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Pellaea calomelanos (Pteridaceae) in Catalonia: is it really a very old disjunction?

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Amb motiu de la celebració de l'any Codina, en el 150è aniversari del seu naixement, els autors d'aquest treball volen retre un modest homenatge al metge i botànic Joaquim Codina i Vinyes (La Cellera de Ter 1867-1934), descobridor de la primera població catalana i europea de la planta estudiada.

On the occasion of the celebration of Codina's year, for his 150 birthday, the authors of this work want to pay a modest homage to the doctor and botanist Joaquim Codina i Vinyes (La Cellera de Ter, 1867–1934), discoverer of the first Catalan and European population of the studied plant.

Abstract

PELLAEA CALOMELANOS (PTERIDACEAE) IN CATALONIA: IS IT REALLY A VERY OLD DISJUNCTION?— *Pellaea calomelanos* is a species discovered in Africa and whose distribution area has been expanding more recently to Asia and to a single European locality, including three populations, in Catalonia. Both, the fact of belonging to ferns and presenting this disjoint distribution fostered the idea of a relict species resulting from an extensive distribution in remote times. The 2C-values range from 16.45 pg for the individual of the Reunion Island to 17.40 pg for the population of Boadella (Catalonia). Although a certain variability exists, no statistically significant differences among them have been found. The phylogenetic analysis reveals a well-supported clade grouping all the individuals of the different populations of *P. calomelanos* but without any kind of internal resolution. The results of this work, based on measures of nuclear DNA amount and also on two regions of chloroplast DNA sequencing, together with the characteristics of its habitat, allow the authors to hypothesize about a recent colonisation of the European continent by this species.

Keywords: Bayesian inference; ferns; nuclear DNA amount; phylogenetic analyses; Pteridaceae; 2C-value.

Resumen

PELLAEA CALOMELANOS (PTERIDACEAE) EN CATALUÑA: ES REALMENTE UNA DISYUNCIÓN ANCESTRAL?— *Pellaea calomelanos* es una especie que fue descubierta en África y cuya área de distribución se ha ido ampliando más recientemente a Asia y a una única localidad europea, que comprende tres poblaciones, en Cataluña. El hecho de pertenecer a los helechos y de presentar esta distribución disyunta alimentaron la idea de una especie relicta resultante de distribución amplia en tiempos remotos. Los valores 2C van desde 16,45 pg para el individuo de la Isla de la Reunión hasta 17,40 pg para la población de Boadella (Cataluña). Aunque existe una cierta variabilidad, no se han encontrado diferencias estadísticamente significativas entre ellos. El análisis filogenético revela un clado bien soportado que agrupa a todos los individuos de las diferentes poblaciones de *P. calomelanos* pero sin ningún tipo de resolución interna. Los resultados del presente trabajo, basado en medidas de cantidad de ADN nuclear y en secuencias de dos regiones del ADN cloroplástico, junto con las características de su hábitat, permiten a los autores hipotetizar sobre una colonización reciente del continente europeo por esta especie.

Palabras clave: análisis filogenéticos; cantidad de ADN nuclear; helechos; inferencia bayesiana; *Pteridaceae*; valor 2C.

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INTRODUCTION

Pellaea calomelanos (Sw.) Link (Fig. 1) was first described by Swartz (1801), under the name of *Pteris calomelanos* Sw., based on a specimen of South Africa that had previously been wrongly attributed to *Pteris hastata* Thunb. (Anthony, 1984). Later, Link (1841) recombined it into the genus *Pellaea* Link.

Regarding the systematic placement of the genus, it has been included in several families such as Adiantaceae, Pteridaceae, Polypodiaceae and Sinopteridaceae (Valentine, 1964; Anthony, 1984; Muñoz Garmendia, 1986; Valentine *et al.*, 1993; Bolòs *et al.*, 2005). Currently, it is placed in the family of Pteridaceae E. D. M. Kirchn. (PPG I, 2016).

The genus *Pellaea*, like others closely related, includes especially apogamous species in which prothallus grows the sporiferous plant without fertilization. In this way, reproduction does not depend on water, what is beneficial for plants that inhabit dry places. Sporangium, with a coarse sporoderma or ornamented with crests, typically produces 32 spores (Tryon, 1957; Gastony & Windham, 1989; Grusz, 2016).

Swartz (1801) considered *P. calomelanos* an only African species, with a distribution area that would encompass territories of Zambeze, Angola, Abyssinia and the island of Reunion, the largest of Mascarene archipelago located in southern Africa. Later this area expanded, as populations were

found on the southern slopes of the Himalayas in the NW of India, specifically in the Simla region (Berthet, 1971; Bhakuni *et al.*, 2013), as well as in Madagascar and the Comores (Anthony, 1984).

In Catalonia, this plant was found for the first time as a population with a few individuals in rocky areas near the Pasteral dam (la Selva district) by the doctor and botanist J. Codina (Codina, 1908), this representing the first European record of the taxon. This botanist informed the specialist C. Christensen, who determined this species as *Pellaea hastata* (Codina, 1908). However, the following year was described as a new species, named *Pteris codinae* Cadevall et Pau (Cadevall, 1909). Finally, Barnola (1915) correctly determined it as *Pellaea calomelanos*.

In spite of confusions about the synonymy of this taxon (Barnola, 1915; Berthet, 1971), it is clear that its specific level is universally accepted and its distribution has been extended according to new locations that have been discovered. Terradas & Brugués (1973) discovered a new station of this species in Catalonia, on the rocky coasts of Sant Llorenç de la Muga, in the Alt Empordà district. This species is represented in this area by several almost continuous populations, which include about 1500 individuals (Sáez *et al.*, 2010) growing on sunny slopes ranging from the dam known as Boadella to Montdavà (municipality of Maçanet de Cabrenys), through Riambau (municipality of Sant Llorenç de la Muga). The current distribution of the species includes, in addition to the African, Asian

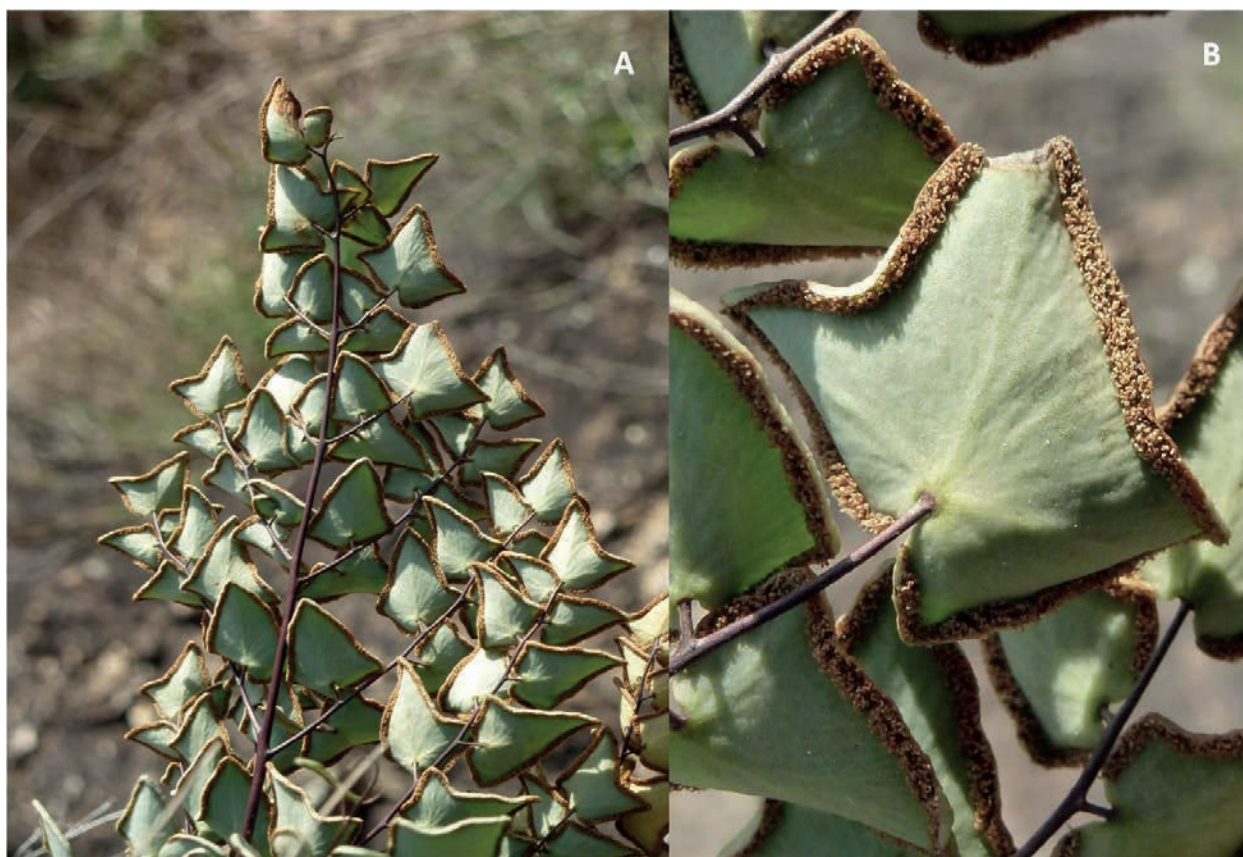


Figure 1. *Pellaea calomelanos*. (A), general appearance; (B), frond and sporangia (detail). Photographs by J. Luque.

and Catalan territories mentioned above—which constitute the only European records for the species, the Azores Islands (Macaronesia) (Sáez *et al.*, 2010). Muñoz Garmendia (1986) considers the species subspontaneous in Macaronesia and Valentine *et al.* (1993) mentions the Azores, indicating that it has a doubtful status in this area; this last work also cites another species of the genus, *P. viridis* (Forskål) Prantl, as naturalized in that archipelago.

Although the distribution of this taxon is more extensive than previously thought, what remains surprising and what has really aroused our interest, is the disjunctions that it presents, as well as the origin and the age of these disjunctions, although other taxa, such as *Crassula campestris* (Eckl. & Zeyh.) Walp., and some *Cheilanthes* Sw., *Andropogon* L. or *Hyparrhenia* Andersson ex E. Fourn. species have a similar distribution (Terradas & Brugués, 1973). This fact, together with the phylogenetic position of this species, placed far from its congeneric species (Kirkpatrick, 2007), and its inclusion in the list of endangered taxa in the *Llibre vermell de les plantes vasculares endèmiques i*

amençades de Catalunya (Sáez *et al.*, 2010) make it even more susceptible to being studied. It should be noted that none of the previous phylogenetic studies including this species have used specimens of the Catalan populations. To date, the sequenced specimens mainly come from South Africa.

On the other hand, neither karyological nor cytogenetic studies have been carried out on this species, and only one chromosomal count revealed that it was a triploid with $2n = 3x = 90$ chromosomes (Berthet, 1971). In general, cytogenetic studies in ferns, including fluorescent *in situ* hybridization and genome size measurements, were scarce, for the high number of chromosomes and the difficulty in obtaining material, mainly due to their reproduction system. Recently, some research has deeply addressed this issue (Ekrt *et al.*, 2009) contributing to increase the number of taxa with genome size data (110 newly studied species have been provided in Clark *et al.*, 2016).

In this context, new questions have arisen, which constitute the general objectives of this work: What are the levels of genetic and cytogenetic

differentiation among the populations of *P. calomelanos*? Do they indicate an ancient fragmentation that could constitute a vestige of a Tertiary Paleo-African flora or should we think that there have been phenomena of relatively recent long-distance dispersion?

MATERIALS AND METHODS

Plant material

The origin of the material studied is shown in Table 1. From each population, samples of fresh material were collected to measure the nuclear DNA amount (see following sections) and part of the same material was kept in silica gel for DNA sequencing. Plant material was collected in the three populations (two of them could be considered subpopulations of a larger population) existing in Catalonia (see Table 1).

In addition, fresh and dried material was obtained from a single individual from the Reunion Island and also dry material from the island of Madagascar and the Comores Islands from the herbarium of the Muséum national d'Histoire naturelle, Paris (P). Unfortunately, it has not been possible to obtain material from India and the Azores, despite we have made several attempts.

Genome size assessments

Nuclear DNA amount has been estimated using the flow cytometry technique. Five individuals from each of the three collected Catalan populations and one individual from the Reunion Island have been processed. Each individual has been measured twice and wheat (*Triticum aestivum* L. 'Chinese Spring' or 'Triple Dirk'; $2C = 30.9$ pg; Marie & Brown, 1993) has been used as the internal standard. Measurements have been carried out in the Scientific and Technological Centres of the University of Barcelona with a XL cytometer (Coulter Corporation, Hialeah, USA) according to the protocol described in Garcia *et al.* (2013). The results obtained have been analysed by one-way ANOVA, carried out using XLSTAT (v2007.5, Addinsoft SARL), to detect possible differences between the amounts of DNA averages obtained for the different populations.

DNA sequencing

Total genomic DNA has been extracted from individuals obtained in the collections and from herbarium materials, and two regions of chloroplast DNA, *rps4-trnS* and *trnL-F*, have been sequenced following the protocols described in Kirkpatrick (2007). One individual from each studied population has been sequenced for both regions and added to a concatenated matrix comprising 122 species provided by Eiserhardt *et al.* (2011). Sequences of *Pellaea calomelanos* from one Asian population (Wang *et al.*, 2015a, b) have also been included in Bayesian analysis. *Calciophlopteris ludens* (Wall. ex Hook.) Yesilyurt & H. Schneid. has been used as outgroup according the previous phylogeny published by Eiserhardt *et al.* (2011).

The General Time Reversible model (GTR + I + G) was chosen for both cpDNA datasets based on AIC criterion implemented in jModeltest v2.1.2 (Darriba *et al.*, 2012). Although the model obtained was the same for both chloroplast regions, two partitions were taken into account to carry out the analysis. Markov Chain Monte Carlo (MCMC) analysis was carried out in MrBayes v3.2.6 (Ronquist *et al.*, 2012) for 10,000,000 generation sampling every 100 generations. The first 25% of trees were discarded as the "burn in" period. Bayesian analyses were conducted within the CIPRES Science Gateway (Miller *et al.*, 2010), and the resulting summary trees were visualized in Figtree v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree>).

RESULTS AND DISCUSSION

Genome size

The results of nuclear DNA assessments are shown in Table 1. The $2C$ -values range from 16.45 pg for the individual of the Reunion Island to 17.40 pg for the population of Boadella, and from 16.68 pg for the population of Montdava to 17.40 of the mentioned population of Boadella, for the Catalan populations. Although we observe a certain variability in these values, the differences obtained are not significant ($F = 2.188$, $df = 13$, $p = 0.158$). These values indicate a homogeneity in ploidy levels and most likely in chromosome numbers of these populations. Attempts to confirm the chromosome number

Table 1. Origin, 2C-value averages and Genbank accession numbers of the studied populations of *Pellaea calomelanos* (Sw.) Link. SD = standard deviation; HPCV = half peak coefficient of variation.

Origin	2C (pg) ± SD	HPCV standard ± SD	HPCV target ± SD	Genbank accession number <i>rps4-trnS</i>	Genbank accession number <i>trnL-F</i>
Spain, Catalonia, Mines de Montdava, <i>Garnatje & Luque</i> , 26.XI.2011	16.68 ± 0.44	1.09 ± 0.67	2.12 ± 0.88	MK476516	MK476514
Spain, Catalonia, Boadella, <i>Garnatje & Luque</i> , 26.XI.2011	17.40 ± 0.45	1.75 ± 0.80	3.08 ± 0.87	MK476518	MK476513
Spain, Catalonia, Pasteral 1, la Cellera de Ter, <i>Garnatje & Luque</i> , 10.XII.2011	16.98 ± 0.62	2.09 ± 0.33	3.20 ± 0.95	MK476517	MK476512
Spain, Catalonia, Pasteral 2, la Cellera de Ter, <i>Garnatje & Luque</i> , 10.XII.2011	16.99 ± 0.66	1.93 ± 0.69	3.21 ± 0.95	–	–
France, Reunion Island, <i>Y. Robert</i> , 2012	16.45	–	–	MK476520	MK476511
Comores Islands [Muséum national d'Histoire naturelle, Paris (P)]	–	–	–	MK476515	MK476509
Madagascar [Muséum national d'Histoire naturelle, Paris (P)]	–	–	–	MK476519	MK476510
China (<i>Wang et al.</i> , 2015a, b)	–	–	–	KP126979	KP085545
South Africa (<i>Lu et al.</i> , 2012)	–	–	–	JF980612	–

of $2n = 3x = 90$, given by Berthet (1971) from Pasteral, the only Catalan population then known, have not been successful in the present study.

Our data are the first report on the genome size in the studied species. Within the genus *Pellaea*, only two taxa had been the object of this type of research, both with smaller genome size than we have found. On the one hand, *P. glabella* Mett. ex Kuhn subsp. *glabella* shows a smaller genome size than *P. calomelanos* [$2C = 13.62$ pg (Bainard *et al.*, 2011); $2C = 13.62$ pg and $2C = 13.78$ pg (Henry *et al.*, 2015)] despite having a larger number of chromosomes [$2n = 4x = 116$; Britton (1953) and Rigby (1973)]. On the other hand, Clark *et al.* (2016) have established $2C = 10.40$ pg for *Pellaea atropurpurea* (L.) Link., with an estimated chromosome number of $2n = 87$. *Pellaea glabella* and *P. atropurpurea*, the species above mentioned, are included together in a very distant clade from the one of *Pellaea calomelanos* in the phylogenetic tree (Fig. 2).

The important differences in genome size in the three *Pellaea* species to date studied from this viewpoint suggest quite different chromosome numbers and ploidy levels in the genus. As stated, Berthet

(1971) counted $2n = 90$ and indicated a triploid level in *P. calomelanos*, for which we present 2C values around 16 pg (see Table 1 for precisions). Data on *P. atropurpurea* and *P. glabella* are of 2C genome sizes around 10 and 13 pg and with estimated chromosome numbers of $2n = 87$ (supposedly $3x$) and 116 (supposedly $4x$), respectively (Clark *et al.*, 2016, and references therein). It is difficult to attribute a so big difference in genome between the supposed triploids with only a difference of three out of 90 chromosomes in the complement. Chromosome counts and genome size assessments are needed in these and other species of the genus in order to clarify its genome evolution and its possible polyploid series.

Phylogenetic analysis

The tree obtained by Bayesian inference (Fig. 2) confirms that *Pellaea* is not a monophyletic genus, as several authors have already pointed in previous studies (Kirkpatrick, 2007; Eiserhardt *et al.*, 2011). It should also be noted that *P. calomelanos* appears in a clade with other species belonging to



Figure 2. Phylogenetic reconstruction based on *rps4-trnS* and *trnL-F*. Posterior probability values are indicated on branches of the tree obtained by Bayesian inference.

the genera *Pellaea* and *Cheilanthes* (PP = 1; Fig. 2). Although, according to Eiserhardt *et al.* (2011), the divergence between *Pellaea calomelanos* and *Cheilanthes viridis* Sw. occurred about 10 million years ago, no differentiation at all occurred in *P. calomelanos*: as we can observe, all the individuals of the different populations of *P. calomelanos* (including those from Catalonia) are grouped in a well-supported clade (PP = 1) regardless of their origin, but without any kind of internal resolution. Not a single mutation (SNPs or indels) was found among the sequences of *Pellaea calomelanos* obtained for this study.

Are there supports for an old disjunction?

The fact that no differences between the African, Asian and Catalan (as stated, the only European) populations have been found does not support an old disjunction as proposed by Terradas & Brugués (1973). However, this cannot be categorically ruled out with available data and additional analyses, including missing populations, are needed. In any case, genome size homogeneity in *P. calomelanos* and the characteristics of the habitat where this species grows in Catalonia also support the hypothesis of a recent origin for the disjunct distribution of this

species. All sampled populations occur in removed soils of very disturbed areas, two of them growing on the banks of recent infrastructures (beginnings and mid-20th century, respectively) in Pasteral and Boadella dams, and the third one occurring in the abandoned mines of Montdava (active from the end of the 18th century to the beginning of the 20th century). Indeed, some arguments rather contradict the hypothesis of an ancient broader distribution area and some current relict populations in refugia: (1) the first to record this taxon in Europe stated that there were just a few stocks in rocks near Pasteral dam (Codina, 1908), although we cannot know the reach of his search, (2) now the species is more abundant there, and also occupies places in close and similar locations, and (3) even though *P. calomelanos* does not require a ruderal habitat, all the stations where this species lives in Catalonia have suffered a serious impact by the construction of dams or mines from the end of 18th to mid-20th centuries.

According to Eiserhardt *et al.* (2011), an enlargement of its ecological niche would have allowed *P. calomelanos* and other related species to colonise new areas from the Cape Floristic region, their putative area of origin. Although this statement is a possible explanation for the colonisation of new environments, it does not explain the particular distribution of the species here considered. In addition, these species occurring in large distribution area show some genetic differentiation.

A similar case of disjunct areas was recorded by Fernández-Brime *et al.* (2014) on *Diploschistes rampoddensis* (Nyl.) Zahlbr., a lichen with a few localities in tropical Asia and Oceania reported for a first time in Sant Llorenç de la Muga (one of the Catalan places where *P. calomelanos* grows) by these authors. Although the only sample from Papua New Guinea appears merged in the same clade with the samples from Catalonia, the authors do not hypothesise about the disjunction age. Other fern species also show similar disjunct distributions as *Pellaea calomelanos*, with isolated occurrences in the Mediterranean area, South and East Africa, as well as West Asia locations (e.g. Pichi Sermolli, 1979). This disjunct distribution mirrors the Rand Flora pattern, precisely named for the first time by Christ (1910) in his *Die Geographie der Farne* (i.e. *The Geography of Ferns*). This biogeographical pattern was traditionally explained by the

fragmentation of an ancient Tertiary African flora, but recent hypotheses support dispersal events between geographically isolated areas (Sanmartín *et al.*, 2010; Pokorný *et al.*, 2015). Indeed, there are other well-documented cases of long distance dispersal of ferns in Europe (e.g. Molnár, 2008; Ekrt & Hrivnák, 2010), suggesting the important role of this mechanism to explain the biogeography on this group of plants (Moran, 2008).

Further studies enlarging the sampling and extending the number of sequenced DNA regions are necessary to determine the validity of the relationships among *P. calomelanos* populations and whether this is a case of disjunction or long distance dispersal, and, whatever process it was, could it also involve other related taxa.

Regardless of the age of colonisation of this species, we believe that protective measures and its inclusion in the catalogue of threatened plants of Catalonia (Sáez *et al.*, 2010) continue to be necessary in order to guarantee the conservation of the only and scarce European localities of this species.

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