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Short communication. Genetic diversity within *Scorpiurus* species from the Iberian Peninsula estimated using ITS DNA sequences

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Abstract

Scorpiurus muricatus and *Scorpiurus vermiculatus* have been proposed as possible subjects for breeding programs to improve their potential as forage crops. However, prior to this genetic diversity between these species and within distinct populations should be assessed. We estimated diversity between populations from the Iberian Peninsula using ITS 1 and ITS 2 nuclear DNA sequences. Twelve haplotypes from 25 individuals were determined, and a network of relationships produced. Our results indicate considerable haplotypic diversity, but no deep subdivisions either within *S. muricatus* or between the two species. This means that no potential barriers to the proposed breeding programs were identified.

Additional key words: forage; prickly caterpillar; *Scorpiurus muricatus; Scorpiurus sulcatus*; *Scorpiurus vermiculatus.*

Resumen

Comunicación corta. Estimación de la diversidad genética entre las especies de *Scorpiurus* de la Península Ibérica utilizando secuencias de ITS DNA

Debido a su potencial como cultivos forrajeros, se han propuesto dos especies, *Scorpiurus muricatus* y *Scorpiurus vermiculatus,* para programas de mejora. Sin embargo, antes de abordar estos programas debe evaluarse la diversidad genética que existe entre las especies y dentro de poblaciones distintas. En este trabajo se estimó la diversidad entre poblaciones de la Península Ibérica con las secuencias de DNA nuclear ITS 1 e ITS 2, se determinaron doce haplotipos a partir de 25 individuos y se creó una red de relaciones. Nuestros resultados indican que existe una considerable diversidad haplotípica, pero sin profundas subdivisiones, ya sea dentro de *S. muricatus* o entre las dos especies. Esto significa que no se han identificado barreras potenciales a los programas de mejora propuestos.

Palabras clave adicionales: forraje; hierba del escorpión; oruga erizada; *Scorpiurus muricatus; Scorpiurus vermiculatus; Scorpiurus sulcatus.*

Scorpiurus (tribe Loteae) is characterised by its fruit, which is a contorted lomentum that gives rise to the common name of «prickly caterpillar». A self re-seeding annual legume, it has potential as a forage crop due to its high nutritive values and its preference by ruminants, but has generally been neglected by researchers and farmers (Abbate *et al*., 2010). Native to Southern and Central Europe, the genus is clearly monophyletic having distinctive pollen morphology (Díez and Ferguson, 1996). However, specific level taxonomy remains controversial. Linnaeus (1753) recognized four species, *Scorpiurus muricatus, Scorpiurus sulcatus, Scorpiurus vermiculatus* and *Scorpiurus subvillosus*. In a study of three of these species, *S. muricatus, S. sulcatus* and *S. subvillosus,* Heyn and Raviv (1966) reported there was «no indication of any genetic crossing barriers bet-

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Abbreviations used: ITS (nuclear ribosomal internal transcribed spacer), PCR (polymerase chain reaction).

ween different taxa» and recommended reorganizing them as one species, *S. muricatus*. In another study, *S. vermiculatus* was easily differentiated from the other forms on the basis of chromosome numbers —2n = 14 while all others have 2n = 28— (Domínguez and Galiano, 1974). Domínguez and Galiano (1974) recognized not only *S. vermiculatus, S. muricatus, S. sulcatus* and *S. subvillosus* as full species, but further identified distinct varieties within each of them. Although later other authors typically recognized only two species, *S. muricatus* and *S. vermiculatus* (*e.g.,* Zieliński, 1991), this is not universal (*e.g.,* Allan and Porter, 2000), and in a recent review Abbate *et al.* (2010) considered the taxonomical issue unresolved. The taxonomic status is important as Abbate *et al.* (2010) also recommended breeding programs to develop *Scorpiurus* as a potentially valuable forage crop. Such programs would involve crossing between forms or «species» to enhance desired characteristics. Given the highly polymorphic nature of *S. muricatus,* an analysis of genetic variability within this species is therefore warranted to assess if distinct genetic units exist.

The aim of this study was to assess variation within and between *S. muricatus* and *S. vermiculatus* using nuclear ribosomal internal transcribed spacer (ITS) sequences. This region of the genome is widely used in «Barcoding» studies in plants to differentiate between species (*e.g.* Chen *et al.,* 2010). Sequences from two individuals of *Scorpiurus*, identified as *S. vermiculatus* and *S. sulcatus* were included from a previous assessment of deeper phylogenetic relationships (Allan and Porter, 2000). This information will be useful to resolve taxonomic issues, and to identify possibly deep genetic differentiation that might be problematic for breeding programs.

Twenty three specimens of *Scorpiurus*, belonging to the species *S. vermiculatus* and *S. muricatus* were collected (Table 1) in Portugal and South Eastern Spain (Fig. 1). DNA was extracted accordingly to a cetyl trimethylammonium bromide (CTAB) based protocol described by Wang *et al.* (1996). The entire ITS1 and ITS2 region was amplified by polymerase chain reaction (PCR) using standard primers (White *et al.,* 1990). Amplifications were performed in a Biometra T3 thermalcycler 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 10 X 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

Table 1. Identification (ID) number, species and coordinates of the collection sites

Species	ID	Collection site		Province and country
Scorpiurus vermiculatus	150	37°55'00" N	6°13'00" W	Sevilla, Spain
	162	37°38'32" N	8°44'44" W	Baixo Alentejo, Portugal
	164	38°01'60" N	8°42'82" W	Ribatejo, Portugal
	165	38°08'00" N	7°22'00" W	Baixo Alentejo, Portugal
	166	37°55'00" N	6°49'00" W	Huelva, Spain
	169	38°13'00" N	7°22'00" W	Baixo Alentejo, Portugal
	170	37°58'00" N	7°13'00" W	Huelva, Spain
Scorpiurus muricatus	172	39°44'59" N	7°25'16" W	Beira Baixa, Portugal
	173	39°44'59" N	7°25'16" W	Beira Baixa, Portugal
	174	39°57'21" N	8°22'18" W	Estremadura, Portugal
	175	38°57'00" N	7°31'04" W	Baixo Alentejo, Portugal
	176	38°42'15" N	9°16'15" W	Estremadura, Portugal
	177	38°49'02" N	7°51'00" W	Alto Alentejo, Portugal
	178	39°24'00" N	8°38'24" W	Ribatejo, Portugal
	179	39°24'00" N	8°38'24" W	Ribatejo, Portugal
	180	38°12'00" N	7°29'00" W	Baixo Alentejo, Portugal
	222	38°36'00" N	7°25'00" W	Alto Alentejo, Portugal
	223	38°48'00" N	4°42'00" W	Ciudade Real, Spain
	224	37°56'00" N	7°26'00" W	Baixo Alentejo, Portugal
	225	37°52'00" N	5°40'00" W	Córdoba, Spain
	226	37°56'00" N	7°26'00" W	Baixo Alentejo, Portugal
	227	38°06'00" N	7°23'00" W	Baixo Alentejo, Portugal
	228	37°52'00" N	6°16'00" W	Sevilla, Spain

Figure 1. Map of the collections sites of the species *S. vermiculatus* and *S. muricatus* used in this study.

95°C for 30 s, 53°C for 30 s 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 (Applied Biosystems, Foster City, USA) using the kit BigDyeTerminator v3.1 from the same supplier.

Sequences were aligned with the two GenBank sequences of *Scorpiurus* (AF218535 and AF218536) using Clustal W with default conditions in the program BioEdit v5.0.9 (Hall, 1999). The program TCS v2.1 (Clement *et al.,* 2000) was used to create a parsimonious network of the aligned haplotypes. All haplotypes could be joined in a single network (95% confidence limit = 10 steps – Fig. 1). New haplotypes have been submitted to GenBank (JF260954 to JF260976).

In total 25 individuals were analysed, including the two previously published, and the aligned sequences were 563 base pairs long. Twelve haplotypes were identified, four unique to *S. vermiculatus*, seven to *S. muricatus*, and one shared haplotype (Fig. 2). All haplotypes could be joined in a single network, with 10 missing haplotypes postulated. Maximum variation within *S. muricatus* (from haplotype 178 to 222, 11 mutations) was greater than the variation between either the most divergent *S. vermiculatus* haplotypes (5 mutations) or between newly sequenced *S. vermiculatus* and *S. muricatus* individuals (*e.g.,* from haplotype 172 to 169, 3 mutations). The two haplotypes from GenBank, identified as *S. vermiculatus* and *S. sulcatus* (= *S. muricatus*), were identical to each other and to one specimen newly sequenced of *S. vermiculatus* from Portugal (162).

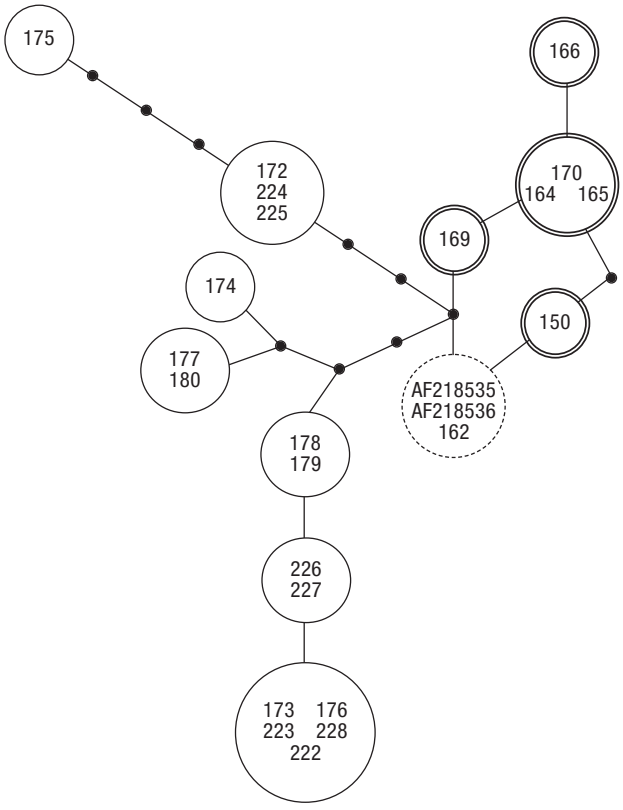

Figure 2. Network of haplotypes estimated using the program TCS. Numbers refer to ID codes in Table 1, or to the two sequences previously published on GenBank. Small filled circles indicate inferred missing haplotypes. Haplotypes unique to *S. vermiculatu*s have a double circle, those unique to *S. muricatus* a single circle, while the single shared haplotype is indicated with a dashed circle.

Abbate *et al*. (2010) suggest that *S. muricatus* could easily be introduced into farming systems where animal husbandry is included, through rotation with traditional arable crops. However, the widespread *S. muricatus* subsp. *subvillosus* has a problematic spiny hard seed pod. *Scorpiurus vermiculatus* and other *S. muricatus* varieties are less spiny, and so Abbate *et al*. (2010) recommend that «a breeding program between *S. muricatus subvillosus* and *S. vermiculatus* or *S. muricatus* could be undertaken». Prior to such a program it is valuable to know how much genetic diversity exists within and between these species.

Our results, based on a neutral nuclear marker, ITS1 and ITS2 DNA sequences, indicate minimal genetic differences between *S. vermiculatus* and *S. muricatus* in the studied locations, and that all the *S. muricatus* sampled from Southern Portugal and Spain form a group without any indications of significant subdivisions. Heyn and Raviv (1966) have already shown that

preliminary crossing experiments between *S. muricatus (*with spineless pods) and *S. muricatus* subsp. *subvillosus* (spiny pod type) were generally successful. Our results indicate that *S. vermiculatus* is genetically closely linked with *S. muricatus*, and thus is likely to be equally viable. On the other hand, haplotypic diversity was relatively high, with 12 haplotypes identified from 25 individuals. Prior to any breeding program involving individuals from other regions, it would be important to assess genetic diversity from these other populations that may harbor additional variants.

To conclude, we can affirm that no deep separation either between species or within *S. muricatus* was found. Therefore no apparent evidence to prevent possible breeding programs was identified, although the high haplotypic diversity indicates that expanding the sampling beyond the Iberian Peninsula would be useful.

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