SEED MICROMORPHOLOGY SUPPORTS SPECIES DELIMITATION OF *ORCHIS CANARIENSIS* (ORCHIDACEAE), AN ENDEMIC ORCHID FROM THE CANARY ISLANDS

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Geographic isolation is one of the primary drivers of speciation, but islands remain a reservoir of overlooked and understudied endemic species. In this study, we collected seed micromorphological data from *Orchis canariensis* (Orchidaceae), a declining species occurring only on the Canary Islands (Spain), whose taxonomy was debated in the past decades. The aim of the study was to detect seed micromorphological traits in support of species delimitation by means of light and scanning electron microscopy. Seeds from a population on Tenerife resulted to be clavate with a cell number in the longitudinal axis varying from five to seven. Seeds showed straight to sinuous anticlinal walls and no ornamentations in the periclinal walls. The average seed length was 313.66 ± 44.78 µm and the average width 184.31 ± 30.26 µm, with a ratio of 1.72 ± 0.25, while the embryo length and width were 157.18 ± 35.21 µm and 125.43 ± 25.92 µm respectively. However, despite the affinities with the sister species, *Orchis patens*, quantitative and qualitative seed traits supported species delimitation recently proposed by means of molecular biology. Taken together, our results confirmed the importance of seed micromorphology in support of taxonomical studies.

Key words: endemic species, island flora, orchid conservation, *Orchis patens*, Red List, scanning electron microscopy, taxonomy, Tenerife

Introduction

With more than 25 000 species, the Orchidaceae is one of the largest families of flowering plants (Christenhusz & Byng, 2016; WCSP, 2022). Although orchids are numerous and widespread, many of them are rare or threatened from extinction, especially those with limited or fragmented ranges (Cribb et al., 2003). Rare species are usually prone to genetic depression, which ultimately leads to vulnerability and to environmental and biological stresses (Leimu et al., 2006; Honnay & Jacquemyn, 2007; Aguilar et al., 2008). Furthermore, small, isolated and declining populations may result in maladaptation in response to climate change (Borrell et al., 2020).

Orchis canariensis Lindl. (Fig. 1a) is an increasingly rare (pers. obs.) and highly localised European orchid. As a subspecies of *O. patens* Desf., *O. canariensis* is red-listed as «Endangered» (Rankou, 2011) and «Vulnerable» (Calevo et al., 2018). It occurs in five out of seven Canary Islands (Spain), with the exception of Lanzarote and Fuerteventura. Several authors have considered *O. canariensis* (syn. *O. patens* subsp. *canariensis* (Lindl.) Asch. & Graebn.) and *Orchis patens* Desf. (Fig. 1b) as sister species (e.g. Bateman et al., 2003; Bernardos et al., 2006; Delforge, 2006; WCSP, 2022), unlike Kretzschmar et al. (2007) and Kühn et al. (2019) who considered *O. canariensis* as the basionym of *O. patens* subsp. *canariensis*. However, a recent study by Calevo et al. (2021a), has provided molecular support for the recognition of *O. canariensis* as rather a sister species to *O. patens*.

The two taxa have morphological differences. Leaves of *O. canariensis* are broader and lower down the stem, more brilliantly green and spotless (Fig. 1a), compared to *O. patens*. Whereas the flower morphology is comparable in size, however, the green centre of the sepals is smaller and unspotted in *O. canariensis*, as shown in Fig. 1a (Kretzschmar et al., 2007), and the lip basal area is larger than in *O. patens* (Kühn et al., 2019). Despite the geographical distance, the two taxa were shown to share the main fungal symbiont (Calevo et al., 2020).

Fig. 1. Inflorescences and leaves of *Orchis canariensis* from Tenerife, Canary Islands (a). The green centre of the sepals is almost undetectable and leaves are brilliant green and unspotted; b: inflorescence of *Orchis patens* from Liguria, Italy. Sepals show the characteristic green and spotted centre. Scale bars = 1 cm.

Given that seed micromorphology has generally been used as a taxonomical tool to investigate species diversity in orchids (Gamarra et al., 2007, 2008, 2012; Calevo et al., 2017; Şeker & Şenel, 2017), we compared new morphological data on one population of *Orchis canariensis* with *O. patens* and other available published information on other *Orchis* species to provide support to species delimitation. In this study, we followed the taxonomy proposed in Kühn et al. (2019) with the exception for *O. canariensis* and *O. mascula* subsp. *tenera* (Landwehr) Del Prete that we consider as a different subspecies of *O. mascula* (L.) L., but not a synonym of *O. mascula* subsp. *mascula*.

Material and Methods

Plant material

Five stems of *Orchis canariensis* (Fig. 1b) were collected on Tenerife at the *locus classicus* Aguamansa (Caldera de la Orotava) in March 2021 after capsule ripening, with the permission of local authorities (Cabildo de Tenerife № Sigma: 2021-00152). This population was previously investigated in a cytotaxonomical study (Bernardos et al., 2006). The habitat, rocky walls with mossy shelves with accumulation of litter and needles in a humid environment (potential vegetation: *Lauro novocanariensis – Perseetum indicae*), with pine plantation of *Pinus canariensis* C. Sm. ex DC. in Buch, *Pinus halepensis* Mill., and *Pinus radiata* D. Don, was characterised by the main presence of *Cistus symphytifolius* Lam., *Erica canariensis* Rivas-Mart., M. Osorio & Wildpret, and *Aeonium* sp.

Seeds from each stem were collected in paper envelops and then approximately 300 seeds from each individual were collected and pooled together for morphological analyses. For a statistical comparison between the two taxa, the same sampling protocol was applied to *O. patens* in May 2021 from a population in Breccanecca, Italy (see Calevo et al., 2021b).

Morphological and data analyses

The characterisation of seed ornamentation was carried out using a scanning electron microscope (SEM) Vega3 Tescan-type LMU on approximately 100 randomly selected seeds, following the protocol described by Calevo et al. (2017), but with a filament voltage of 20 kV. Light microscopy analysis of seeds was undertaken with a Leica DM2000 microscope. Data concerning seed length and width, embryo length and width, and cell number along the longitudinal axis were collected by analysing 100 seeds as described in Calevo et al. (2017).

Data were analysed using the R Studio v1.4 (RStudio Team, 2021). Seed morphological data of *O. canariensis* were compared with those obtained from 100 randomly selected seeds of *O. patens* with a paired t-test by using the packages rstatix (Alboukadel, 2021) and tidyverse (Wickham et al., 2019). Seed morphological data for *O. canariensis* and *O. patens* were deposited in Zenodo open repository (Calevo et al., 2022).

Quantitative and qualitative seed data were used to perform a comparative analysis between *O. canariensis* and 14 other closely related taxa (namely *O. purpurea* Huds., *O. militaris* L., *O. italica* Poir., *O. anthropophora* (L.) All., *O. provincialis* Balb. ex Lam. & DC., *O. pallens* L., *O. spitzelii* Saut. ex W.D.J.Koch, *O. spitzelii* subsp. *cazorlensis* (Lacaita) D.Rivera & Lopez Velez, *O. patens* Desf., *O. olbiensis* Reut. ex Gren., *O. mascula*, *O. mascula* subsp. *laxifloriformis* Rivas Goday & B.Rodr., *O. mascula* subsp. *ichnusae* Corrias, *O. mascula* subsp. *tenera*, and *Dactylorhiza romana* (Sebast.) Soó) previously studied by Gamarra et al. (2012), Calevo et al. (2017), and Şeker & Şenel (2017). We used a binary matrix based on 14 morphological class characters accounting for seed L/W ratio (L/W < 2, 2 < L/W < 2.5, or L/W > 2.5), cell number in the longitudinal axis (n = 5–6, n = 7–8, or n = 9–10), seed shape (clavate or fusiform), shape of the anticlinal walls (highly undulate, sinuous, or straight to sinuous), and ornamentation of the periclinal walls (slanting ridges, reticulated, or absent), to obtain Jaccard distances among species. A neighbour-joining tree was built on Jaccard distances by using the packages ape 5.0 (Paradis & Schliep, 2019) and phangorn 2.7.1. (Schliep, 2011). *Dactylorhiza romana* was used as out-group for the tree.

Results

The seed shape of *O. canariensis* is clavate and the cell number in the longitudinal axis varies from five to seven (Fig. 2a). The anticlinal walls were straight to sinuous and no ornamentations were observed in the periclinal walls (Fig. 2a,b), which is in line with Kretzschmar et al. (2007). The average seed length (313.66 ± 44.78 μm; mean ± standard deviation) did not differ significantly ($t = -0.035732$, $df = 99$, $p = 0.9716$) from *O. patens* (313.4 ± 52.73 μm), whereas both average seed width (184.31 ± 30.26 μm) and seed length/width ratio (1.72 ± 0.25) significantly differed ($t = -5.4555$, $df = 99$, $p < 0.001$ and $t = 5.7128$, $df = 99$, $p < 0.0001$, respectively) from *O. patens* (161.49 ± 29.12 μm and 1.97 ± 0.29 μm, respectively). In addition, significant differences were found in the embryo length (157.18 ± 35.21 μm) and width (125.43 ± 25.92 μm) compared to embryo length (132.97 ± 26.33 μm) and embryo width (89.79 ± 22.82 μm) in *O. patens* ($t = -5.2806$, $df = 99$, $p < 0.0001$ and $t = -9.9898$, $df = 99$, $p < 0.0001$, respectively).

Neighbour-joining tree based on Jaccard distances of seed traits (Fig. 3) divided the genus *Orchis* into two main clusters, corresponding to the subgenera *Orchis* and *Masculae* (*sensu* Kretzschmar et al., 2007). A further branching of the subgenus *Masculae* distinguished the section *Provinciales* (namely *O. provincialis* and *O. pallens*) from the sections *Robustocalcare* (*O. spitzelii* together with the subspecies *cazorlensis*, as well as *O. patens* and *O. canariensis*) and *Masculae* (namely *O. olbiensis*, *O. mascula*, and the subspecies *laxifloriformis*, *ichnusae*, and *tenera*).

Discussion

The IUCN Red List of Threatened Species (Baillie et al., 2004) is a species-driven global resource for extinction risk assessments and a practical tool that provides incentives for additional conservation measures of organisms, for example at regional and national levels. However, the fundamental assumption is that the taxa under consideration are well-defined species. Therefore, taxonomic studies are central in circumscribing species and in identifying high priorities for conservation in order to conserve biodiversity effectively (Fay, 2018). Taxonomic uncertainty and species complexes on the IUCN Red Lists overestimate the distribution and underestimate the threat level of the one species (nomen), to which the assessment purportedly applies, as well as all of the complex's unnamed members, resulting in an inaccurate/incorrect assessment (Scherz et al., 2019). In this study, we provide new information concerning the taxonomic relationships and reproductive traits of the relatively unknown *Orchis canariensis*, endemic to the Canary Islands which is currently included as subspecies of *O. patens* in the IUCN Red Lists (Rankou, 2011).

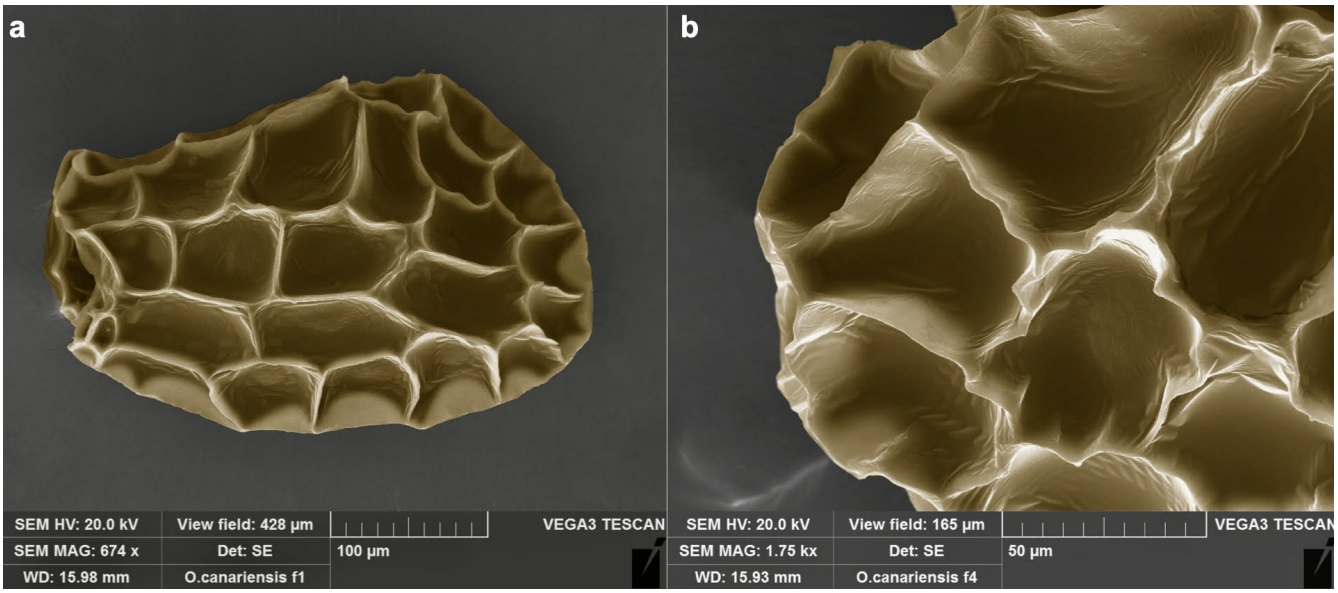

Fig. 2. Scanning electron microscopy (SEM) micrographs of *Orchis canariensis* seed (a). Clavate shape, short basal, and apical cells and elongated medial cells are visible; scale bar = 100 µm. Detailed view of seed surface (b), showing a lack of ornamentation in the periclinal walls and straight to sinuous anticlinal walls; scale bar = 50 µm.

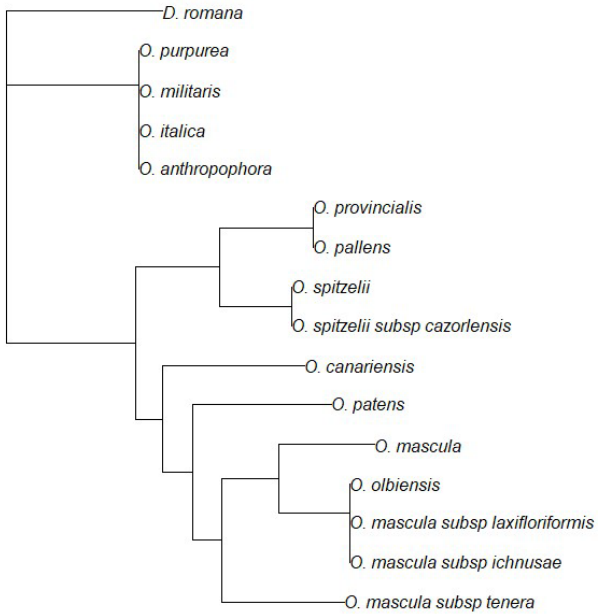

Fig. 3. Neighbour-joining tree analysis based on 14 morphological classes of seed characters of related taxa from the genus *Orchis*; data were obtained from Gamarra et al. (2012), Calevo et al. (2017), and Şeker & Şenel (2017). *Dactylorhiza romana* was used as outgroup.

Micromorphological analyses highlighted the distinguishable characters between seeds of *O. canariensis* and *O. patens* (see Calevo et al., 2017). One of the most evident and discriminating characters is that the anticlinal walls of *O. canariensis* are straighter compared to the more sinuous/undulate ones of *O. patens* (and the other members of the section *Robustocalcare* such as *O. spitzelii* and *O. spitzelii* subsp. *cazorlensis*). Furthermore, as also confirmed by a significantly lower L/W ratio, seeds of *O. canariensis* appear larger compared to *O. patens* while the seed length is similar. Moreover, the embryo of *O. canariensis* is significantly larger compared to *O. patens*, both in length and width.

The importance of seed micromorphological analyses for clarifying the taxonomic controversies within the subtribe Orchidinae has already been underlined by several authors (e.g. Gamarra et al., 2007, 2008, 2012; Güler, 2016; Calevo et al., 2017; Şeker & Şenel, 2017; Şeker et al., 2021). Our phylogenetic analysis based on seed morphological characters revealed a clusterisation of the two subgenera *Orchis* and *Masculae*, and a separation into two branches of *O. patens* and *O. canariensis*. Indeed, while no differences were detected between species/subspecies in many sections (e.g. in *Provinciales* between *O. provincialis* and *O. pallens*; in *Robustocalcare* between *O. spitzelii* and *O. spitzelii* subsp. *cazorlensis*; in *Masculae* between *O. olbiensis*, *O. mascula* subsp. *laxifloriformis* and *O. mascula* subsp. *ichnusae*), the separation of *O. patens* and *O. canariensis* from the other taxa of the section *Robustocalcare*, probably due to their different ploidy level (see Calevo et al., 2021a), and between themselves offers a morphological support to the recent species delimitation suggested by molecular markers (Calevo et al., 2021a) which is worth further investigations including more populations. *Orchis canariensis*, currently listed as «Endangered» in the IUCN European Red List

as subspecies of *O. patens* (Rankou, 2011), would therefore need the first assessment as a separate taxon, while *O. patens* should be re-assessed in order to correctly inform and catalyse action for their conservation.

Conclusions

In conclusion, scanning electron microscopy showed the qualitative characteristics of *O. canariensis* seeds such as a clavate shape, straight to slightly sinuous anticlinal walls and absence of ornamentations in the periclinal walls. Even if only considering one population from Tenerife, these qualitative data, together with quantitative results from seed micromorphology seems to support species delimitation between *O. canariensis* and *O. patens* recently proposed by means of molecular markers (Calevo et al., 2021a). These results show once again that seed morphology could be a useful tool for taxonomical studies. However, future investigations should include *O. patens* from North Africa and more populations from Canary Islands. Taken together, the phylogenetic separation based on seed micromorphology here obtained and the recently published molecular data (Calevo et al., 2021a), highlights the need of assessing *O. canariensis* for the first time according to the guidelines of the IUCN Red List as a separate taxon and to update the assessment of *O. patens*. Further studies on the reproductive fitness and for the conservation of *Orchis canariensis* should be encouraged.

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МИКРОМОРФОЛОГИЯ СЕМЯН ПОДТВЕРЖДАЕТ ВЫДЕЛЕНИЕ ВИДА *ORCHIS CANARIENSIS* (ORCHIDACEAE), ЭНДЕМИЧНОЙ ОРХИДЕИ КАНАРСКИХ ОСТРОВОВ

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Географическая изоляция является одним из основных факторов видообразования. Но острова остаются резервуаром незамеченных и недостаточно изученных эндемичных видов. В этом исследовании мы представили микроморфологические данные для семян *Orchis canariensis* (Orchidaceae), исчезающего вида, встречающегося только на Канарских островах (Испания), таксономия которого обсуждалась в последние десятилетия. Цель исследования заключалась в выявлении микроморфологических признаков семян в поддержку разграничения видов с помощью световой и сканирующей электронной микроскопии. Семена из популяции *O. canariensis* на Тенерифе – булавовидные с числом клеток по продольной оси от пяти до семи. Семена показали наличие антиклинальных стенок (от прямолинейных до извилистых) и отсутствие орнаментации на периклинальных стенках. Средняя длина семени была 313.66 ± 44.78 µm, средняя ширина семени – 184.31 ± 30.26 µm с отношением 1.72 ± 0.25; длина и ширина зародыша были 157.18 ± 35.21 µm и 125.43 ± 25.92 µm, соответственно. Однако, несмотря на сходство с сестринским видом, *Orchis patens*, количественные и качественные признаки семян подтверждают разграничение видов, недавно предложенное с помощью методов молекулярной биологии. Принимая во внимание выше сказанное, наши результаты подтвердили важность микроморфологии семян для таксономических исследований.

Ключевые слова: *Orchis patens*, Красный список, островная флора, сканирующая электронная микроскопия, таксономия, сохранение орхидей, Тенерифе, эндемичный вид