base of epichile	2.30 ± 0.53	2.21 ± 0.35	2.85 ± 0.36	2.25 ± 0.46	2.80 ± 0.48	2.57 ± 0.50	2.61 ± 0.50	2.67 ± 0.64	2.38 ± 0.49	4.21 ± 0.85	3.53 ± 0.39	3.10 ± 0.36	11.26***
Maximum width of epichile	3.27 ± 0.83	3.12 ± 0.35	3.85 ± 0.53	3.25 ± 0.46	3.96 ± 0.64	3.76 ± 0.53	3.61 ± 0.50	3.37 ± 0.76	3.45 ± 0.57	6.20 ± 1.35	4.80 ± 1.03	4.43 ± 0.62	11.21***

All lengths are in mm. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 3. PCA of *S. politisii* (x), *S. parviflora* (o), and *S. vomeracea* ssp. *laxiflora* (+) based on floral morphometric (**A**) and microsatellite allelic (**B**) data.

(ranging from .39 to .59), intermediate in *S. parviflora* (.50 to .65) and highest in *S. politisii* (.75 to .78).

F_{IS} values varied among species. Mean F_{IS} was highest in *S. parviflora* (mean $F_{IS} = .142$), medium in *S. vomeracea* ssp. *laxiflora* (mean $F_{IS} = .039$), and lowest in *S. politisii* (mean $F_{IS} = -.005$). The occurrence of inbreeding in the widespread species is in contrast with the high outcrossing level showed by the rare *S. politisii*, a result probably relying on the unpredictable behavior of the pollinators due to the peculiar pollination strategy of the genus.

Populations were genetically well differentiated. F_{ST} across all populations and loci was .229. This estimate includes differentiation among populations within species, and differentiation between species. F_{ST} values within species were lower. Across *S. vomeracea* ssp. *laxiflora* populations, F_{ST} was .141, and across *S. parviflora* populations, F_{ST} was .113.

Table 4. Allele frequencies at five nuclear microsatellite loci in *Serapias*, with mean allele numbers (A ± SD), and observed (Ho ± SD) and expected heterozygosities (He ± SD) per locus and population.

	PAPUG	PATOS	PASAR	PASIC	PACAM	PACAL	PAKER	POPUG	POKER	VLCAL	VLSIC	VLKER
Sv01												
235	0	0	0	0	0	0	0	0.375	0.375	0	0	0
237	0.250	0.437	0.438	0.500	0.375	0.312	0.438	0	0.062	0	0.625	0
239	0.375	0.437	0.187	0.250	0.125	0.250	0.312	0.188	0.188	1.000	0.375	1.000
241	0.375	0.126	0.375	0.250	0.500	0.438	0.250	0.062	0.062	0	0	0
243	0	0	0	0	0	0	0	0.312	0.312	0	0	0
Sv02												
210	0.125	0	0.312	0	0	0	0	0	0	0	0.375	0
212	0.375	0	0	0.250	0.313	0.250	0.313	0.188	0.188	0.389	0	0.437
216	0	0	0.313	0	0	0	0.062	0.562	0.438	0.111	0	0.125
218	0	0.562	0.125	0.312	0.313	0.438	0.375	0	0.125	0.333	0.562	0.250
222	0.375	0.188	0.125	0	0.062	0	0	0	0	0.111	0.623	0.125
236	0	0.062	0.125	0	0	0	0	0	0	0.056	0	0.063
238	0	0.188	0	0	0	0	0	0	0.062	0	0	0
240	0	0	0	0	0	0	0	0.188	0.062	0	0	0
242	0.125	0	0	0.438	0.312	0.312	0.250	0.062	0.125	0	0	0
Sv03												
127	0	0	0	0	0.126	0	0.188	0	0	0.389	0	0.500
129	0	0	0	0	0.437	0.563	0.312	0	0	0	0.250	0
131	0.250	0.188	0.125	0.562	0.437	0.375	0.312	0	0	0.222	0.375	0.313
133	0.500	0.437	0.500	0.250	0	0.062	0	0.375	0.250	0.389	0.375	0.187
137	0	0	0	0	0	0	0	0.125	0.312	0	0	0
139	0	0	0	0	0	0	0	0.188	0.126	0	0	0
141	0	0	0	0	0	0	0	0.250	0.312	0	0	0
143	0	0.125	0.250	0	0	0	0	0.062	0	0	0	0
145	0.250	0.250	0.125	0.188	0	0	0.188	0	0	0	0	0
Sv04												
311	0	0	0	0	0	0	0	0	0	0.056	0.125	0.250
313	0	0	0	0	0	0	0	0	0	0.112	0.188	0.375
315	0	0	0	0	0	0	0	0	0	0	0.062	0
317	0	0	0	0	0	0	0	0	0	0.833	0.625	0.375
321	0	0.375	0.250	0	0	0	0	0	0	0	0	0
325	0.375	0.313	0.250	0.312	0.312	0.188	0.062	0	0	0	0	0
329	0.500	0.062	0.312	0.250	0.500	0.624	0.375	0	0	0	0	0
331	0.125	0.250	0.188	0.438	0.188	0.188	0.563	0	0.188	0	0	0
339	0	0	0	0	0	0	0	0.312	0.061	0	0	0
341	0	0	0	0	0	0	0	0.250	0.125	0	0	0
343	0	0	0	0	0	0	0	0	0.061	0	0	0
345	0	0	0	0	0	0	0	0.312	0.125	0	0	0
347	0	0	0	0	0	0	0	0.126	0	0	0	0
349	0	0	0	0	0	0	0	0	0.125	0	0	0
351	0	0	0	0	0	0	0	0	0.310	0	0	0
Sv06												
145	0	0	0	0	0	0	0	0	0	0.555	0.188	0.437
147	0	0	0	0	0	0	0	0	0	0	0.250	0
149	0	0	0	0	0	0	0	0	0	0.388	0.312	0.438
151	0	0	0	0	0	0	0	0	0	0.056	0.250	0.125
153	0.062	0	0	0	0	0	0.250	0.062	0	0	0	0
155	0.376	0.188	0	0.062	0.125	0.374	0.250	0	0	0	0	0
157	0.250	0.375	0.125	0.250	0.312	0.188	0.250	0	0	0	0	0
159	0.062	0.062	0.312	0.188	0.250	0.125	0.062	0.125	0.062	0	0	0
161	0.250	0.062	0.125	0.375	0.125	0.125	0.188	0	0	0	0	0
163	0	0	0	0.125	0.188	0.188	0	0.062	0	0	0	0
165	0	0.313	0.438	0	0	0	0	0.312	0.125	0	0	0
167	0	0	0	0	0	0	0	0.125	0.062	0	0	0
169	0	0	0	0	0	0	0	0.062	0.125	0	0	0
171	0	0	0	0	0	0	0	0.250	0.250	0	0	0
175	0	0	0	0	0	0	0	0	0.376	0	0	0
A	3.6	4.0	4.0	3.4	3.6	3.4	3.8	5.0	5.6	3.0	3.2	3.0
± SD	0.89	0.71	0.70	0.89	0.89	0.89	0.84	1.22	1.14	1.11	0.84	0.11
Ho	0.65	0.58	0.60	0.55	0.61	0.50	0.63	0.75	0.78	0.54	0.39	0.59
± SD	0.06	0.07	0.10	0.07	0.11	0.13	0.09	0.01	0.06	0.11	0.16	0.19
He	0.68	0.69	0.71	0.67	0.68	0.65	0.70	0.74	0.77	0.45	0.61	0.53
± SD	0.06	0.06	0.07	0.06	0.08	0.09	0.09	0.07	0.04	0.20	0.10	0.18

Table 5. AMOVA based on five microsatellite loci for populations of *S. politisii*, *S. parviflora*, and *S. vomeracea* ssp. *laxiflora*, with degrees of freedom (d.f.), variance components (Va), percentages of variation (%) and probability of component being equal to zero (P)

Source of variation	d.f.	Va	%	P
Among taxa	2	0.417	18.61	<0.001
Among populations within taxa	9	0.189	8.42	<0.001
Within populations	376	1.635	72.97	<0.001

Pairwise F_{ST} between the two *S. politisii* populations was lowest ($F_{ST} = .046$). AMOVA indicated that 18.61% of the variation was distributed among species, 8.42% among populations within species, and 72.97% within populations (Table 5). No evidence for isolation by distance was found when all species and populations were included in the analysis. Among the seven *S. parviflora* populations, weak evidence for isolation by distance was found, but the correlation was only marginally significant ($P = .054$, results not shown).

In the assignment test, 143 out of 194 individuals (74%) were assigned correctly to the population of origin. The remaining 51 individuals were assigned to other populations of the same species. This was observed in particular for populations PACAM and PACAL, which were genetically very similar.

The ordination diagram derived from the PCA based on microsatellite data showed three distinct groups. The first principal component (PC1) explained 45.7% of the total variance, and axis PC2 explained 15.2% of the total variance. All individuals of a single species are well separated from the individuals of the other two species, with the exception of one individual each of *S. parviflora* and *S. politisii* (Figure 3B).

In the UPGMA dendrogram, based on Nei's genetic distances (data not shown), populations clearly grouped in three distinct, well supported clades that correspond to the three species. *S. parviflora* populations cluster in two geographically distinct groups, one including populations from southern Italy and the Corfu, and the other one including populations from Sardinia and Tuscany.

Discussion

Pollinator-mediated floral isolation occurs most likely in plant species that are visited by distinct and often specialized pollinators, although it may also occur between plant species that are visited by generalist pollinators (Grant 1994). In the latter case, floral isolation occurs when variable floral traits act as mechanical and/or ethological isolation and thus maintain or reinforce species boundaries through prezygotic mechanisms (Grant 1994; Johnson and Steiner 2000; Kephart and Theiss 2003; Ollerton 1996; Waser 1998; Waser et al. 1996).

Although many orchid species show striking variation in flower shape or color and are visited by species-specific pollinators, the situation is different in *Serapias*. These plants have a generalized pollination system in which a wide range of insect species use the flowers as shelters. Although nothing is known about specific pollen placement mechanisms, the weak differentiation in floral traits among many *Serapias* species and the low rate of speciation in the genus contrast sharply with plant lineages showing floral isolation (Grant 1949).

In the presence of generalized pollination and weak or absent floral isolation, congeneric species, although distinct in floral traits, are sometimes nearly indistinguishable in terms of neutral genetic markers, which indicates that gene flow across species boundaries does occur (Rieseberg and Soltis 1991; Wolf and Soltis 1992; Wolfe and Elisens 1994).

Our analysis of microsatellite DNA variation in *Serapias*, however, reveals clear and significant genetic differentiation between species, suggesting that they are reproductively well isolated although they have overlapping distribution areas and flowering times and often grow in sympatry. Our results therefore suggest that reproductive isolation between the investigated *Serapias* species is not primarily due to floral isolation, but may have another, as yet unidentified basis. Hence, these species represent an interesting model to investigate how reproductive isolation occurs.

The strength of differentiation between *Serapias* species contrasts with that reported for sympatric *Ophrys* species, where only 4.02% of the variation was found between sympatric species (Soliva and Widmer 2003). The latter result was taken as evidence for ongoing gene flow between sympatric species. Similar to *Ophrys*, however, where DNA sequence variation among species is typically low (Soliva et al. 2001), nrITS sequence variation (Bateman et al. 2003; Pridgeon et al. 1997) and noncoding chloroplast DNA sequence variation (Widmer and Kocyan unpublished data) are also low or absent between *Serapias* species. These results suggest that species in both groups have diverged recently and presumable rapidly. In contrast to *Ophrys*, however, where genetic differentiation as estimated with microsatellites between sympatric species of the *O. sphegodes* group is very weak, clear genetic differentiation has emerged between *Serapias* species. This result is surprising. Floral differences among *Ophrys* species are often clear and pollination in *Ophrys* has typically been considered highly species-specific (Kullenberg 1961; Schiestl et al. 1999), but molecular data suggest that pollinators are either less specific than suggested or occasionally make mistakes (Soliva and Widmer 2003). In contrast, molecular data suggest that reproductive isolation is well established among *Serapias* species, although floral traits overlap considerably (this study; Gölz and Reinhard 1993; Lorenz 2001) and pollination seems to be fairly unspecific (Dafni et al. 1981; Pellegrino et al. unpublished data).

Consequently, we suggest that other reproductive barriers most likely exist in *Serapias*. Some *Serapias* taxa are polyploids (e.g., *S. lingua*, D'Emerico et al. 2000), but polyploidy cannot explain our results because we chose diploid taxa for this study. Reproductive barriers that act postzygotically might explain the observed reproductive isolation. Giemsa C-banded karyotypes suggest that *Serapias* species are all very similar (D'Emerico et al. 2000), but genic barriers need not be visible at this level, although they may exist. Clearly, further studies including controlled pollination experiments within and between species of *Serapias* are needed.

Although differentiation is strong between *Serapias* species, it is relatively weak between conspecific populations. Given the small number of populations analyzed for the other two species, we here focus on *S. parviflora*. Assignment tests repeatedly assigned *S. parviflora* individuals to allopatric populations of the same species, indicating that populations are weakly differentiated. The analysis of isolation by distance supported this result. Although a weak effect of geographic isolation on genetic differentiation was found, this correlation was only marginally significant. Potential explanations for this weak differentiation are either a recent origin of the populations or ongoing gene flow. The former hypothesis is not very likely. The areas from which samples were collected for this study were not covered by ice during the most recent glaciation and thus have long been accessible for plants. But the most recent glaciation may nevertheless have affected the plant's population structure. Sea level changes most likely have occurred, and land bridges between western Greece and southeastern Italy may have existed (Van Andel and Tzedakis 1996). This would have allowed for more extensive gene flow in the recent evolutionary history of these populations and may explain the weak genetic differentiation observed. In addition, present gene flow may occur at a low but sufficient rate to balance the effect of genetic drift. Over large geographic distances, gene flow is most likely due to seed dispersal. Orchids have thousands of minute, dust-like seeds that are wind-dispersed and may allow gene flow over large distances, whereas pollinators have more limited flight ranges and are most likely to promote gene flow through pollen over short distances, such as between neighboring populations.

Our results have interesting implications not only for evolutionary studies on *Serapias* but also for taxonomic issues. Our microsatellites proved useful for identifying and grouping *Serapias* species and could be helpful for assessing evolutionary and thus also taxonomic relationships. In the UPGMA tree based on genetic distances calculated from microsatellite data, all *Serapias* species were resolved as monophyletic groups with relatively high bootstrap support. This dendrogram emphasizes that the main factor affecting differentiation among investigated *Serapias* populations is their taxonomy. Even the two geographically well-separated populations of *S. politisii* group together (100% bootstrap), although other *Serapias* species may be growing nearby. This result also contrasts with the situation observed in *Ophrys*, where some populations group according to their geographic provenience, rather than their taxonomy (Soliva and Widmer 2003).

Our results obtained from the morphometric data were similar to those reported previously by Gölz and Reinhard (1993) and Lorenz (2001) from the same geographic region. Samples of *S. politisii* from Italy are included for the first time in a morphometric analysis. The species was first reported from Italy by Bianco et al. (1992), after the work of Gölz and Reinhard (1977). Lorenz (2001) considered the report of *S. politisii* from Italy as erroneous and did not include samples in his analysis. Our results confirm that *S. politisii* does occur in Italy and is genetically very close to the Greek population analyzed.

Our results thus show that microsatellites are useful not only to estimate population genetic parameters but also to identify *Serapias* species. Future work on *Serapias* should thus not rely on morphometric analyses alone, because many traits overlap considerably between species, but should apply a combined approach, where morphometric and molecular microsatellite data are used in combination to address the many open questions in this fascinating orchid group.

Acknowledgments

We thank P. Grünanger and S. Cozzolino for stimulating discussions and encouragement, and A. Kocyan for valuable comments on an earlier version of the manuscript. This research was supported by a grant from the Ministero Istruzione Università e Ricerca.

References

Bateman RM, Hollingsworth PM, Preston J, Yi-Bo L, Pridgeon AM, and Chase MW, 2003. Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). Bot J Linn Soc 142:1–40.

Baumann H and Künkele S, 1989. Die Gattung *Serapias* L—eine taxonomische Übersicht. Mitteilungsblatt Arbeitskreis Heimische Orchideen Baden-Würtemberg 20:10–651.

Bianco P, D'Emerico S, Medagli P, Ruggiero I, and Liverani P, 1992. *Serapias politisii* Renz. (Orchidaceae), nuova per la Flora Italiana. Webbia 46:219–223.

Camus EG, Bergon P, and Camus A, 1908. Monographie des Orchidées de l'Europe, de l'Afrique septentrionale, de l'Asie mineure et des Provinces Russes transcaspiennes. Paris.

Cornuet JM, Pyri S, Luikart G, Estoup A, and Solignac M, 1999. New methods employing multilocus genotypes to select or exclude populations as origins of individuals. Genetics 153:1989–2000.

Dafni A, Ivri Y, and Brantjes NMB, 1981. Pollination of *Serapias vomeracea* Briq (Orchidaceae) by imitation of holes for sleeping solitary males bees (Hym). Acta Bot Neerl 30:69–73.

Delforge P, 1994. Guide des Orchidées d'Europe, d'Afrique du Nord et du Proche-Orient. Lausanne: Delachaux et Niestlé.

D'Emerico S, Pignone D, and Scrugli A, 2000. Giemsa C-banded karyotypes in *Serapias* L. (Orchidaceae). Bot J Linn Soc 133:485–492.

Doyle JJ and Doyle JL, 1990. Isolation of plant DNA from fresh tissue. Focus 12:13–15.

Dressler RL, 1993. Phylogeny and classification of the orchid family. Portland, OR: Timber Press.

Felicioli A, Strumia F, Filippi L, and Pinzauti M, 1998. Observations on the relation between orchids of the genus *Serapias* and their pollinators in an area of central Tuscany. Frustula Entomol 21:103–108.

Gölz P and Reinhard HR, 1977. Statistische Untersuchungen uber einige Arten der Orchideegattung *Serapias*. Die Orchidee 28:108–116.

Gölz P and Reinhard HR, 1993. *Serapias*—Probleme unter besonderer Berucksichtigung der Serapiasflora von Kerkira (Korfu) (1. Teil). J Eur Orch 25:1–58.

Grant V, 1949. Pollination system as isolation mechanisms in angiosperm. Evolution 3:82–97.

Grant V, 1994. Modes and origins of mechanical and ethological isolation in angiosperms. Proc Natl Acad Sci USA 91:3–10.

Grünanger P, 2001. Orchidee d'Italia. Quaderni di Botanica Ambientale Applicata 11:3–80.

Gumprecht R, 1977. Seltsame Bestaubungsvorgange bei Orchideen. Die Orchidee 28:1–23.

Gustafsson S, 2000. Patterns of genetic variation in *Gymnadenia conopsea*, the fragrant orchid. Mol Ecol 9:1863–1872.

Gustafsson S and Sjögren-Gulve P, 2002. Genetic diversity in the rare orchid, *Gymnadenia odoratissima* and a comparison with the more common congener *G. conopsea*. Conserv Genet 3:225–234.

Gustafsson S and Thorén PA, 2001. Microsatellite loci in *Gymnadenia conopsea*, the fragrant orchid. Mol Ecol Notes 1:81–82.

Hedrén M, Fay MF, and Chase MW, 2001. Amplified fragment length polymorphisms (AFLP) reveal details of polyploid evolution in *Dactylorhiza* (Orchidaceae). Am J Bot 88:1868–1880.

Johnson SD and Steiner KE, 2000. Generalization versus specialization in plant pollination systems. Trends Ecol Evol 15:140–143.

Kaptein den Boumeester D and Willing E, 1988. Aktuelle Verbreitung der Orchideen auf Kerkira (Korfu/Griechenland). Berichte Arbeitskreis Heimische Orchideen Beiheft 2:4–128.

Kephart S and Theiss K, 2003. Pollinator-mediated isolation in sympatric milkweeds (*Asclepias*): do floral morphology and insect behavior influence species boundaries?. New Phytol 161:265–277.

Krzanowski WJ, 1997. Recent trends and developments in computational multivariate analysis. Stat Comput 7:87–99.

Kullenberg B, 1961. Studies in *Ophrys* pollination. Zoologiska Bidrag från Uppsala 34:1–340.

Lorenz R, 2001. Die Gattung *Serapias* in Italien: Arten und Verbreitung. J Eur Orch 33:235–368.

Miller MP, 1997. Tools for population genetic analyses (TFPGA) 1.3: A Windows program for the analyses of allozyme and molecular population genetic data. Computer software distributed by author.

Nei M, 1972. Genetic distance between populations. Am Nat 106:283–292.

Nelson E, 1968. Monographie und Ikonographie der Orchidaceen-Gattungen *Serapias*, *Aceras*, *Loroglossum*, *Barlia*. Chernez-montreux.

Ollerton J, 1996. Reconciling ecological processes with phylogenetic patterns: the apparent paradox of plant-pollinator systems. J Ecol 84:767–769.

Parlatore F, 1837. Nova serapiadis species ex orchidarum famiglia. Giorn Sci Lett Sicil 59:66–67.

Pellegrino G, Cafasso D, Widmer A, Soliva M, Musacchio A, and Cozzolino S, 2001. Isolation and characterization of microsatellite loci from the orchid *Serapias vomeracea* (Orchidaceae) and cross-priming to other *Serapias* species. Mol Ecol Notes 1:279–280.

Pridgeon AM, Bateman RM, Cox AV, Hapeman JR, and Chase MW, 1997. Phylogenetics of Subtribe Orchidinae (Orchidoideae, Orchidaceae) based on nuclear ITS sequences. 1. Intergeneric relationships and polyphily of *Orchis sensu lato*. Lindleyana 12:89–109.

Raymond ML and Rousset F, 1995. GENEPOP (12): Population genetics software for exact tests and ecumenicism. J Hered 86:248–249.

Renz J, 1928. Zur Kenntnis der griechischen Orchideen. Feddes Rep 25:225–270.

Rieseberg LH and Soltis DE, 1991. Phylogenetic consequences of cytoplasmic gene flow in plants. Evol Trends Plants 5:65–84.

Roques S, Duchesne P, and Bernatchez L, 1999. Potential of microsatellites for individual assignment: the North Atlantic redfish (genus *Sebastes*) species complex as a case study. Mol Ecol 9:1703–1717.

Schiestl FP, Ayasse M, Paulus HF, Löfstedt C, Hansson BS, Ibarra F, and Francke W, 1999. Orchid pollination by sexual swindle. Nature 399:421–422.

Schneider S, Roessli D, and Excoffier L, 2001. Arlequin ver 2.0: A software for population genetics data analysis. Genetics and Biometry Laboratory, University of Geneva, Switzerland.

Sneath PHA and Sokal RR, 1973. Numerical taxonomy. San Francisco: WH Freeman.

Soliva M and Widmer A, 2003. Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. Evolution 57.

Soliva M, Gautschi B, Salzmann, Tenzer I, and Widmer A, 2000. Isolation and characterization of microsatellite loci in the orchid *Ophrys araneola* (Orchidaceae) and a test of cross-species amplification. Mol Ecol 9:2178–2179.

Soliva M, Kocyan A, and Widmer A, 2001. Molecular phylogenetics of the sexually deceptive orchid genus *Ophrys* (Orchidaceae) based on nuclear and chloroplast DNA sequences. Mol Phylogenet Evol 20:78–88.

Squirrel J, Hollingsworth PM, Bateman RM, Tebbitt MC, and Hollingsworth ML, 2002. Taxonomic complexity and breeding system transitions: conservation genetics of the *Epipactis leptochila* complex (Orchidaceae). Mol Ecol 11:1957–1964.

Van Andel TH and Tzedakis PC, 1996. Palaeolithic landscapes of Europe and environs, 150,000–25,000 years ago: an overview. Quat Sci Rev 15:481–500.

Van der Pijl L and Dodson CH, 1966. Orchid flowers: their pollination and evolution. Coral Gables, FL: University Miami Press.

Waser NM, Chittka L, Price MV, Williams N, and Ollerton J, 1996. Generalization in pollination systems, and why it matters. Ecology 77:279–296.

Waser NM, 1998. Pollination, angiosperm speciation, and the nature of species boundaries. Oikos 81:198–201.

Wolf PG and Soltis PS, 1992. Estimates of gene flow among populations, geographic races, and species in *Ipomopsis aggregata* complex. Genetics 130:652–662.

Wolfe AD and Elisens WJ, 1994. Nuclear ribosomal DNA restriction-site variation in *Penstemon* section *Peltanthera* (Scrophulariaceae): an evaluation of diploid hydrid speciation and evidence for introgression. Am J Bot 81:1627–1635.

Yeh FC, Young RC, Boyle TBJ, Ye ZH, and Mao JX, 1997. POPGENE, the user-friendly shareware for population genetic analysis. Molecular Biology and Biotechnology Centre, University of Alberta, Edmonton, Alberta, Canada.

Received December 11, 2003
Accepted July 21, 2004

Corresponding Editor: John M. Burke