

## The section *Pseudophrys* (*Ophrys*, Orchidaceae) in the Iberian Peninsula: a morphometric and molecular analysis

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Section *Pseudophrys* is a taxonomically critical species complex that has a history of circumscriptional uncertainty, suggesting the need for further evaluation. Its taxonomy in the Iberian Peninsula has been controversial, with treatments ranging from three species and three subspecies to ten species. Extensive observations in the field and analysis of morphological characters based on 50 populations sampled show that most of the characters used by previous authors for distinguishing the species are variable among or even within populations. In the present study, representatives from the Iberian Peninsula and Morocco were investigated using morphometric and molecular (nuclear ribosomal ITS sequences) analyses. Seventeen floral characters and, in addition, four ratios, were measured from 642 live plants belonging to 50 populations of representatives of sect. *Pseudophrys* (*Ophrys arnoldii*, *O. bilunulata*, *O. dianica*, *O. fusca* and *O. lupercalis*). To determine characters distinguishing different species and to examine their circumscription, we performed morphometric analyses on three different subsets of sect. *Pseudophrys*, one with all the currently recognized species, another containing the closely related *O. bilunulata* and *O. dianica*, and finally the populations of *O. arnoldii* and *O. lupercalis*. These populations were treated as Operational Taxonomic Units (OTUs) and studied using cluster analysis, principal component analysis (PCA) and canonical discriminant analysis (CDA); box plots of selected quantitative characters were also made. Finally, variation in nrITS sequences among eight members of sect. *Pseudophrys* was analysed phylogenetically. Our results indicate that sect. *Pseudophrys* is monophyletic and well supported. We maintain the recognition of all taxa studied except *O. arnoldii*, and do not recognize any new taxa based on our examinations of the different populations of *O. lupercalis*. © 2005 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2005, 148, 359–375.

ADDITIONAL KEYWORDS: ITS sequences – Portugal – Spain – taxonomy – variation gradient.

### INTRODUCTION

Geographical variation in organisms attracts the attention of biologists since it provides clues about evolutionary processes. Such variation may be caused by chance events, such as genetic drift or founder effects, or it may be the result of natural selection due to variation in environmental conditions. There is often speculation about how the variation among species may have come about, as once speciation is complete, any selective pressures that led to divergence

may no longer be operative. Studying geographical variation within species where evolutionary divergence may still be in progress provides an opportunity to observe the speciation process in action (Boyd, 2002).

The genus *Ophrys* has been studied by numerous authors (e.g. Godfery, 1928; Greilhuber & Ehrendorfer, 1975; Baumann & Künkele, 1986; Bianco *et al.*, 1989, 1991; Devillers & Devillers-Terschuren, 1994; Arnold, 1999; Paulus, 2001; Bernardos, Amich & Gallego, 2003), all of whom have contributed to an understanding of the evolution of this group. Most recently, molecular analysis (cpDNA and ITS sequencing) has

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also been performed (e.g. Bateman, Pridgeon & Chase, 1997; Pridgeon *et al.*, 1997; Soliva, Kocyan & Widmer, 2001; Bateman *et al.*, 2003; S. Bernardos, M.A. Santos, J.L. Revuelta, F. del Ray, D. Tyteca & F. Amich, unpubl. data).

However, the studies indicated above included very few species of sect. *Pseudophrys* and have not elucidated the evolutionary processes underlying its reproductive and morphological diversity. Research within the Iberian Peninsula, considered one of the major European Pleistocene refugia (Comes & Kadereit, 1998), should help provide insight into these processes.

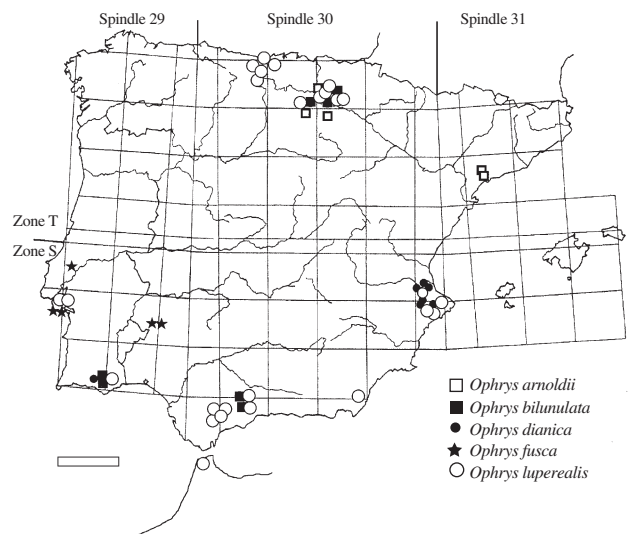
Sect. *Pseudophrys* Godfrey (genus *Ophrys* L., Orchidaceae) comprises several taxonomically critical species complexes, which show different patterns of karyological variation, at least in the Central Western Mediterranean (Greilhuber & Ehrendorfer, 1975; Bernardos *et al.*, 2003). It includes several closely related diploid and tetraploid taxa distributed throughout most of the Mediterranean Basin (Delforge, 2002). The section is represented in the Iberian Peninsula by between three (Aldasoro & Sáez, 2005) and ten species (Delforge, 2002). These taxa are quite difficult to distinguish morphologically, and several were not described until recently (Devillers & Devillers-Terschuren, 1994; Delforge, 1999; Lowe, Piera & Crespo, 2001; Tyteca, Benito Ayuso & Walravens, 2003). Misidentifications are therefore common (e.g. Lowe *et al.*, 2001; Galán & Gamarra, 2003). To date, only a few detailed morphological/circumscriptional studies have been made.

In this paper we present a combined morphometric and molecular study focusing on the Iberian representatives of sect. *Pseudophrys*, based on extensive sampling of all taxa throughout their respective ranges. Emphasis was put on: (1) exploring morphological variation within and among populations; (2) resolving the taxonomic position of populations ascribed to the critical taxa *Ophrys arnoldii* and *O. dianica*, and (3) testing the monophyly of sect. *Pseudophrys*.

## MATERIAL AND METHODS

### TAXONOMIC SAMPLING

Fifty populations belonging to five putative species of sect. *Pseudophrys* were sampled for morphometric data. The study populations were situated in several provinces of Portugal, Spain and Morocco (Table 1, Fig. 1) in order to eliminate noise from clinal variation. Each population sample consisted of 12–32 individuals (642 in total); as some populations were small, fewer individuals (7–10) were sampled. Twenty-six populations were sampled for *Ophrys lupercalis* (represented by 368 plants), seven for *O. dianica* (81 plants), seven for *O. bilunulata* (65 plants), five for *O. fusca* (48 plants) and five for *O. arnoldii* (80 plants).



**Figure 1.** Map showing distribution of the study populations of sect. *Pseudophrys* in Morocco and the Iberian Peninsula. Scale bar = 150 km.

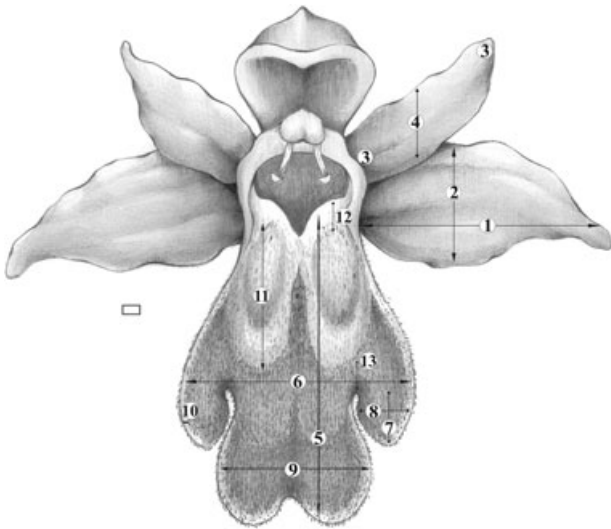
The states of 17 exomorphological characters (variables) and four ratios were recorded and coded. Vouchers for each population were prepared in the field and typically deposited at SALA. The data matrix used in this study is available on request from S. B.

Eight species of sect. *Pseudophrys* were sampled for sequencing (Table 2). To the three ITS sequences analysed by S. Bernardos, M.A. Santos, J.L. Revuelta, F. del Ray, D. Tyteca & F. Amich (unpubl. data) a further five were added during the present study. *Epipactis duriensis* (Epidendroideae), *Spiranthes spiralis* (Orchidoideae: Cranichideae) and *Disa uniflora* (Orchidoideae: Diseae) were chosen as the outgroup (GenBank accession numbers AY351377 and AY364888, deposited by Bernardos *et al.*, 2003; AJ000112, Douzery *et al.*, 1999, respectively), and *Anacamptis pyramidalis*, *O. apifera* and *O. scolopax* (Orchidoideae: Orchideae) (GenBank accession numbers AY364870, AY364887 and AY364876, deposited by Bernardos *et al.*, 2003) were included in the ingroup.

A Garmin e-map GPS was used for geographically locating the populations using  $1 \times 1$  km UTM coordinates. A representative voucher specimen from each population was collected and deposited at SALA.

### MORPHOMETRIC ANALYSES

The characters selected (Table 3, Fig. 2) include variables of continuous variation (characters 1–13), two-state characters (14, 15) and multistate discontinuous characters (16, 17). In addition, four ratios were computed (Table 3: characters 18–21). The criteria used for the multistate discontinuous characters are as follows. Hairiness of labellum: slight (hirtellous, 1), mod-



**Figure 2.** Quantitative morphological characters examined in this study (see Table 3). Scale bar = 1 cm.

erate (pubescent, 2) and great (strongly pubescent, 3); protuberances: little developed (1), moderately developed (2), and well developed (3).

Morphological characters were measured in the field from the most recently opened flower on the stem. All measurements were taken using an electronic digital rule which allowed precision, especially with respect to labellum characteristics. Only well-developed plants without missing values were considered. The characters evaluated in this study were selected following some previous studies in sect. *Pseudophrys* (Arnold, 1999; Lowe *et al.*, 2001) and our initial field observations, aiming to include the characters that might have discriminatory value.

The analyses were performed in three steps: (1) all the Iberian material of sect. *Pseudophrys* was evaluated in order to reveal the overall pattern of variation; (2) samples of *O. bilunulata* and *O. dianica* were compared in greater detail; (3) samples of *O. arnoldii* and *O. lupercalis* were analysed independently to evaluate the variation among populations.

Cluster analysis (UPGMA, unweighted pair-group method using arithmetic averages; Everitt, 1986), principal components analysis based on a correlation

**Table 1.** Material used for the morphological study of sect. *Pseudophrys*. Abbreviations: MO, Morocco; PO, Portugal; SPA, Spain. Collectors: FA, F. Amich; SB, S. Bernardos; JFD, J. Fernández-Díez; DT, D. Tyteca. *N*, number of individuals in the sample

Number of populations/Taxon/localities, collectors and voucher at SALA	<i>N</i>
<i>Ophrys arnoldii</i> P. Delforge	
01 SPA. Burgos: Villafranca-Montes de Oca, 960 m, 30TVM7491, 31.v.2004, FA & SB (SALA 108556)	15
02 SPA. La Rioja: Ezcaray, 875 m, 30TVM9886, 31.v.2004, FA & SB (SALA 108559)	15
03 SPA. La Rioja: Puerto de Herrera, 875 m, 30TWN2815, 22.v.2004, FA, SB & DT (SALA 108526)	8
04 SPA. Tarragona: Mont Ral, 805 m, 31TCF4172, 30.v.2004, FA & SB (SALA 108570)	22
05 SPA. Tarragona: La Febro-Vilaplana, 975 m, 31TCF3786, 30.v.2004, FA & SB (SALA 108567)	20
<i>O. bilunulata</i> Risso	
06 PO. Algarve: between Prego and Moreno, 320 m, 29SNB7621, 28.iii.2003, FA & SB (SALA 108516)	10
07 PO. Algarve: Morgado, 285 m, 29SNB9311, 28.iii.2003, FA & SB (SALA 108520)	7
08 SPA. Álava: Labastida, 480 m, 30TWN1516, 3.iv.2003, FA & SB (SALA 108535)	10
09 SPA. Burgos: Bugedo, 575 m, 30TVN9820, 3.iv.2003, FA & SB (SALA 108538)	10
10 SPA. La Rioja: Villalba de Rioja, 525 m, 30TWN0816, 3.iv.2003, FA & SB (SALA 108541)	9
11 SPA. Málaga: Antequera, 780 m, 30SUF6693, 26.iii.2003, FA & SB (SALA 108531)	9
12 SPA. Málaga: Casabermeja, 640 m, 30SUF7183, 27.iii.2003, FA & SB (SALA 108558)	10
<i>O. dianica</i> M.R. Lowe, Piera, M.B. Crespo & J.E. Arnold	
13 PO. Algarve: Boliqueime, 145 m, 29SNB7212, 28.iii.2003, FA & SB (SALA 108519)	11
14 SPA. Alicante: between Gata and Líber, 125 m, 31SBC4394, 4.iii.2004, FA & SB (SALA 108508)	14
15 SPA. Alicante: Coll de Rates, 630 m, 30SYH5590, 4.iii.2004, FA & SB (SALA 108507)	8
16 SPA. Alicante: Líber, Jalón, 280 m, 31SBC3990, 4.iii.2004, FA & SB (SALA 108511)	10
17 SPA. Valencia: Pla de Corral, 180 m, 30SYJ2723, 4.iii.2004, FA & SB (SALA 108524)	20
18 SPA. Valencia: Quatretonda, 240 m, 30SYJ2719, 4.iii.2004, FA & SB (SALA 108523)	12
19 SPA. Valencia: Xátiva, 170 m, 30SYJ1417, 28.ii.2003, FA & SB (SALA 108509)	6
<i>O. fusca</i> Link	
20 PO. Estremadura: Vila Nogueira, 145 m, 29SNB0163, 29.iii.2003, FA & SB (SALA 108540)	10
21 PO. Estremadura: Pragança, Montejunto, 320 m, 29SMD9638, 30.iii.2003, FA & SB (SALA 108527)	8

**Table 1.** *Continued*

Number of populations/Taxon/localities, collectors and voucher at SALA	<i>N</i>
22 PO. Estremadura: Sesimbra, 170 m, 29SMC9056, 29.iii.2003, FA & SB (SALA 108515)	11
23 SPA. Badajoz: Alconera, 555 m, 29SQC1951, 26.iii.2003, FA & SB (SALA 108542)	7
24 SPA. Badajoz: Los Santos de Maimona, 590 m, 29SQC3059, 26.iii.2003, FA & SB (SALA 108536)	12
<i>O. lupercalis</i> Devillers-Tersch. & Devillers	
25 MO. Tetouan: Zinat, 565 m, 30STE8609, 27.iii.2004, FA, SB & JFD (SALA 108522)	12
26 PO. Algarve: between Prego and Moreno, 325 m, 29SNB7622, 28.iii.2003, FA & SB (SALA 108517)	11
27 PO. Estremadura: Sesimbra, 170 m, 29SMC9056, 29.iii.2003, FA & SB (SALA 108514)	10
28 PO. Estremadura: Serra de Arrábida, 130 m, 29SMC0560, 29.iii.2003, FA & SB (SALA 108513)	11
29 SPA. Álava: Alegría, 570 m, 30TWN4042, 4.iv.2003, FA & SB (SALA 108532)	10
30 SPA. Álava: Lanciego, 520 m, 30TWN4010, 3.iv.2003, FA & SB (SALA 108529)	10
31 SPA. Alicante: Beniarrés, 515 m, 30SYJ2701, 2.iii.2003, FA & SB (SALA 108506)	15
32 SPA. Alicante: between Gata and Llíber, 125 m, 31SBC4394, 4.iii.2004, FA & SB (SALA 108512)	10
33 SPA. Alicante: near Coll de Rates, 540 m, 30SYH5288, 4.iii.2004, FA & SB (SALA 108549)	11
34 SPA. Alicante: La Nucia, 180 m, 30SYH5277, 1.iii.2003, FA & SB (SALA 108510)	7
35 SPA. Almería: Sorbas, 285 m, 30SWG8511, 2.iii.2003, FA & SB (SALA 108553)	20
36 SPA. Asturias: Arena de Cabrales, 225 m, 30TUN9156, 13.iv.2004, FA & SB (SALA 108560)	17
37 SPA. Asturias: Panes, 65 m, 30TUN7398, 13.iv.2004, FA & SB (SALA 108546)	14
38 SPA. Burgos: Buggedo, 575 m, 30TVN9820, 3.iv.2003, FA & SB (SALA 108534)	8
39 SPA. Cádiz: Benamahoma, 450 m, 30STF7971, 6.iii.2004, FA & SB (SALA 108550)	12
40 SPA. Cádiz: Grazalema, Pinsapar, 850 m, 30STF8871, 10.iv.2003, FA (SALA 108521)	10
41 SPA. La Rioja: Casalareina, 510 m, 30TWN0510, 3.iv.2003, FA & SB (SALA 108528)	10
42 SPA. La Rioja: Cidamón, 600 m, 30TWN1005, 12.iv.2004, FA & SB (SALA 108525)	24
43 SPA. León: Desfiladero de La Hermida, 215 m, 30TUN7187, 14.iv.2004, FA & SB (SALA 108545)	32
44 SPA. León: Potes, Naroba, 360 m, 30TUN6775, 14.iv.2004, FA & SB (SALA 108544)	19
45 SPA. Málaga: Antequera, 780 m, 30SUF6693, 26.iii.2003, FA & SB (SALA 108530)	10
46 SPA. Málaga: near Ronda, 760 m, 30STF9673, 6.iii.2004, FA & SB (SALA 108548)	16
47 SPA. Málaga: Casabermeja, 660 m, 30SUF7183, 5.iii.2004, FA & SB (SALA 108554)	31
48 SPA. Málaga: Mijas, 480 m, 30SUF5552, 5.iii.2004, FA & SB (SALA 108551)	12
49 SPA. Navarra: Torres del Río, 500 m, 30TWN5811, 3.iv.2003, FA & SB (SALA 108537)	10
50 SPA. Santander: Cotera, 210 m, 30TUN8591, 13.iv.2004, FA & SB (SALA 108547)	15

matrix (PCA; Sneath & Sokal, 1973; Krzanowski, 1990) and canonical discriminant analysis (CDA; Klecka, 1980) were performed using populations characterized by mean values as operational taxonomic units (OTUs, i.e. objects). Prior to clustering, data were standardized by zero mean and unit standard deviation, and City-block (Manhattan) distances were used for computing pairwise similarities between OTUs. Descriptive statistical parameters of the measured characters (mean, median, standard deviation and percentiles) for all populations were also computed, and those of most discriminating characters are presented in the form of box-plots. Morphometric analyses were performed using SPSS v.10.0 (SPSS, 1999) and STATISTICA v. 6.0 (StatSoft, 2001).

#### ITS ANALYSES, DNA EXTRACTION, PCR AND SEQUENCING

Total genomic DNA was isolated from fresh and silica dried leaf samples, according to the 2 × CTAB protocol

of Doyle & Doyle (1987). Some of the ITS sequences used in our analysis had been previously published (S. Bernardos, M.A. Santos, J.L. Revuelta, F. del Ray, D. Tyteca & F. Amich, unpubl. data). New sequences were amplified and sequenced using the same primers as previously (ITS4 and ITS5, White *et al.*, 1990) and following the procedures used by Bernardos *et al.* (2004).

#### SEQUENCE ALIGNMENT AND PHYLOGENETIC RECONSTRUCTION

Sequences were aligned with CLUSTAL W (Thompson, Higgins & Gibson, 1994) and visually adjusted as necessary, following the guidelines of Kelchner (2000). All ITS sequences analysed in this study were deposited in GenBank under the accession numbers provided in Table 2. The aligned matrix is available on request from S. B.

Prior to phylogenetic reconstruction we tested for homogeneity of base frequencies among taxa using the

**Table 2.** Voucher information and GenBank numbers for the 14 taxon ITS sequences

Species	Accession no. (GenBank)	Collection location	Collectors and voucher
Ingroup			
<i>Anacamptis pyramidalis</i> (L.) Rich.	AY364870	–	Deposited by Bernardos <i>et al.</i> (2003)
<i>Ophrys apifera</i> Huds.	AY364887	–	Deposited by Bernardos <i>et al.</i> (2003)
<i>O. arnoldii</i> P. Delforge	AY699952	SPAIN. Burgos: Villafranca, 960 m, 30TVM7491, 31.v.2004	<i>Amich &amp; Bernardos s.n.</i> (SALA 108556)
<i>O. bilunulata</i> Risso	AY699963	SPAIN. La Rioja: Villalba de Rioja, 525 m, 30TWN0816, 3.iv.2003	<i>Amich &amp; Bernardos s.n.</i> (SALA 108541)
<i>O. dianica</i> M. R. Lowe, Piera, M. B. Crespo & J. E. Arnold	AY699955	SPAIN. Valencia: Quatretonda, 240 m, 30SYJ2719, 4.iii.2004	<i>Amich &amp; Bernardos s.n.</i> (SALA 108523)
<i>O. dyris</i> Maire	AY364874	–	Deposited by Bernardos <i>et al.</i> (2003)
<i>O. fusca</i> Link	AY364875	–	Deposited by Bernardos <i>et al.</i> (2003)
<i>O. lupercalis</i> Devillers-Tersch. & Devillers	AY699962	SPAIN. Málaga: Casabermeja, 660 m, 30SUF7183, 5.iii.2001	<i>Amich &amp; Bernardos s.n.</i> (SALA 108554)
<i>O. lutea</i> Cav.	AY699953	SPAIN. La Rioja: Ezcaray, 875 m, 30TVM9886, 23.v.2004	<i>Amich &amp; Bernardos s.n.</i> (SALA 108557)
<i>O. scolopax</i> Cav.	AY364876	–	Deposited by Bernardos <i>et al.</i> (2003)
<i>O. vasconica</i> (O. Danesch & E. Danesch) P. Delforge	AY364878	–	Deposited by Bernardos <i>et al.</i> (2003)
Outgroup			
<i>Epipactis duriensis</i> Bernardos, D. Tyteca, Revuelta & Amich	AY351377	–	Deposited by Bernardos <i>et al.</i> (2003)
<i>Disa uniflora</i> Berg.	AJ000112	–	Deposited by Douzery <i>et al.</i> (1999)
<i>Spiranthes spiralis</i> (L.) Chev.	AY364888	–	Deposited by Bernardos <i>et al.</i> (2003)

chi-square test as implemented in PAUP\* 4.0b10 (Swofford, 2002), which ignores correlation due to phylogenetic structure. All reconstruction was performed with PAUP. We performed maximum parsimony (MP) (Fitch, 1971) and maximum likelihood (ML) reconstructions. Parsimony reconstruction was conducted assuming unordered characters states and equal character weighting. Gaps were treated as a 5th character state (Giribet & Wheeler, 1999; Simmons & Ochoterena, 2000; Simmons, Ochoterena & Carr, 2001). Parsimony analyses were completed with heuristic searches, with random addition of taxa for ten replications and tree bisection-reconnection (TBR) branch swapping. Branches were collapsed if maximum

length was zero. Consistency index (CI, excluding uninformative characters) (Kluge & Farris, 1969; Sanderson & Donoghue, 1989), retention index (RI) (Farris, 1989) and rescaled consistency index (RC) (Farris, 1989) were computed to assess homoplasy. The ML tree was calculated with the parameter estimates obtained under the Hasegawa–Kishino–Yano model of sequence evolution (Hasegawa, Kishino & Yano, 1985). A heuristic search was made with ten replicates of random stepwise addition and nearest-neighbour interchange (NNI) branch swapping.

Relative branch support was evaluated with 1000 bootstrap pseudoreplicates. More pseudoreplicates are preferable for larger analyses because increasing the

**Table 3.** Morphological characters used in morphometric analyses of *Ophrys* sect. *Pseudophrys* in the Iberian Peninsula

Morphological character	Character states
1 Length of sepals (LS)	(mm)
2 Width of sepals (WS)	(mm)
3 Length of petals (LP)	(mm)
4 Width of petals (WP)	(mm)
5 Length of labellum (LL)	(mm)
6 Width of labellum (WL)	(mm)
7 Length of lateral lobe of labellum (LLL)	(mm)
8 Width of lateral lobe of labellum (WLL)	(mm)
9 Width of central lobe of labellum (WCL)	(mm)
10 Width of yellowish margin (WYM)	(mm)
11 Length of speculum (LSP)	(mm)
12 Distance base speculum to throat (SPT)	(mm)
13 Distance base speculum to sinus (SPS)	(mm)
14 Convexity of labellum at base (CLB)	no (1), yes (2)
15 Convexity of labellum at apex (CLA)	no (1), yes (2)
16 Hairiness of labellum (HL)	hirtellous (1), pubescent (2), strongly pubescent (3)
17 Protuberances (PRO)	little developed (1), moderately developed (2), well developed (3)
18 Ratio length/width of labellum (LL/WL)	
19 Ratio length of labellum/length of lateral lobe (LL/LLL)	
20 Ratio length of labellum/length of speculum (LL/LSP)	
21 Ratio width of labellum/length of speculum (WL/LSP)	

number of replicates gives more consistent results (Mort *et al.*, 2000; heuristic search with random sequence addition for ten replicates, TBR branch swapping) for MP and 100 replicates for ML analysis (heuristic search, random addition of taxa with ten replicates, NNI branch swapping). Levels of bootstrap support for particular clades in Figures 9 and 10 are summarized as poor (< 50%), weak (50–74%), moderate (75–84%) and strong (> 85%), in accordance with Zomlefer *et al.* (2003). Nucleotide frequencies, the proportion of invariable sites, and the transition/transversion ratio were estimated via ML.

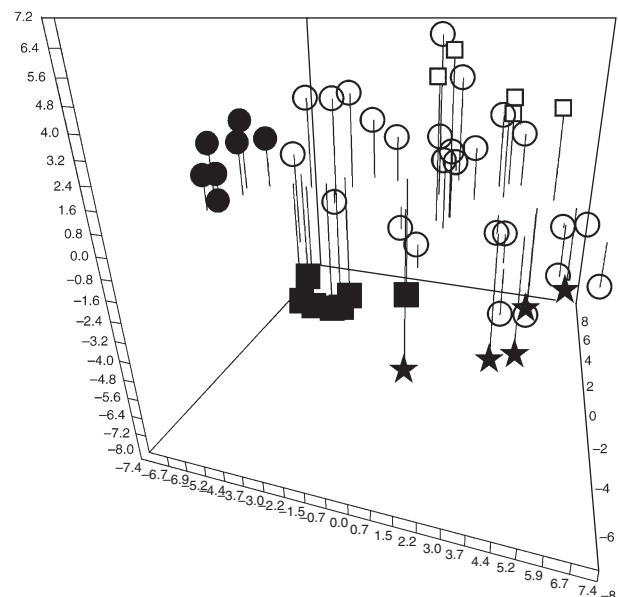
Confidence in the phylogenetic signal for this molecular data set was assessed with a permutation-tailed-probability (PTP) test conducted (MP settings identical to those described above; randomized ingroup taxa only) as suggested by Faith & Cranston (1991), with 100 replicates.

## RESULTS

### MORPHOMETRIC ANALYSES

#### *Iberian representatives of Pseudophrys*

Table 4 shows the values obtained for the basic statistical parameters defining each species. Both the PCA (Fig. 3) and cluster analysis (Fig. 4) performed on all



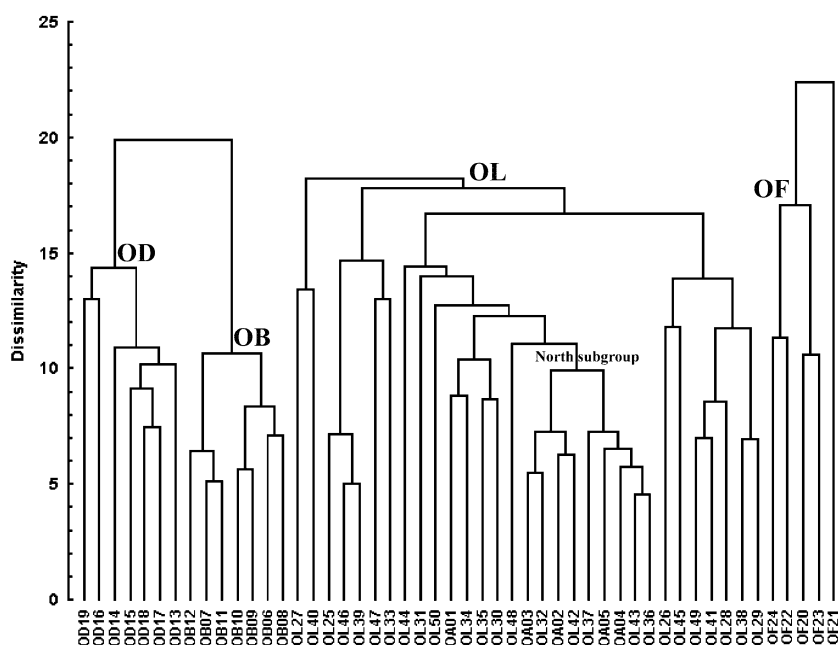
**Figure 3.** Principal components analysis of Iberian and Moroccan populations of sect. *Pseudophrys* based on 21 morphological characters (see Table 5). (□) *Ophrys arnoldii*; (■) *O. bilunulata*; (●), *O. dianica*; (★) *O. fusca*; (○) *O. lupercalis*. Axes 1–3 explain 38.77, 31.12 and 12.72% of the total variation, respectively.

**Table 4.** Summary statistics for 17 characters examined in *Ophrys arnoldii* (OA), *O. bilunulata* (OB), *O. dianica* (OD), *O. fusca* (OF), *O. lupercalis* (OL)

Character	Taxa	Mean	Median	Standard deviation	10–90 percentile	Character	Taxa	Mean	Median	Standard deviation	10–90 percentile
LS	OA	12.12	12.30	1.03	10.81–13.51	WYM	OA	0.03	0.02	0.03	0.00–0.08
	OB	8.85	8.73	0.55	8.26–9.62		OB	0.53	0.52	0.14	0.33–0.70
	OD	9.42	9.30	0.67	8.72–10.43		OD	0.55	0.48	0.20	0.33–0.80
	OF	14.06	14.35	0.93	12.43–14.65		OF	0.49	0.50	0.32	0.16–0.86
	OL	12.03	12.11	0.84	10.85–13.05		OL	0.20	0.16	0.15	0.02–0.36
	OA	6.53	6.64	0.52	5.72–7.06		OA	7.64	7.57	0.42	7.15–8.16
WS	OB	4.95	4.83	0.41	4.41–5.58	OB	7.22	7.28	0.20	6.89–7.47	
	OD	5.04	5.27	0.51	4.22–5.58	OD	5.36	5.28	0.33	4.96–5.92	
	OF	8.00	8.09	0.58	7.21–8.63	OF	10.55	10.83	0.55	9.62–10.93	
	OL	6.53	6.45	0.56	6.01–7.35	OL	7.87	7.93	0.74	6.84–8.73	
	OA	7.77	7.90	0.45	6.98–8.11	OA	1.86	1.97	0.24	1.55–2.08	
	OB	6.05	5.97	0.41	5.57–6.65	OB	0.23	0.20	0.09	0.12–0.35	
LP	OD	6.19	6.18	0.49	5.36–6.88	OD	1.39	1.49	0.31	0.87–1.76	
	OF	10.35	9.91	0.96	9.55–11.90	OF	0.74	0.67	0.56	0.00–1.43	
	OL	8.26	8.31	0.61	7.43–9.18	OL	1.78	1.81	0.42	1.22–2.33	
	OA	2.44	2.49	0.16	2.18–2.58	OA	0.52	0.57	0.19	0.28–0.75	
	OB	2.00	1.97	0.25	1.69–2.36	OB	1.38	1.47	0.38	0.93–1.83	
	OD	1.96	1.97	0.35	1.32–2.41	OD	0.87	0.98	0.32	0.22–1.18	
WP	OF	3.36	3.15	0.43	2.98–3.94	OF	1.45	1.16	0.56	0.98–2.38	
	OL	2.58	2.59	0.31	2.03–2.99	OL	0.74	0.79	0.23	0.45–0.98	
	OA	2.44	2.49	0.16	2.18–2.58	OA	0.52	0.57	0.19	0.28–0.75	
	OB	2.00	1.97	0.25	1.69–2.36	OB	1.38	1.47	0.38	0.93–1.83	
OD	1.96	1.97	0.35	1.32–2.41	OD	0.87	0.98	0.32	0.22–1.18		
OF	3.36	3.15	0.43	2.98–3.94	OF	1.45	1.16	0.56	0.98–2.38		
OL	2.58	2.59	0.31	2.03–2.99	OL	0.74	0.79	0.23	0.45–0.98		

Table 4. *Continued*

Character	Taxa	Mean	Median	Standard deviation	10–90 percentile	Character	Taxa	Mean	Median	Standard deviation	10–90 percentile
LL	OA	14.78	14.49	0.73	13.97–15.82	CLB	OA	1.30	1.31	0.05	1.23–1.36
	OB	12.04	11.60	0.39	11.60–12.63		OB	1.26	1.26	0.02	1.23–1.30
	OD	10.80	10.82	0.64	9.86–11.72		OD	1.18	1.19	0.03	1.14–1.22
	OF	18.60	18.90	1.56	16.18–20.51		OF	1.25	1.24	0.04	1.19–1.29
	OL	15.09	15.24	1.05	13.66–16.25		OL	1.26	1.26	0.05	1.21–1.31
WL	OA	11.42	11.44	0.29	11.05–11.73	CLA	OA	1.39	1.37	0.03	1.36–1.47
	OB	9.53	9.46	0.18	9.32–9.77		OB	1.27	1.27	0.03	1.23–1.32
	OD	9.16	9.12	0.47	8.61–9.86		OD	1.36	1.37	0.06	1.26–1.46
	OF	14.95	14.71	1.32	13.09–16.48		OF	1.33	1.33	0.07	1.23–1.41
	OL	11.99	11.77	0.87	10.97–13.28		OL	1.38	1.38	0.06	1.30–1.44
LLL	OA	3.05	3.10	0.19	2.75–3.24	HL	OA	1.96	1.96	0.03	1.93–2.01
	OB	2.59	2.58	0.08	2.48–2.72		OB	1.68	1.69	0.06	1.59–1.75
	OD	2.37	2.36	0.19	2.10–2.62		OD	2.05	2.05	0.07	1.92–2.13
	OF	3.97	4.03	0.43	3.28–4.38		OF	1.77	1.75	0.10	1.68–1.91
	OL	3.29	3.21	0.47	2.82–3.71		OL	1.94	1.93	0.08	1.83–2.07
WLL	OA	7.19	7.26	0.21	6.88–7.42	PRO	OA	1.52	1.48	0.08	1.45–1.63
	OB	5.64	5.65	0.28	5.34–6.15		OB	1.31	1.32	0.09	1.12–1.38
	OD	5.66	5.75	0.56	4.82–6.38		OD	1.69	1.72	0.16	1.36–1.87
	OF	10.04	9.84	0.67	9.14–10.79		OF	1.42	1.36	0.10	1.33–1.54
	OL	7.37	7.32	0.96	6.39–8.68		OL	1.54	1.52	0.10	1.44–1.69
WCL	OA	10.67	10.56	0.39	10.27–11.08		OA	1.52	1.48	0.08	1.45–1.63
	OB	9.47	9.25	0.43	9.00–10.07		OB	1.31	1.32	0.09	1.12–1.38
	OD	7.98	8.05	0.71	7.00–9.00		OD	1.69	1.72	0.16	1.36–1.87
	OF	14.01	13.66	0.96	13.05–15.46		OF	1.42	1.36	0.10	1.33–1.54
	OL	11.02	10.78	0.90	9.95–12.44		OL	1.54	1.52	0.10	1.44–1.69



**Figure 4.** Cluster analysis of Iberian and Moroccan populations of sect. *Pseudophrys* based on 21 morphological characters (see Table 5). Samples are numbered as in Table 1.

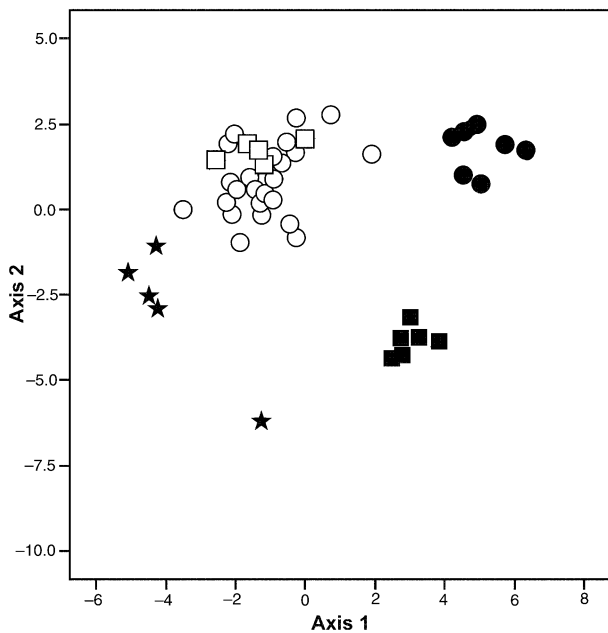
the Iberian material gave similar results. Four main groups, corresponding to *O. bilunulata*, *O. dianica*, *O. fusca* and *O. lupercalis* as previously classified, were resolved. The three former taxa were clearly separated along the first axis in the ordination graph, which represents 38.77% of the total variation among populations. *O. lupercalis* is separated from the others along the second and the third axes (representing 31.12% and 12.72% of the total variation, respectively). The populations previously identified as *O. arnoldii* (population numbers OA1–OA5, see Table 1) were placed among samples of *O. lupercalis*. The majority of the quantitative floral characteristics (excluding ratios) contributed almost equally to division along the first axis, as seen from the eigenvector values (Table 5).

Canonical discriminant analysis (CDA, Fig. 5) based on four groups resulting from PCA and cluster analysis shows that there is enough information in quantitative characters for clear and unequivocal separation of all taxa. The first canonical axis (49.41% of variation) is most closely correlated with quantitative characters LL, LSP, WL and WCL (Table 6), the second (29.00% of variation) with SPT, SPS, LSP and WYM (Table 6).

In order to show the variability of the most discriminant characters, we have performed graphic tests (box-plots) for median comparisons (Fig. 6). LL and SPT produce efficient discrimination among the species.

**Table 5.** Eigenvectors expressing correlation of characters with principal components (axes 1–3) in morphometric analysis of Iberian populations of *Ophrys arnoldii*, *O. bilunulata*, *O. dianica*, *O. fusca* and *O. lupercalis*

Character	Axis 1	Axis 2	Axis 3
LS	0.941	0.259	0.144
WS	0.969	0.072	0.128
LP	0.981	0.044	0.142
WP	0.964	-0.102	0.123
LL	0.961	0.045	0.263
WL	0.983	-0.024	0.148
LLL	0.939	0.088	0.245
WLL	0.980	-0.107	0.098
WCL	0.956	-0.135	0.255
WYM	-0.405	-0.882	-0.069
LSP	0.898	-0.136	0.412
SPT	0.175	0.737	-0.587
SPS	-0.074	-0.906	0.276
CLB	0.460	0.343	0.754
CLA	-0.137	0.951	-0.050
HL	-0.400	0.559	-0.716
PRO	-0.414	0.303	-0.850
Ratio LL/WL	-0.267	0.603	0.096
Ratio LL/LLL	0.686	0.328	0.019
Ratio LL/LS	0.924	-0.238	-0.081
Ratio WL/LS	-0.669	0.055	-0.183



**Figure 5.** Canonical discriminant analysis of Iberian and Moroccan *Ophrys* populations, based on 21 morphological characters (see Table 6). Symbols per Fig. 3. Axes 1 and 2 explain 49.41 and 29.00% of the total variation, respectively.

#### Iberian *O. bilunulata* and *O. dianica*

In the PCA of individual plants, two groups corresponding to the two species were clearly separated along the first axis in the ordination graph, which represents 55.44% of the total variation among samples; very few specimens overlapped (Fig. 7). Floral quantitative characters showed the highest correlation with the first axis (Table 7).

#### Iberian Peninsula and Moroccan representatives of *O. lupercalis*

The PCA of *O. lupercalis* (including *O. arnoldii*) populations revealed a virtually complete overlap between taxa (Fig. 8). The floral quantitative characteristics showed the highest correlation with the first axis (Table 8). The same was seen in the cluster analysis dendrogram (not shown).

#### ITS ANALYSES, DNA MATRIX FEATURES AND SEQUENCE DIVERGENCE

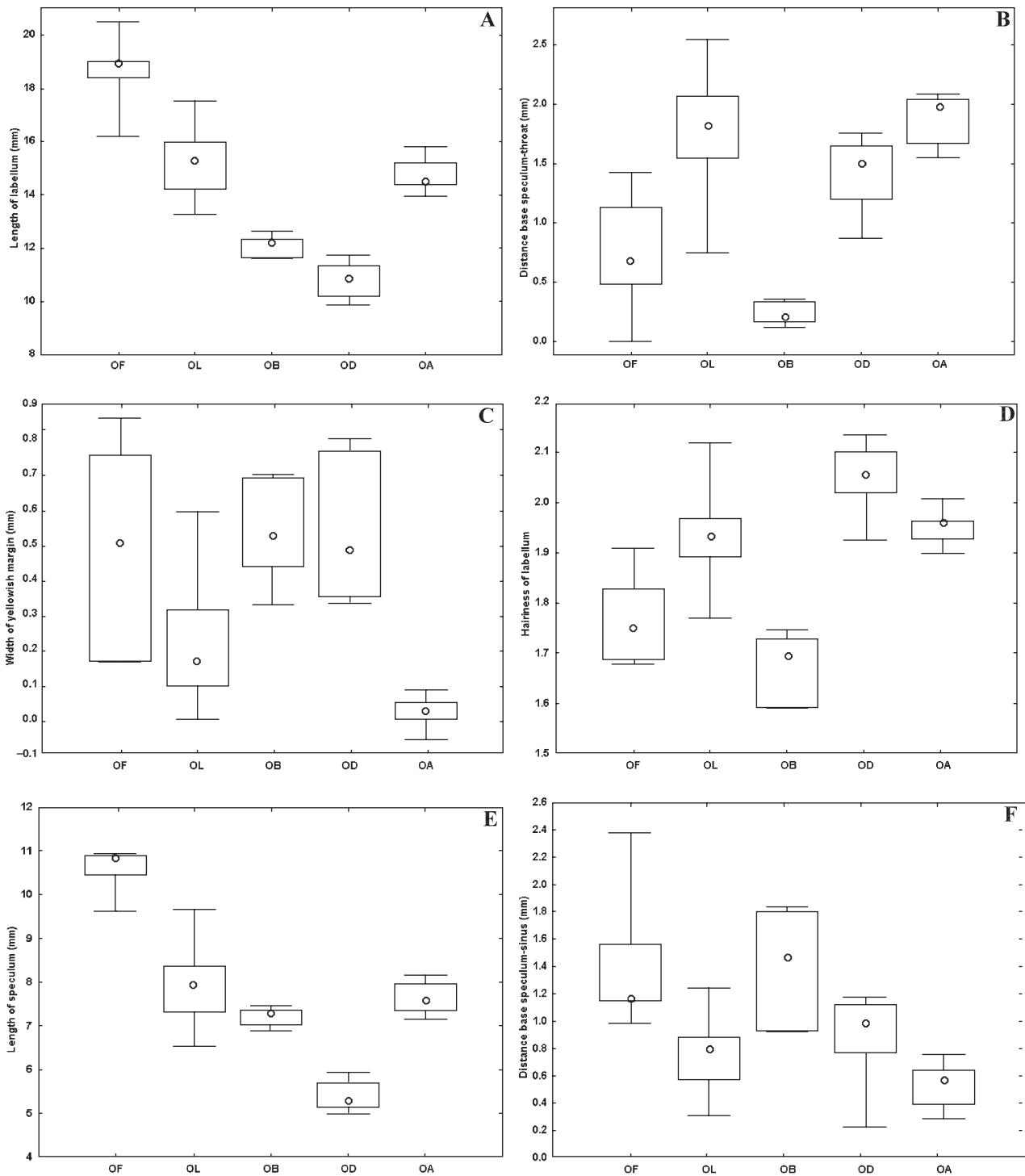
Length of the ITS sequences ranged between 608 and 655 bp; aligned sequences for the same region were 670 bp. Sequence divergence values among the 14 taxa ranged between 23.66 and 35.89. For ingroup taxa the range was 0.0–16.42; among all the *Ophrys* taxa it was 0.0–2.24, with no divergence within taxa.

**Table 6.** Total canonical structure expressing correlation of morphological characters with canonical axes in discriminant analysis of Iberian populations of sect. *Pseudophrys*

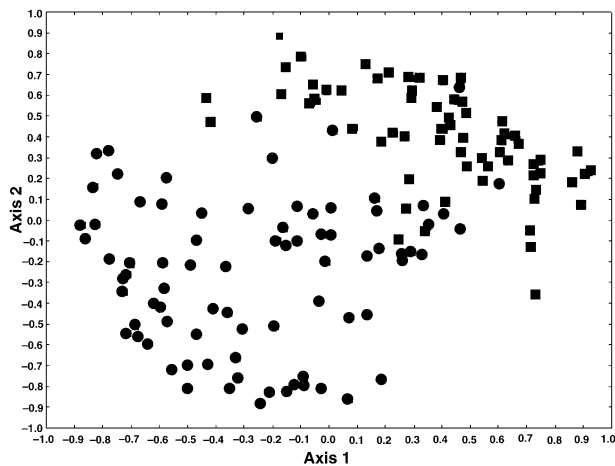
Character	Axis 1	Axis 2	Axis 3
LS	-0.530	0.086	0.132
WS	-0.476	-0.118	0.163
LP	-0.539	-0.018	0.229
WP	-0.467	-0.091	0.394
LL	-0.804	-0.110	0.235
WL	-0.660	-0.069	0.259
LLL	-0.531	-0.035	0.099
WLL	-0.477	-0.083	0.346
WCL	-0.597	-0.229	0.227
WYM	0.242	-0.296	0.310
LSP	-0.701	-0.428	0.192
SPT	-0.043	0.479	0.003
SPS	0.052	-0.446	0.180
CLB	-0.325	-0.151	0.010
CLA	-0.221	0.244	-0.036
HL	0.055	0.699	0.163
PRO	0.205	0.411	0.153
Ratio LL/WL	0.019	0.176	-0.044
Ratio LL/LLL	-0.129	0.110	-0.088
Ratio LL/LS	-0.148	-0.153	0.002
Ratio WL/LS	-0.058	0.103	0.084

**Table 7.** Eigenvectors expressing correlation of characters with principal components (axes 1, 2) in morphometric analysis of Iberian populations of *Ophrys bilunulata* and *O. dianica*

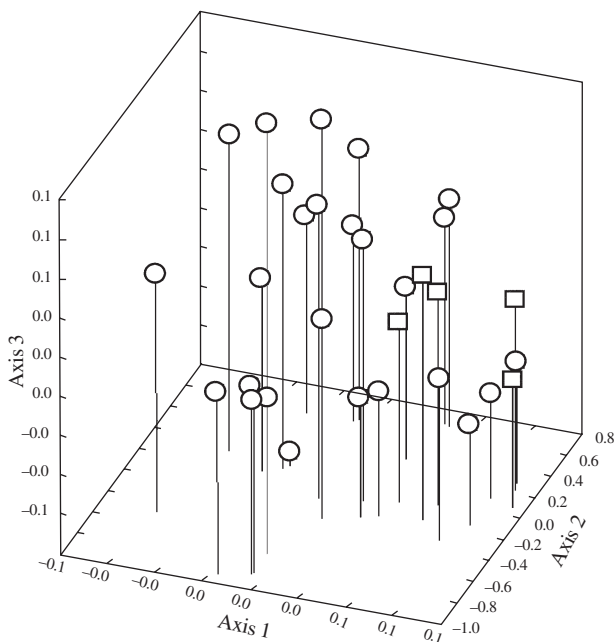
Character	Axis 1	Axis 2
LS	-0.061	0.120
WS	-0.007	0.168
LP	-0.016	0.173
WP	0.020	0.156
LL	0.081	0.020
WL	0.071	0.064
LLL	0.079	0.022
WLL	0.009	0.152
WCL	0.081	0.020
WYM	0.004	0.161
LSP	0.085	-0.022
SPT	-0.085	0.048
SPS	0.075	0.041
CLB	0.084	-0.016
CLA	-0.080	-0.026
HL	-0.085	0.048
PRO	-0.084	0.0369
Ratio LL/LS	0.034	-0.133
Ratio WL/LS	-0.078	0.012



**Figure 6.** Variation in selected morphological characters of sect. *Pseudophrys*. Rectangles show 25th and 75th percentiles, circles indicate medians and whiskers 10th to 90th percentiles. A, length of labellum (mm); B, distance between base of speculum and stigmatic cavity (mm); C, width of yellowish margin of labellum (mm); D, hairiness of labellum; E, length of speculum (mm); F, distance between base of speculum and sinus (mm).



**Figure 7.** PCA of individuals of (■) *Ophrys bilunulata* and (●) *O. dianica* based on 21 morphological characters (see Table 7). Axes 1 and 2 explain 55.44% and 40.17% of the total variation, respectively.



**Figure 8.** PCA of individuals of (□) *Ophrys arnoldii* and (○) *O. lupercalis* based on 21 morphological characters (see Table 8). Axes 1–3 explain 23.74, 20.48 and 14.14% of the total variation, respectively.

PHYLOGENETIC RESULTS

The analysed sequences produce a matrix of 670 characters; 258 sites were constant, 236 were variable and 176 were parsimony informative.

When all characters were included, we found no significant deviation from the homogeneity of base fre-

**Table 8.** Eigenvectors expressing correlation of characters with principal components (axes 1–3) in morphometric analysis of Iberian populations of *Ophrys lupercalis* (including *O. arnoldii*)

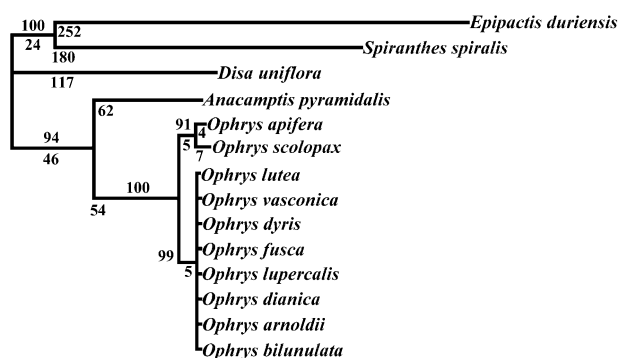
Character	Axis 1	Axis 2	Axis 3
LS	0.024	0.130	0.048
WS	0.126	-0.044	0.066
LP	0.085	0.054	0.001
WP	0.153	-0.117	0.035
LL	0.092	0.067	0.034
WL	0.131	-0.043	0.008
LLL	0.123	-0.002	0.138
WLL	0.117	-0.075	-0.034
WCL	0.077	0.032	-0.065
WYM	0.080	-0.076	-0.019
LSP	0.082	0.080	0.031
SPT	-0.085	0.007	-0.235
SPS	0.100	-0.197	0.104
CLB	-0.053	0.216	0.064
CLA	-0.010	0.062	0.223
HL	-0.052	-0.109	-0.016
PRO	0.003	-0.189	-0.045
Ratio LL/WL	0.039	-0.097	0.210
Ratio LL/LLL	0.074	-0.152	0.095
Ratio LL/LS	0.054	-0.038	-0.141
Ratio WL/LS	0.037	-0.036	0.231

quencies among taxa ( $\chi^2 = 19.23$ ,  $P = 1.00$ , d.f. = 39). The same was true for the parsimony-informative sites only and without constant sites.

The PTP test resulted in a significant difference ( $P = 0.01$ ) between the most parsimonious tree and trees generated from random permutations of the data matrix, which, according to Faith & Cranston (1991), demonstrates the presence of a significant phylogenetic signal.

The heuristic search of the MP analysis produced a single most-parsimonious tree (tree length = 691, CI = 0.8828, CI minus autapomorphies = 0.7686, HI = 0.1172, RI = 0.6721). Our ML search resulted in a single tree with a  $-\ln L$  value of 2962.64658.

Both the phylogram of the MP tree and the best ML tree are shown in Figures 9 and 10. Both MP and ML methods produced quite similar topologies, with both trees showing strong bootstrap support for a monophyletic clade containing all *Ophrys* taxa (MP: 100, ML: 99). Within the strongly supported *Ophrys* clade two monophyletic groups are conspicuous: the first contains the two sampled taxa of sect. *Ophrys* (MP: 91, ML: 78), the second all the sampled taxa of sect. *Pseudophrys* (MP: 99, ML: 93).



**Figure 9.** Maximum parsimony tree (MP) obtained from PAUP\* searches. Numbers above nodes represent bootstrap proportions for 1000 pseudoreplicates. Numbers below branches indicate branch lengths.

## DISCUSSION

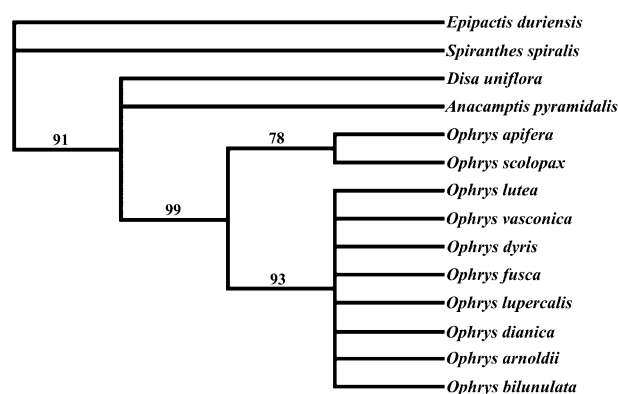
As we have previously indicated, the morphological characters were measured from the most recently opened flower on each stem, carefully checking that it was always well-shaped and that there were no significant differences in relation to the lowest flowers on the stem.

The PCA supported the existence of discrete groups within sect. *Pseudophrys*, and it indicated morphological trends within the section. As a result of the PCA, cluster and canonical analyses, four clear clusters can be recognized; they correspond well with the four species recognized by Delforge (2002). The only universally distinguishing trait was the length of the labellum, although there are several characters useful in differentiating the taxa by pairs.

### *OPHRYS ARNOLDII*, *O. FUSCA* AND *O. LUPERCALIS*

The current tendency to attribute taxonomic value to relatively small differences in phenology or pollinating agent has led to the complex systematics of this group of species. The present analysis confirms this complexity. *O. fusca* seems to be well defined on the basis of the size of its floral organs; in particular, the labellum, at 16–20.5 mm, is clearly longer than those of *O. arnoldii* and *O. lupercalis* (Table 4, Fig. 6). The distribution of *O. fusca* is also different; it appears to be restricted to the south-western quadrant of the Iberian Peninsula. Although other authors (Hermosilla & Sabando, 1996; Medrano *et al.*, 1997) have reported it from other parts of the Peninsula, these populations were subsequently reported to belong to a different species (Benito Ayuso, Alejandre & Arizaleta, 1999).

*Ophrys arnoldii* was described by Delforge (1999) from the north-eastern part of the Peninsula (the



**Figure 10.** Maximum likelihood tree (ML) obtained from PAUP\* searches. Numbers above nodes represent bootstrap proportions for 100 replicates.

Province of Tarragona). It was said to be distinguished from *O. lupercalis* by its slightly longer labellum, later flowering, and subtle differences in the position, angle and hairiness of labellum. However, as most of the quantitative and qualitative characters studied in the present work also fall clearly within the range of variability of *O. lupercalis* (Table 4, Fig. 6), consistent separation of *O. arnoldii* is hardly possible. The only feature that separated populations of *O. arnoldii* (along with other populations from the north and south of the Peninsula) was the consistent lack of a yellow rim around the labellum. In *O. lupercalis* s.s. this character is very variable.

*Ophrys lupercalis* is a controversial taxon. Though some authors consider it conspecific with *O. fusca* (Arnold, 1999; Paulus & Gack, 1999), the present analysis shows that the taxa differ in all the quantitative traits examined (Table 4): the sepals, petals and labellum of *O. fusca* are larger, the distance between the base of the speculum and the stigmatic cavity is shorter, and the distance between the base of the speculum and the apical sinus clearly greater. Furthermore, *O. lupercalis* is found all over the Peninsula (Fig. 1); the present results hardly show any patterning in its geographical distribution. The dendrogram in Figure 4 reveals a small subgroup made up of mainly northern populations (OA02–OA05 and OL36, OL37, OL42, OL43), although it also contains populations from the south (OL48) and from eastern Spain (OL32).

### *OPHRYS BILUNULATA* AND *O. DIANICA*

PCA (Figs 3, 7), cluster analysis (Fig. 4) and CDA (Fig. 5) clearly separated these two taxa, which have smaller flowers and labellae than the other species.

*Ophrys bilunulata* is distributed in the west of the Mediterranean region and is well characterized by its small labellum (12–13 mm) and its speculum which continues into the stigmatic cavity. The present results do not support its treatment as a subspecies of *O. fusca* (Aldasoro & Sáez, 2005), but as a morphologically distinguished species in its own right. The indicated characters (no specimens were found with intermediate characters) are quite constant among the study populations. While, as mentioned above, the systematics of sect. *Pseudophrys* are complex, we believe that specific status is fully justified.

Plants from eastern Spain similar to *O. bilunulata*, but with a smaller labellum, were identified by Delforge (1999) as *O. luentina*. The identity and circumscription of *O. luentina* is, however, controversial. Several authors have suggested that in fact material belonging to various entities of the group containing *O. fusca* was examined (Lowe *et al.*, 2001; Galán & Gamarra, 2003). Lowe *et al.* (2001) therefore describe it as *O. dianica* (= *O. luentina* P. Delforge pro parte, typo excluso). The present analyses clearly distinguish the eastern Spanish populations of *O. dianica*, which have a small labellum (9.5–11.5 mm) and a speculum that does not continue into the stigmatic cavity. Other features distinguishing *O. dianica* from *O. bilunulata* are, in the former, the shorter distance between the speculum and apical sinus, a denser covering of hairs on the labellum, and more marked protuberances on the base of the labellum. Delforge (1999) indicated that *O. dianica* might be present in Portugal; the present work confirmed the existence of a population in the Algarve (Boliqeime, OD13).

Though only eight taxa were included in the ITS sequence analysis, some preliminary conclusions can be drawn. Sect. *Pseudophrys* appears to be clearly monophyletic (MP: 99, ML: 93, Figs 9, 10). This agrees with the results of earlier analyses (Soliva *et al.*, 2001; Bateman *et al.*, 2003; S. Bernardos, M.A. Santos, J.L. Revuelta, F. del Ray, D. Tyteca & F. Amich, unpubl. data), and reinforces its unequivocal morphological (Devillers & Devillers-Terschuren, 1994) and karyological ( $2n = 72$ ; Greilhuber & Ehrendorfer, 1975; Bernardos *et al.*, 2003) cohesion.

#### TAXONOMIC AND PHYLOGENETIC CONCLUSIONS

The generally weak morphological differentiation of the study taxa supports the hypothesis of a group of close allies. This is also reflected in the results of the molecular analysis. Various characters have been identified that distinguish readily between the different species of sect. *Pseudophrys* as currently recognized. According to our morphological analysis, *O. dianica* and *O. fusca* are the least variable taxa.

They also have the most restricted geographical distributions: *O. dianica* is found in eastern Spain, with a disjunct population in Algarve (Portugal), while *O. fusca* is found in the south-west of the Iberian Peninsula. These species can be distinguished by a set of features, the most diagnostic being the size of the labellum. Their recognition as independent species is not supported, however, by the present molecular data.

*Ophrys bilunulata* is quite homogeneous. The features that most usefully characterize it are the small labellum and speculum that continues into the stigmatic cavity. We have not found morphological differences between specimens and populations growing in the north and south of the Peninsula. The molecular analysis showed this species to have the same nrITS sequence as the other taxa studied.

*Ophrys lupercalis* (including *O. arnoldii*) appears to be the most variable species. It is widely distributed within the Peninsula and Morocco. The wide morphological variability displayed by the specimens studied here agrees well with the fact that *O. lupercalis* is a polyploid complex (S. Bernardos, M. García-Barriuso, A. Crespi & F. Amich, unpubl. data). We have not found relationships between any morphological group within this species and a particular ploidy level, or between such groups and their geographical distribution. Although it is polymorphic, there are some characters that separate it well from the other species, such as the size of labellum and the distance from the base of speculum to the stigmatic cavity. Its nrITS sequence was identical to those of *O. fusca* and *O. dianica*.

Although floral characters in sect. *Pseudophrys* are diverse, DNA sequences did not reflect this diversity and no genetic differentiation was detected. While Soliva *et al.* (2001) consider the low genetic diversity of the genus *Ophrys* to reflect hybridization (i.e. sequence divergence followed by homogenization), Bateman (2001) suggests this may be due to conspecificity, i.e. a historical absence of divergence. The lack of phylogenetic resolution observed for the taxa in this study suggests a recent and rapid colonization of the Mediterranean Basin without sufficient time for molecular divergence.

Our results suggest that additional molecular markers are needed to resolve more fully the relationships among sect. *Pseudophrys* taxa and illustrate species diversification. High resolution markers, such as the external transcribed spacers (ETS) region of nrDNA (Baldwin & Markos, 1998), have not been employed in studies of Orchidaceae, but have revealed their usefulness in studies of other families, such as Asteraceae (e.g. Roberts & Urbatsch, 2004) and Crassulaceae (e.g. Acevedo-Rosas *et al.*, 2004). They may assist in producing a more complete picture of the unique evolu-

KEY TO THE IBERIAN REPRESENTATIVES OF THE STUDIED TAXA OF SECT. *PSEUDOPHRYS*

1. Base of labellum flat . . . *O. atlantica* and *O. omegaifera* groups (*O. atlantica*, *O. algarviensis*, *O. dyris*, *O. vasconica*)  
Base of labellum shaped like a V . . . . . 2
2. External border of the lateral lobe of the labellum at an angle of  $\pm 65^\circ$  to the longitudinal axis. . . . . *O. lutea*  
Angle of  $< 65^\circ$  . . . . . 3
3. Labellum longer than (13.5–) 14 mm. . . . . 4  
Labellum shorter than 13 (–13.5) mm . . . . . 5
4. Labellum of (13.5–) 14–16 (–17) mm; distance between the base of the speculum and the stigmatic cavity generally more than 1.5 mm . . . . . *O. lupercalis*  
Labellum of (16.5–) 17–20 (–21) mm; distance between the base of the speculum and the stigmatic cavity generally less than 1 mm . . . . . *O. fusca*
5. Labellum of (11.5–) 12–13 mm; distance between the base of the speculum and the stigmatic cavity almost nonexistent. . . . . *O. bilunulata*  
Labellum of 9.5–11.5 mm; distance between the base of the speculum and the stigmatic cavity generally greater than 1 mm . . . . . *O. dianica*

tionary patterns suggested by ITS phylogenies. Plastid *psaB* gene sequences have been successfully used while investigating intrafamilial relationships within Orchidaceae (Cameron, 2004). Their use in *Pseudophrys* can also help to clarify the relationships within the section.

This study focused on taxa in the western Mediterranean Basin (Iberian Peninsula and Morocco), a region in which *Ophrys* sect. *Pseudophrys* achieves a high diversity (Delforge, 2002). It would be interesting to extend such a study to a broader distribution range in order to check whether the indicated differences remain stable outside the range prospected in this study.

The results of the present morphological study (qualitative and quantitative data), support the recognition of four species that can be identified according to the following key, which also includes other Iberian species of sect. *Pseudophrys*.

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