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Short communication. Phylogeny and genetic diversity within Iberian populations of *Ornithopus* L. and *Biserrula* L. estimated using ITS DNA sequences

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Abstract

Genetic diversity within Iberian populations of *Ornithopus pinnatus, O. compressus, O. sativus* and *Biserrula pelecinus* were assessed using ITS1 and ITS2 DNA sequences from sixty four specimens, and a phylogeny between *Ornithopus* species was estimated. Generally within-species variation was low, particularly within *Ornithopus*. The Mediterranean species of *Ornithopus* form a sister clade relative to the South American *O. micranthopus.* The sometimes considered a full species, *O. sativus isthmocarpus*, was not distinct from *O. sativus.* Between some species there is limited genetic divergence using these markers, although the situation of *O. perpusillus* requires additional specimens to be examined before firm conclusions can be drawn.

Additional key words: *Biserrula pelecinus*; forage; *Ornithopus compressus*; *Ornithopus pinnatus*; *Ornithopus sativus.*

Resumen

Comunicación corta. Filogenia y diversidad genética dentro de poblaciones ibéricas de *Ornithopus* L. y *Biserrula* L. utilizando secuencias ITS de ADN

Se evaluó la diversidad genética entre 64 poblaciones ibéricas de *Ornithopus pinnatus, O. compressus, O. sativus* y *Biserrula pelecinus* utilizando secuencias ITS1 e ITS2 de ADN, y se estimó la filogenia entre las especies de *Ornithopus.* En general la variación intraespecífica fue baja, especialmente dentro de *Ornithopus.* Las especies mediterráneas de *Ornithopus* forman un clado hermano del *O. micranthopus* sudamericano. La considerada a veces como una especie completa, *O. sativus isthmocarpus*, no fue distinta de *O. sativus.* Utilizando estos marcadores existe una divergencia genética limitada entre algunas especies, aunque la situación de *O. perpusillus* requiere el examen de muestras adicionales antes de que se puedan establecer conclusiones firmes.

Palabras clave adicionales: *Biserrula pelecinus*; forraje; *Ornithopus compressus*; *Ornithopus pinnatus*; *Ornithopus sativus.*

Biserrula pelecinus and species of *Ornithopus* (family Fabaceae) are important annual legumes used as valuable pasture species. *Biserrula* is monotypic, with *B. pelecinus* distributed around the Mediterranean basin, in the Canary Islands and in the highlands of Ethiopia, in Northeast Africa. *Ornithopus* is typically considered to include four species —*O. compressus, O. perspusillus, O. pinnatus* and *O. sativus*—

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Abbreviations used: ITS (nuclear ribosomal internal transcribed spacer); ML (maximum likelihood); PCR (polymerase chain reaction).

from the Mediterranean basin, and *O. micranthus* from South America (Fu *et al.,* 1994). *Ornithopus sativus isthmocarpus* is also sometimes recognized as a distinct species (Allan & Porter, 2000). All the species are adapted to sandy soils and are highly palatable. *Biserrula pelecinus* and some *Ornithopus* (*O. compressus, O. sativus* and *O. pinnatus*) in particular have become used for forage in disparate regions with Mediterranean climate, especially Australia (Nichols *et al.,* 2010) and Chile (Del Pozo & Ovalle, 2009). Sowing *O. sativus* pasture between cropping sequences in Australia has shown to benefit grain production through restoring soil fertility (Doole *et al.,* 2009), and during the last 15 years both *O. sativius* and *B. pelecinus* have been commercialised in Australia (Nichols *et al.,* 2010).

Given this agricultural importance surprisingly little is known regarding relationships between *Ornithopus* species or levels of genetic variation within species. Biodiversity of root-nodule bacteria has been assessed for both *B. pelecinus* (Vicente *et al.,* 2009) and *O. compressus* (Loi *et al.,* 1999). Determination of morphological differences between species of *Ornithopus* indicated considerable differences of important characteristics such as stem length or time of first flowering (Fu *et al.,* 1994), so that Loi *et al.* (1997) concluded there was sufficient morphological variation to initiate a selection program for Southern Australia. Such programs benefit from an assessment of genetic variation within species, and of the phylogenetic relationships between them.

The aim of this study was to determine genetic variation within *Biserrula pelecinus* and the species of *Ornithopus* from the Iberian Peninsula using ribosomal internal transcribed spacer (ITS) sequences. At the same time by incorporating data from GenBank for *O. micranthus*, as well as some additional samples of these species, a phylogeny for the genus can be determined. This gene region is widely used both in phylogeny reconstruction in plants (Allan & Porter, 2000), and in barcoding studies to differentiate species (Chen *et al.,* 2010). It has also been recently used to assess variation within other species from the region, *Scorpiurus muricatus* and *S. vermiculatus* (Visnevschi-Necrasov *et al.,* 2011). Thus direct comparisons of relative within-species genetic diversity can be made. This should help resolving the taxonomic issue, such as the distinctiveness or not of *O. s. isthmocarpus*, and to identify genetically divergent groups that might be important for breeding programs.

Sixty four specimens of *Biserrula and Ornithopus* were collected (Table 1) in Portugal and Western Spain (Fig. 1). In order to collect only wild germplasm seeds were collected in natural occurring plants from field borders and road sides. DNA was extracted using a cetyl trimethylammonium bromide (CTAB)-based protocol following Wang *et al.* (1996). The ITS1 and ITS2 region was amplified by polymerase chain reaction (PCR) using standard primers (White *et al.,* 1990). Amplifications were performed in 20 μL reactions consisting of approximately 10 ng DNA template, 1 μM of each primer, 200 μM of each dNTP, 0.5 U EcoTAQ DNA polymerase, 2 μL of 10X PCR buffer and 1.5 mM $MgCl_2$. The amplification protocol consisted of an initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30s, 53°C for 30s and 72°C for 1 min. A final extension step at 72°C for 7 min was performed. PCR products were purified using the JetQuick (Genomed, Löhne, Germany) micro spin kit and sequenced using the same primers on an ABI 3730 DNA sequencer using BigDyeTerminator v3.1 from the same supplier.

Sequences were aligned with the available sequences from GenBank for these genera using Clustal W with default conditions in the program BioEdit v5.0.9 (Hall, 1999). Within closely related groups the program TCS v2.1 (Clement *et al.,* 2000) was used to create a parsimonious network of the aligned haplotypes. To estimate phylogenetic relationships of *Ornithopus* all unique haplotypes were aligned. Maximum likelihood (ML) analysis with random sequence addition (100 replicate heuristic searches) was used to estimate their evolutionary relationships, using the program PAUP v4.0b10 (Swofford, 2002). Support for nodes was estimated using the bootstrap technique (Felsenstein, 1985) with 1000 replicates. The model of evolution employed was chosen using the Akaike Information Criteria carried out in Modeltest 3.06 (Posada & Crandall, 1998). Bayesian analysis was implemented using Mr. Bayes v.3.1 (Huelsenbeck & Ronquist, 2001) with parameters estimated as part of the analysis. The analysis was run for 1×10^7 generations, saving one tree every 1000 generations. The log-likelihood values of the sample point were plotted against the generation time and all the trees prior to reaching stationary were discarded as burn-in samples. Remaining trees were combined in a 50% majority consensus tree (Huelsenbeck & Ronquist, 2001). New haplotypes have been submitted to GenBank (JQ042900 toJQ042909).

Table 1. Identification (ID) number, species and coordinates of the collection sites for the 64 samples used in this study. Haplotype refers to Figure 2

ID number	Species	Haplotype	Collection site		Province and country
1	*Biserrula pelecinus*	7	39º44'00'' N	7º25'16'' W	Beira Baixa, Portugal
2	*Biserrula pelecinus*	7	39º06'49'' N	7º16'82'' W	Alto Alentejo, Portugal
3	*Biserrula pelecinus*	10	37º58'00'' N	5º54'00'' W	Andalucía, Spain
4	*Biserrula pelecinus*	11	39º09'89'' N	8º39'15'' W	Ribatejo, Portugal
5	*Biserrula pelecinus*	7	41º26'29'' N	7º10'14'' W	Trás-os-Montes, Portugal
6	*Biserrula pelecinus*	7	41º25'35'' N	7º22'14'' W	Trás-os-Montes, Portugal
7	*Biserrula pelecinus*	7	38º05'00'' N	7º09'00'' W	Alto Alentejo, Portugal
9	*Biserrula pelecinus*	7	37º52'00'' N	5º40'00'' W	Andalucía, Spain
10	*Biserrula pelecinus*	7	38º27'00'' N	7º26'00'' W	Alto Alentejo, Portugal
13	*Biserrula pelecinus*	9	39º01'51'' N	8º41'10'' W	Ribatejo, Portugal
14	*Biserrula pelecinus*	8	38º58'00'' N	7º04'00'' W	Alto Alentejo, Portugal
15	*Biserrula pelecinus*	7	38º01'00'' N	5º42'00'' W	Andalucía, Spain
16	*Biserrula pelecinus*	7	37º52'00'' N	5º40'00'' W	Andalucía, Spain
17	*Biserrula pelecinus*	7	37º52'00'' N	5º41'00'' W	Andalucía, Spain
18	*Biserrula pelecinus*	11	37º59'00'' N	5º58'00'' W	Andalucía, Spain
19	*Biserrula pelecinus*	11	37º52'00'' N	6º16'00'' W	Andalucía, Spain
115	*Ornithopus compressus*	1	41º12'44'' N	7º32'00'' W	Trás-os-Montes, Portugal
117	*Ornithopus compressus*	1	39º51'00'' N	7º21'49'' W	Beira Baixa, Portugal
118	*Ornithopus compressus*	1	40º44'00'' N	7º20'21'' W	Beira Alta, Portugal
119	*Ornithopus compressus*	1	38º49'02'' N	7º51'00'' W	Alto Alentejo, Portugal
112	*Ornithopus compressus*	1	39º49'54'' N	7º12'42'' W	Beira Baixa, Portugal
121	*Ornithopus compressus*	4	40º49'18'' N	7º16'57'' W	Beira Alta, Portugal
122	*Ornithopus compressus*	4	40º50'51'' N	7º14'88'' W	Beira Alta, Portugal
123	*Ornithopus compressus*	6	38º23'13'' N	8º34'00'' W	Estremadura, Portugal
124	*Ornithopus compressus*	1	40º49'18'' N	7º17'00'' W	Beira Alta, Portugal
125	*Ornithopus compressus*	1	38º46'00'' N	4º46'00'' W	Castilla-La Mancha, Spain
126	*Ornithopus compressus*	1	38º12'00'' N	7º29'00'' W	Baixo ALentejo, Portugal
127	*Ornithopus compressus*	1	38º08'00'' N	7º22'00'' W	Baixo ALentejo, Portugal
128	*Ornithopus compressus*	1	37º59'00'' N	5º58'00'' W	Andalucía, Spain
129	*Ornithopus compressus*	1	37º52'00'' N	6º16'00'' W	Andalucía, Spain
130	*Ornithopus compressus*	1	37º59'00'' N	5º34'00'' W	Andalucía, Spain
131	*Ornithopus compressus*	1	37º59'00'' N	5º58'00'' W	Andalucía, Spain
132	*Ornithopus compressus*	6	37º57'00'' N	6º03'00'' W	Andalucía, Spain
133	*Ornithopus compressus*	1	37º57'00'' N	6º55'00'' W	Andalucía, Spain
134	*Ornithopus compressus*	1	38º09'00'' N	6º59'00'' W	Baixo ALentejo, Portugal
135	*Ornithopus compressus*	1	37º52'00'' N	6º30'00'' W	Andalucía, Spain
136	*Ornithopus compressus*	1	37º59'00'' N	5º60'00'' W	Andalucía, Spain
140	*Ornithopus compressus*	1	37º58'00'' N	5º54'00'' W	Andalucía, Spain
141	*Ornithopus compressus*	1	38º49'00'' N	4º43'00'' W	Castilla-La Mancha, Spain
144	*Ornithopus compressus*	1	37º52'00'' N	5º40'00'' W	Andalucía, Spain
145	*Ornithopus compressus*	1	38º08'10'' N	7º02'00'' W	Baixo ALentejo, Portugal
147	*Ornithopus compressus*	1	38º09'00'' N	7º01'00'' W	Baixo ALentejo, Portugal
148	*Ornithopus compressus*	1	38º42'00'' N	5º04'00'' W	Andalucía, Spain
150	*Ornithopus compressus*	1	38º36'00'' N	7º25'00'' W	Alto Alentejo, Portugal
152	*Ornithopus compressus*	6	37º55'00'' N	6º49'00'' W	Andalucía, Spain
153	*Ornithopus compressus*	6	37º54'00'' N	6º12'00'' W	Andalucía, Spain
156	*Ornithopus compressus*	1	38º48'00'' N	4º53'00'' W	Castilla-La Mancha, Spain
157	*Ornithopus compressus*	1	37º58'00'' N	7º27'00'' W	Baixo ALentejo, Portugal
158	*Ornithopus compressus*	5	37º52'00'' N	5º46'00'' W	Andalucía, Spain
159	*Ornithopus compressus*	6	38º12'00'' N	7º32'00'' W	Alto Alentejo, Portugal
161	*Ornithopus compressus*	5	39º41'00'' N	7º24'00'' W	Beira Baixa, Portugal
162	*Ornithopus compressus*	1	38º57'03'' N	8º58'30'' W	Estremadura, Portugal
163	*Ornithopus compressus*	1	39º09'89'' N	8º39'15'' W	Ribatejo, Portugal

Table 1 (cont.). Identification (ID) number, species and coordinates of the collection sites for the 64 samples used in this study. Haplotype refers to Figure 2

ID number	Species	Haplotype	Collection site		Province and country
164	*Ornithopus compressus*	1	40º20'09'' N	7º24'10'' W	Beira Baixa, Portugal
165	*Ornithopus compressus*	6	41º10'11'' N	7º02'32'' W	Trás-os-Montes, Portugal
167	*Ornithopus pinnatus*	1	40º20'09'' N	7º24'10'' W	Beira Baixa, Portugal
168	*Ornithopus pinnatus*	1	41º17'25'' N	5º44'25'' W	Castilla y León, Spain
169	*Ornithopus pinnatus*	1	38º21'19'' N	8º32'35'' W	Alto Alentejo, Portugal
170	*Ornithopus pinnatus*	1	38º39'54'' N	8º43'85'' W	Estremadura, Portugal
171	*Ornithopus pinnatus*	1	37º35'32'' N	8º44'41'' W	Baixo Alentejo, Portugal
172	*Ornithopus pinnatus*	1	40º42'64'' N	7º21'54'' W	Beira Alta, Portugal
173	*Ornithopus pinnatus*	1	40º20'05'' N	7º24'10'' W	Beira Baixa, Portugal
175	*Ornithopus sativus*	2	39º09'88'' N	8º39'15'' W	Ribatejo, Portugal
179	*Ornithopus sativus*	3	39º14'23'' N	7º23'19'' W	Alto Alentejo, Portugal

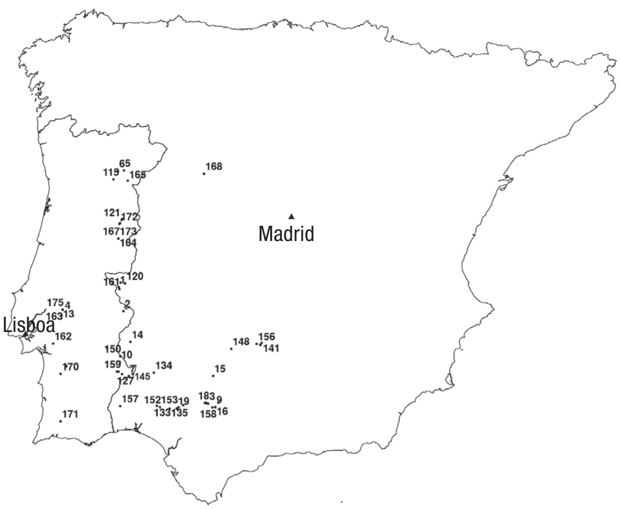

Figure 1. Map of the collections sites of the species of *Ornithopus* and *Biserrula* used in this study. Numbers refer to Table 1.

For *Biserrula* 16 new specimens were sequenced (625 bp aligned length) and compared with a single specimen from GenBank (AB287409, 50 bp shorter). Six haplotypes were recovered, with a single common haplotype (nine individuals), four unique haplotypes, and one haplotype shared by three individuals. The greatest distance between haplotypes was three differences (Fig. 2C). For *Ornithopus* 48 new specimens were sequenced. Within *O. pinnatus* seven individuals were sequenced (600 bp aligned length) and compared with a single specimen from GenBank (AY325278). All shared the same haplotype, distinct from the remaining Mediterranean *Ornithopus*. Within the remaining *Ornithopus* from the Mediterranean 41 new specimens were sequenced (600 bp aligned length), and compared with five sequences from GenBank (AF450226-8, AF218533-4, Fig. 2B). There was haplotype sharing between species, and a single haplotype network was recovered, with six unique haplotypes in total (Fig. 2B).

For the estimate of phylogenetic relationships, the unique haplotypes (seven from the Mediterranean species, plus one *O. micranthopus* – AY325277) were aligned with a specimen of *Lotus wrangelianus* (AF450174) as outgroup. The resulting alignment was 603 bp long. Both ML (GTR+I+G model of evolution) and Bayesian estimates of relationships strongly suggested the monophyly of the Mediterranean species relative to *O. micranthopus*. Within the Mediterranean species *O. sativus* (including *O. s. isthmocarpus* – AF218534) was closely related to, but distinct from, *O. perspusillus* (including AF450226) and *O. compressus* which share haplotypes (1, Fig. 2B). Estimates of relationships were well supported (Fig. 2A).

Our results in general revealed low levels of intraspecific genetic variation. Within *Biserrula*, where six haplotypes were recovered, this is considerably less than recovered in *Scorpiurus muricatus* (Visnevschi-Necrasov *et al.,* 2011). On the other hand morphologically distinct North African populations (Loi *et al.,* 1997) were not assessed. Within *Ornithopus* variation was even lower. This may mean that the extensive morphological variants known to occur in *Ornithopus* (Fu *et al.,* 1994) have arisen in a relatively short evolutionary time, so that minimal neutral genetic variation (such as within the ITS region) has had time to occur. It may also be that population sizes of these species were considerably smaller during the Pleistocene, when climatic conditions were colder, and this has led to a genetic bottleneck effect. The estimate of phylogenetic relationships indicates, as expected, that the South

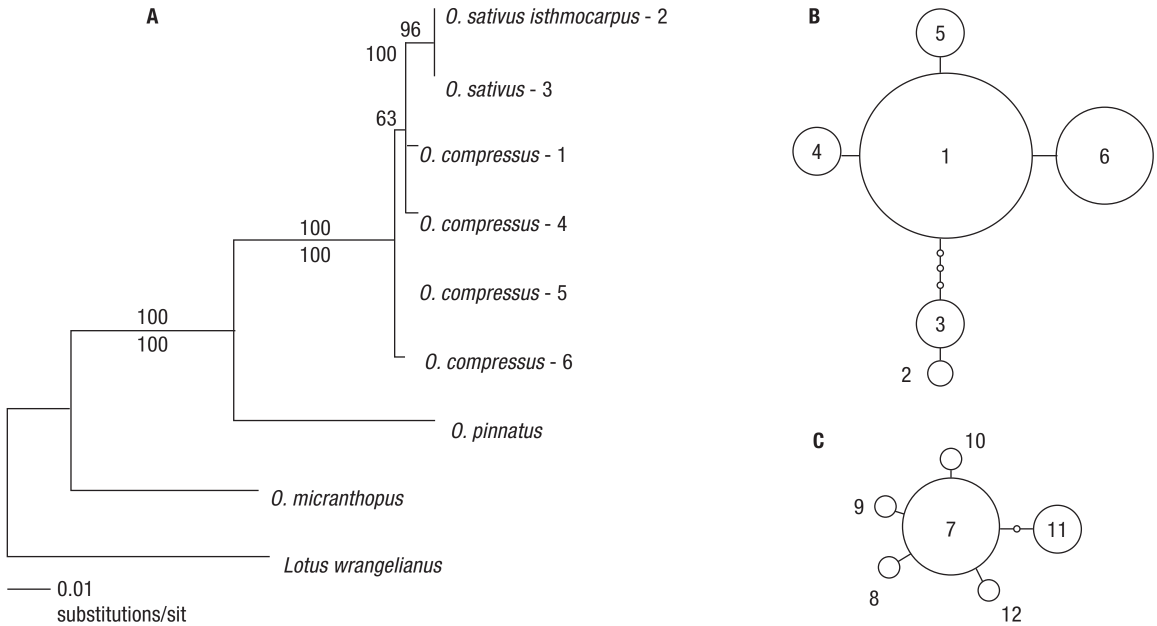

Figure 2. A. Estimate of relationships within *Ornithopus* based on maximum likelihood analysis. Numbers above nodes indicate bootstrap support; those below nodes correspond to Bayesian posterior probabilities. B. Network of haplotypes within the clade of *O. compressus* and *O. sativus*. Numbers refer to haplotype codes in Table 1 and in Fig. 2A. Small circles indicate inferred missing haplotypes. C. Network of haplotypes within *B. pelecinus*.

American species *O. micranthopus*, is sister taxa to all the remaining Mediterranean species. Within the Mediterranean species it is clear that *O. pinnatus* is genetically distinct from the other species that form a closely related clade. Although *O. s. isthmocarpus* is sometimes considered a distinct species, the genetic data presented here shows that it shares the same haplotype as *O. sativus*. As seen in the network of haplotypes, *O. sativus* is distinct from the other species included in this work, but only by three mutations which is a low degree of genetic separation. Within *O. compressus* there is a single common haplotype, with three other haplotypes that differ from this by a single mutation. Surprisingly a sequence from *O. perpusillus* (AF450226) shares the common haplotype (1) of *O. compressus*. This clearly warrants further investigation.

To conclude, none of the examined species shows high levels of intraspecific variation. This implies that the morphological variants do not reflect deep genetic divergences, and that no genetic barriers to breeding programs were identified. Even between some species there is limited genetic divergence, although the situation of *O. perpusillus* requires additional specimens to be examined before firm conclusions can be drawn. It will also be important in the future to assess North African populations, especially for *Biserrula*.

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