Ophrys fusca and Ophrys dyris (Orchidaceae) – constancy of tetraploidy amongst populations in Central Portugal

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Ophrys fusca and *O. dyris* (Orchidaceae) - constancy of tetraploidy amongst populations in Central Portugal

Ophrys is amongst the best known orchid genera, and is an established system for the study of pollinator-mediated floral evolution. Two species, *Ophrys fusca* s.l. and *Ophrys dyris* (= *O. omegaifera* subsp. *dyris*) belonging to *Ophrys* section *Pseudophrys* are the focus of this study. In the context of an integrative study of morphological and genetic diversity of *O. fusca* and *O. dyris*, genome size (GS) and cytotype diversity were surveyed from Portuguese populations. Flow cytometry methods were used to assess GS, and subsequently determine the ploidy level of 67 specimens, including the species and putative hybrids. Cytotypes were also confirmed based on chromosome counts from the roots of two specimens, one of each species. Constancy of nuclear DNA content (1C = 11.19 pg) and ploidy level ($2n = 4x = 72, 74$) was documented among all the individuals analysed. Implications are considered, in terms of interpreting the origin and predicting the persistance of putative hybrids.

Keywords: central Portugal; chromosome numbers; cytotype; genome size; *Ophrys fusca, Ophrys dyris*; polyploidy

Introduction

Ophrys is a distinctive genus supported by morphological and molecular characters, but there is a lack of agreement about the number of species in the genus that has been attributed to interspecific hybridisation and introgression (Devey *et al.*, 2008; Stebbins and Ferlan, 1956). Better documentation of the extent of reproductive isolation between species could inform taxonomy and conservation strategies and contribute to our understanding of pollinator-mediated floral evolution in these charismatic, sexually deceptive orchids. Cytotype characterisation of hybridising species at the population level can be an important component of studies of hybridisation and introgression (interspecific gene exchange, through repeated hybridisation and backcrossing, following Anderson 1949), contributing to the robust interpretation of the co-dominant

molecular marker data used to measure gene flow (Pellicer *et al.*, 2012). Furthermore, in a hybridisation scenario, isolation barriers due to genetic incompatibilities attributed to the ploidy of the parental taxa may account for low incidence of introgressed individuals. Since homoploid hybrid species may show only weak post-zygotic isolation, ploidy influences the frequency of introgressed individuals. Discriminating between polyploidy and other variables influencing reproductive isolation of hybrids (fertile or partially fertile F1 individuals resulting from the interbreeding between the two species), such as pollinator behaviour, could be important in these plants.

Ophrys dyris Maire and *Ophrys fusca* Link are one pair of species found in sympatry and offering opportunity for the study of hybridisation in the context of a specialised orchid-pollinator system. These two species are respectively included in the groups *omegaifera* and *fusca* of section *Pseudophrys* Godfery (Orchidaceae) and are closely related (Bernardos *et al.*, 2005; Cotrim *et al.*, 2016; Devey *et al.*, 2008). Despite the different approaches and reported relationships pointed out below, we refer these taxonomic entities as separate species, as our preliminary morphological analysis seem to maintain the segregration between specimens presenting trait expression of the diagnostic characters in the mean values of its description range.

These species are found across the Iberian Peninsula and northern Africa, *O. dyris* having a narrow and more localised distribution in this region, and *O. fusca* being more common and widespread, with a geographical range that reaches western Asia. Flowers of the latter species are extremely variable in morphology, leading to the segregation of the species into more than ten different species (Delforge, 2002), though other authors have taken a more conservative approach, recognising these two species in the broad sense, with the morphological variants being treated at lower taxonomic levels (Pederson and Faurholdt, 2007). *Ophrys dyris* is less variable in floral morphology and less abundant throughout

its distribution range. Both species are listed in protected habitats, as components and bioindicators of habitat 6210 of Directive 92/43/CEE, under which habitats are prioritised when any listed species is numerous.

Ophrys dyris and *O. fusca* have similar morphological characters, and careful inspection is needed for field identification (Figure 1), particularly as intermediate individuals are known, some of which have been characterised as hybrids using co-dominant molecular marker data (Cotrim *et al*., 2016).In 1981 the name *O. ×brigittae* H.Baumann was used to refer to the hybrids between *Ophrys dyris* and *Ophrys fusca.* Aside from *O. ×brigittae*, several other species considered to result from introgression between *O. dyris* and *O. fusca* have been described for Portugal. These are *O. algarvensis* D.Tyteca, Benito & M.Walravens, *O. vasconica* (O.Danesch & E.Danesch) P.Delforge and *O. lenae* M.R.Lowe & D.Tyteca (Lowe and Tyteca, 2012). They were referred to as "paleohybrids" by Lowe and Tyteca (2012), as neither parent is present in the populations, such absence suggesting that hybridization occurred in the past. As far as we know, there are no molecular studies focused on the origin of these putative hybrids which form part of a "partly stabilized hybrid complex" between the *omegaifera* and *fusca* groups (Pederson and Faurholdt, 2007). *Ophrys dyris* itself might be of hybrid origin. Devey *et al.* (2008) postulated that the plant which represented *O. dyris* in their study was an intersectional hybrid between sections *Pseudophrys* and *Ophrys*, and based on ITS and AFLP data it seems likely that the specimen in cause was an F1 hybrid. However, as we are referring to one particular sample, more data are needed to interpret on the potential hybrid origin of the species.

In Portugal, *O. fusca* and *O. dyris* overlap in at least three limestone regions: in the Aire and Candeeiros Mountains, in Arrábida and in the Algarve. The distribution of putative hybrids and introgressed individuals varies (Abreu *et al.*, in prep.), but it is not known whether post-zygotic isolation due to incompatible parental ploidy could account for these differences. O*phrys fusca* is known to exist at diploid ($2n = 2x = 36$) and tetraploid ($2n = 4x = 72$) levels (Greilhuber and Ehrendorfer, 1975), whereas tetraploid and pentaploid ($2n = 4x = 90$) cytotypes of *O. dyris* have been described. Greilhuber and Ehrendorfer (1975) first described tetraploids for Mallorcan specimens of the *O. fusca* aggregate, but also reported diploid Italian plants. In 2005, D'Emerico *et al.* reported that Italian *O. fusca* is diploid. In 2007 and 2010, chromosome counts of $2n = 4x = 72$, 76 and $2n = 4x = 72$ were reported for *O. fusca* from Arrábida region, Portugal, by García-Barriuso *et al.* (2010). Cotrim *et al.* (2009, 2016) inferred that specimens from the western Iberian Peninsula with four alleles per individual for some microsatellite loci were tetraploid. Considering *O. dyris*, following an early report of tetraploid plants (Kullenberg 1961), Bernardos *et al.* (2003), later cited by Aedo and Herrero (2005) and Amich *et al.* (2007), reported counts of $2n = 4x = 72$ and $2n = 5x = 90$. A confirmation of $2n = 4x = 72$ was reported by García-Barriuso *et al.* (2010) for specimens from Montejunto region, Portugal.

This study is part of a wider project describing the genetic and morphometric diversity of *O. fusca* and *O. dyris* in central Portugal (Figure 2) and documenting hybridisation and introgression between these species. Here we focus on describing the ploidy of these plants, characterising the accessions to be included in genetic and morphometric surveys with a view to integrative analysis in the future. Our new characterisations will permit robust interpretation of microsatellite data in preparation. Presently, knowledge of ploidy

levels will clarify whether variants described in the literature are found in the Portuguese populations, may shed light on the hypothesis of hybrid origin for the species *O. dyris*, and on the possible dynamics of the *Ophrys fusca-O. dyris* populations known in Portugal.

Methods

Flow cytometry

As indicated above, nuclear DNA contents were estimated from a subset (67) of the plants that were measured for morphology and collected for DNA analysis in the scope of the broader study. Samples came from the six populations being monitored (Figure 2): *O. fusca* - 35 from *Arruda dos Vinhos* (north of Lisbon, AV) and 5 from *Casal Facho* (Arrábida, CF); *O. dyris* - 9 from *Montejunto* (Montejunto mountain, Mj) and 10 from *Serra de Sto António* (Santo António Mountain, SA); samples from populations where both species are present - 3 from *Pinheirinhos* (Arrábida, Pi) and 5 from *Mendiga* (Aire and Candeeiros Mountains, Me). Analyses were carried out by flow cytometry using pollinaria instead of leaf tissue, in order to avoid potential misinterpretation of the results given the differential release of nuclei in orchids (Pellicer and Leitch, 2014). To test the performance of pollinaria under different storage conditions, we analysed (i) fresh, (ii) fixed in 3:1 alcohol:acetic acid and (iii) silica-dried pollinaria after collection in the field. Although fresh samples provided good quality results, after several days of storage the quality of the results decayed significantly, so we used silica-dried pollinaria instead, which provided better quality flow histograms and comparable relative fluorescence. Measurements were performed on the pollinaria of 67 plants (Table 1) that had been previously screened with microsatellites. Genome size was also assessed

using fresh leaves. For the cytotype screening, one to three pollinaria were co-chopped with the selected internal standard [*Pisum sativum* 'Ctirad' (2C = 9.09 pg) or *Petroselinum crispum* 'Curled Moss' (2C = 4.45 pg)] in a Petri dish containing 1ml of Ebihara buffer (Ebihara *et al*., 2005) following the one-step procedure described in Doležel *et al.* (2007). The nuclei suspension was filtered through a 30 µm nylon mesh and stained with 100 µl propidium iodide (1mg/ml). Samples were kept on ice for 15 min and analysed using a Partec Cyflow SL3 flow cytometer fitted with a 100 mW green solid state laser (Cobolt Samba). For each run, 3,000 particles were analysed. Measurements from leaves followed the same procedures, with three replicates run for each of the accessions and analysing 5,000 particles per run.

Chromosome counts

Knowing that all the plants have approximate values for genome size, we chose reference samples from all the populations to assess ploidy level and chromosome numbers. To count the number of chromosomes, root tips of those plants, with genome sizes previously assessed (seven plants from all the populations sampled: two from Pi, one from AV, one from CF, one from Me, one from Mj and one from SA) and collected from the field were used. To promote active root growth, plants had been re-potted one week before pre-treatment with colchicine. After roots were harvested, c. 10-15 mm root tips were cut and placed into a tube of cold distilled water. Samples were then placed into a 0.05% colchicine (w/v) solution and placed at 21ºC for three hours, from where they were transferred to a freshly prepared fixative of 3:1 ethanol:acetic acid. After 48 hours at 4ºC, they were moved to Eppendorf tubes in 70% ethanol. Roots were then washed in distilled water for 5-10 mins using a shaker at room temperature, and transferred to 1M hydrochloric acid (HCl) at 60º C to be hydrolysed for 5-6 min. For the following staining phase, we used Schiff's reagent for 20 min, and subsequently used

acetic orcein for a further 20 mins. The root tips were excised with a razor blade under a stereo microscope and mounted on a microscope slide in one drop of 2 % aceto-orcein to be squashed. Plates were then observed on a Zeiss Axioplan Imaging microscope and the metaphase plates photographed with a digital camera (SPOT RT; Diagnostic Inc.). Images were edited with the software ProgRes Capture Pro v2.9.1 (Jenoptik Optical Systems GmbH, Jena, Germany).

Results and discussion

***The use of alternative tissues to estimate GS and infer ploidies in* Ophrys**

In recent times, flow cytometry has become the preferred method to estimate nuclear DNA contents in plants, not only because it is fast, reproducible and enables analysis of thousands of particles, but also because it only requires small amounts of tissue (Doležel *et al.*, 2007). Nonetheless, this is not always straightforward, and several taxonomic groups pose challenges due to either the presence of cytosolic compounds or unbalanced numbers of nuclei in G_1-phase of mitosis, which may lead to misinterpretation of the resulting flow histograms (Trávníček *et al.*, 2015). Orchids are sometimes challenging due to frequent rounds of endoreplication (including partial endoreplication), which might be tissue-specific, hence efforts have focused in searching for alternatives in order to overcome this problem (e.g. Pellicer and Leitch, 2014; Trávníček *et al.*, 2015). In our study, although *Ophrys* does not seem to represent a major challenge in this respect, we investigated the use of pollinaria for estimating nuclear DNA contents and allocating DNA-ploidies instead of leaves. We found that the results obtained not only were highly similar (Tables 1 and 2), but the number of particles released were always higher when using pollinaria [e.g. out of 5,000 particles

(including debris): leaf (337 nuclei), pollinarium (780 nuclei)], thus making our inferences more robust. In addition, since fresh and silica-dried pollinaria resulted in similar relative fluorescences [e.g. ratio standard-sample 1.235(dried)-1.261(fresh)], we also overcome efficiently one of the limitations of flow cytometry, i.e. the need for fresh leaves for high-quality estimations. As reported in Trávníček *et al.* (2015), pollinaria did not show multiple rounds of endopolyploidy, with most samples only displaying G_1 peaks (= 1C-value peak, since these are haploid cells). By contrast, our tests on leaves showed higher levels of endopolyploidy with 2C, 4C and 8C peaks, with 4C peaks (G_2) nuclei more than three times more abundant than 2C peaks (G_1) (i.e. 300/2C *vs*. 1045/4C, Fig. 3).

Tetraploid cytotypes in Ophrys fusca *and* O. dyris *across central Portugal*

Chromosome counts for the two plants from different populations (CF and Pi) and different species, *O. fusca* (CF02) and *O. dyris* (Pi15), revealed the same ploidy levels, with chromosome numbers of $2n = 4x = 72$, 74, respectively (Fig. 4). Since genome size from pollinaria and from leaves revealed approximately the same nuclear DNA content for all plants (average 1C-value = 11.19 pg), including typical *O. fusca* and *O. dyris*, specimens with intermediate morphotypes and putative hybrids following molecular analysis (data not shown), we inferred a tetraploid cytotype across central Portugal for the species *O. fusca* and *O. dyris* (Table1),and their putative hybrids (Table1). The difference between the chromosome numbers ($2n = 72$, 74) could be due to aneuploidy events (as identified previously in *Ophrys* by Greilhuber and Ehrendorfer, 1975), although chromosome breakage during preparation cannot be discounted.

Within species of section *Pseudophrys*, García-Barriuso *et al.* (2010) observed a restricted geographic distribution of tetraploids, and interpreted their findings as suggestive of a young polyploid complex *sensu* Stebbins (1971). Our results provide

support the view that polyploidy may have played an important role in shaping the evolutionary diversification of section *Pseudophrys* in the Iberian Peninsula (Amich *et al.,* 2007; García-Barriuso *et al.,* 2010), and that this area is a hotspot of polyploidisation in the section (García-Barriuso *et al.,* 2010). It is notable that the remarkable cytotype consistency amongst the plants analysed here contrasts with the diversity of phenotypes and different genetic groups found (Abreu *et al.*, in prep.). It is also relevant to any discussion of possible fate of hybrids between *O. fusca* and *O. dyris*. Hybridisation in the case of species of the same ploidy could have a range of outcomes, including the establishment of a hybrid swarm, the transfer of traits through introgressive hybridisation, and the origin of new homoploid hybrid species (Yakimowski and Reiseberg, 2014), as already referred as a likely evolutionary mode in *Ophrys* by Paulus in 1990. Since Stebbins and Ferlan (1956) reported solitary or few fully fertile F1 hybrid individuals in (another pair of) sympatric *Ophrys* populations but no hybrid swarms, the view has been that the breakdown of species-specific pollinator relationships in *Ophrys* hybrids would mediate against the establishment of complex hybrid populations. The fact that both species studied here have the same ploidy levels suggests that cytological isolation is not acting as a post-zygotic barrier, at least in this case, and a homoploid hybrid speciation process might be in the beginning. Future dissection of the hybridising populations to identify the frequency of F1, F2 and other hybrid progeny can be interpreted in this light.

The origin of Ophrys dyris

In 2008, when studying phylogenetic relations between several species of *Ophrys,* Devey *et al*. (2008) suggested that either the particular accession used in their analysis was of hybrid origin or the species itself could be of hybrid origin. If the later

hypothesis holds true, the likely parents would be from sections *Pseudophrys* and *Ophrys* (*fuciflora* aggregate), based on the phylogenetic results obtained by the authors.

Despite being impossible to confirm the identification of such specimen (Bateman, *com. pers.*) and the uncertainty of this conclusion, as assumed by the authors, our current molecular dataset under analysis supports the idea of a hybrid origin for *O. dyris*. To clarify this idea, plastidial genes were analised in the scope of this study (data not publ.), which seem to indicate *O. lutea* (section Ophrys) as one of the parents. In addition, Cotrim *et al.* (2016), supported by plastid haplotypes, reported introgressed individuals and hybridising populations between *fusca-lutea*, bearing out the tight relationship between these species previously pointed out by Soliva *et al.* (2001).

The different hypotheses illustrate different scenarios of breakage of reproductive barriers: the hybrid would be either the outcome of an intersectional cross or a plant with both parents from the same section. As the strength of reproductive isolation mechanisms (mainly postzygotic in sexual deceptive orchids) does not seem to be related with genetic distance or species divergence (Scopece, 2007), both scenarios would be equally plausible. In the light of previous counts and the results presented here, in either case the outcoming hybrid would be tetraploid with a diploid and a tetraploid parent. Such crosses are known to result from fusion of an unreduced $2n$ gamete from the diploid parent and a normally reduced $2n$ gamete from the tetraploid parent (Carroll and Borrill, 1965; VanSanten *et al.*, 1991; Petit, 1999). Unreduced gametes are the result of abnormal meiotic division and, despite ease of production in controlled experiments, their frequency in natural populations is still largely unknown. The viability of the resulting hybrid would have been influenced by paternal:maternal ratios, the direction of the cross and by endosperm development (Burton and Husband, 2000; van Santen *et al.*, 2001; Sabara *et al.*, 2013). Maintainance is likely to be

grounded on competitive ability, higher fecundity, selfing and habitat segregation between cytotypes, as documented by Rodriguez (1996) and cited by Petit (1999). As theorised by the latter author, the establishment of a species such as *O. dyris* might reflect the maintainance of viable populations for successive generations, or the rapid colonisation of new areas. Pre-zygotic isolating mechanisms, such as pollinator-mediated isolation, are of major importance for the establishment of such species (Petit 1999). The constancy of cytotypes, recurrent hybridisation events and gene flow between *O. dyris* and *O. fusca* suggest that isolation - at least from one of the putative parents – is not complete. To achieve a more comprehensive understanding of the evolution of this group including clarifying the origin of *O. dyris*, further genetic analyses are being conducted..

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Table captions:

Table 1: Nuclear DNA contents from all the plants assessed, from pollinaria (67).

Plant	Fluorescence Ratio (Sample/Standard)	Relative nuclear DNA content	DNA Ploidy	Internal standard
AV02	2.429	10.9305	4x	Petroselinum
AV03	2.518	11.331	4x	Petroselinum
AV04	FAIL			
AV05	2.398	10.791	4x	Petroselinum
AV06	2.586	11.637	4x	Petroselinum
AV07	2.488	11.196	4x	Petroselinum
AV08	2.514	11.313	4x	Petroselinum
AV09	2.598	11.691	4x	Petroselinum
AV10	2.411	10.8495	4x	Petroselinum
AV11	2.554	11.493	4x	Petroselinum
AV12	2.521	11.3445	4x	Petroselinum
AV13	2.594	11.673	4x	Petroselinum
AV14	2.649	11.9205	4x	Petroselinum
AV15	1.249	11.35341	4x	Pisum
AV16	2.561	11.5245	4x	Petroselinum
AV17	2.524	11.358	4x	Petroselinum
AV18	2.264	10.188	4x	Petroselinum
AV19	2.604	11.718	4x	Petroselinum
AV20	1.224	11.12616	4x	Pisum
AV22	2.531	11.3895	4x	Petroselinum
AV24	2.549	11.4705	4x	Petroselinum
AV25	2.578	11.601	4x	Petroselinum
AV26	2.567	11.5515	4x	Petroselinum
AV27	2.63	11.835	4x	Petroselinum
AV28	2.549	11.4705	4x	Petroselinum
AV29	2.547	11.4615	4x	Petroselinum
AV30	2.53	11.385	4x	Petroselinum
AV31	2.6	11.7	4x	Petroselinum
AV32	2.452	11.034	4x	Petroselinum
AV33	2.61	11.745	4x	Petroselinum
AV34	2.66	11.97	4x	Petroselinum
AV35	2.637	11.8665	4x	Petroselinum
AV36	2.587	11.6415	4x	Petroselinum
AV37	2.591	11.6595	4x	Petroselinum
AV38	2.629	11.8305	4x	Petroselinum
CF02	2.565	11.5425	4x	Petroselinum
CF37	2.574	11.583	4x	Petroselinum
CF42	1.244	11.30796	4x	Pisum
CF43	2.567	11.5515	4x	Petroselinum
CF58	2.541	11.4345	4x	Petroselinum

Me68	2.649	11.9205	4x	Petroselinum
Me69	2.617	11.7765	4x	Petroselinum
Me70	1.245	11.31705	4x	Pisum
Me71	2.658	11.961	4x	Petroselinum
Me72	2.625	11.8125	4x	Petroselinum
Mj02	FAIL			
Mj10	2.541	11.4345	4x	Petroselinum
Mj19	2.632	11.844	4x	Petroselinum
Mj28	2.591	11.6595	4x	Petroselinum
Mj31	1.194	10.85346	4x	Pisum
Mj54	FAIL			
Mj56	2.521	11.3445	4x	Petroselinum
Mj60	2.554	11.493	4x	Petroselinum
Mj61	1.167	10.60803	4x	Pisum
SA04	2.542	11.439	4x	Petroselinum
SA05	2.564	11.538	4x	Petroselinum
SA06	1.254	11.39886	4x	Pisum
SA07	1.249	11.35341	4x	Pisum
SA08	2.484	11.178	4x	Petroselinum
SA12	1.236	11.23524	4x	Pisum
SA13	1.285	11.68065	4x	Pisum
SA21	2.622	11.799	4x	Petroselinum
SA22	2.578	11.601	4x	Petroselinum
SA23	2.653	11.9385	4x	Petroselinum
Pi15	1.231	11.18979	4x	Pisum
Pi80	1.289	11.71701	4x	Pisum
Pi87	1.239	11.26251	4x	Pisum

Table 2. Summary of the nuclear DNA contents from flow cytometry using silica-dried pollinaria and fresh leaves of *Ophrys fusca* and *O. dyris*. Chromosome numbers for each individual are provided.

	Pollinaria [1C-value (S.D.)]	Leaves [2C-value (S.D.)]	Chromosome number (2*n*)
CF02 (O. fusca)	11.125 pg	21.716 pg	72
Pi15 (O. dyris)	11.247 pg	22.120 pg	74

Figure 1. *Ophrys fusca* Link subsp. *fusca* (A) and *Ophrys dyris* Maire (= *O. omegaifera* H.Fleischm. subsp. *dyris* (Maire) Del Prete) (B)

Figure 2. Distribution map of populations sampled in central Portugal. 1. *Arruda dos Vinhos* (AV); 2. *Casal Facho* (CF); 3. *Montejunto* (Mj); 4. *Serra de Sto António* (SA); 5. *Pinheirinhos* (Pi); 6. *Mendiga* (Me)

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Figure 3. Representative fluorescence histograms in *Ophrys* (* = 2C and 4C peaks of the internal standard used (*Pisum sativum* 'Ctirad'). A. *Ophrys dyris* leaf analysis (2C ~ 21.99 pg). Note that an additional histogram run on logarithmic scale is shown for illustrating the impact of endoreplication (2C, 4C and 8C peaks displayed). B. *Ophrys dyris* pollinarium analysis (1C ~11.17 pg)
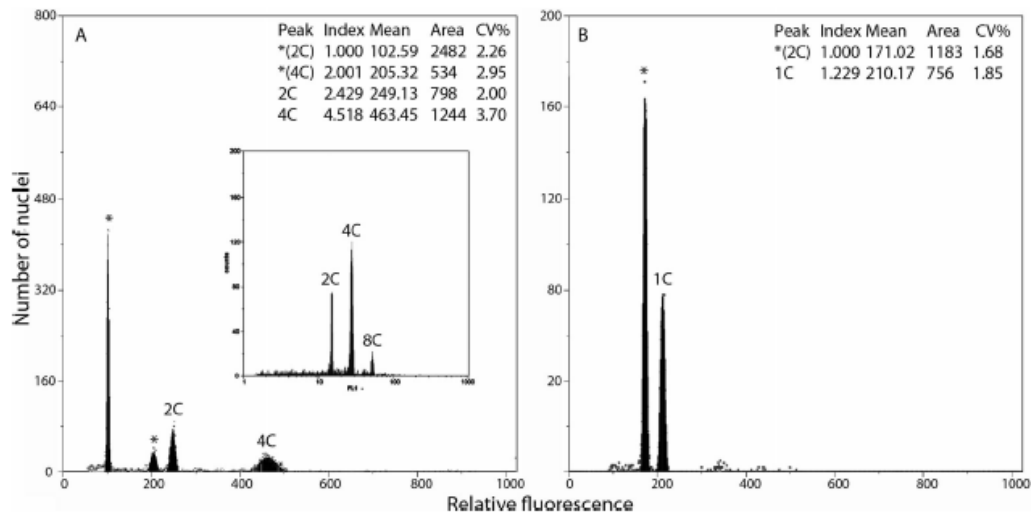
Figure 4. Metaphase cells from root-tips of *Ophrys*, observed on a Zeiss Axioplan Imaging microscope. A. *Ophrys fusca* (CF02) $2n = 72$, B. *Ophrys dyris* (Pi15) $2n = 74$. Scale bar = 10 µm.