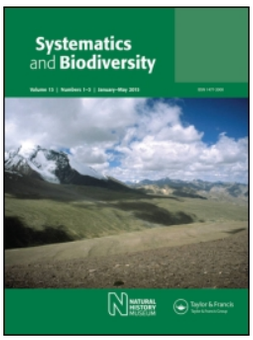

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Research Article

Retrotransposon-based molecular markers as a tool in delimiting species in section *Rynchotolus*, a recent radiation group of Macaronesian *Lotus*

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Island species are particularly vulnerable to extinction and decline due to a range of factors, including isolation, small population sizes, climate change, or the introduction of alien species. Given their high levels of biodiversity, the preservation and protection of island endemic species are fundamental to reducing the loss of global diversity. Therefore, species delimitation, description and identification are among the most important tasks in conservation biology. However, determining species boundaries in some cases can be challenging, especially in groups that have radiated recently, where frequently used molecular markers do not have enough discrimination power. Thus, it is important to look for new approaches with higher resolution. In the present study, we test the use of retrotransposon-based molecular markers to investigate the taxonomic status of five endemic and endangered *Lotus* species in the Canary Islands. Our analysis revealed that the five species conform different entities, in concordance with their morphological differences, and shown that the technique named inter-Primer Binding Site (iPBS) is a reliable molecular marker system that allows to discriminate among *Lotus* and has a potential value for taxonomy and conservation.

Key words: conservation priorities, DNA fingerprint, inter-primer binding site, mobile DNA elements, recent radiation, species resolution

Introduction

The legume family is one of the largest family of angiosperms (Wojciechowski et al., 2004) and the cosmopolitan genus *Lotus* is the most species-rich with about 130 species and taxonomically problematic genera of tribe *Lotea* (Allan et al., 2003; Kramina et al., 2016). This genus includes important agricultural and ornamental plants, as well as the model legume *L. japonicus* (Sandral et al., 2006). It has a major center of diversity in the Mediterranean Region, including portions of Europe, Africa and Western Asia (Allan et al., 2004); whereas phylogenetic data support the separation of the native American species from *Lotus* in some new genera (Arambarri et al., 2005; Brouillet, 2008; Degtjareva et al., 2006; Kramina et al., 2016). Since the recognition of the genus *Lotus* by Linnaeus (1753), different authors throughout the years have changed the generic delimitation (Arambarri et al., 2005). Several infrageneric classifications have been proposed but none are fully congruent with the phylogenies inferred from different molecular markers (Kramina et al., 2016).

In addition to the Mediterranean Region, species of *Lotus* are distributed on several archipelagoes. These include the Macaronesian Islands (Allan et al., 2004). Macaronesia is globally recognised for its species richness, and it is included in one of the 35 World Biodiversity Hotspots (Marchese, 2015; Myers et al., 2000). This phytogeographical region comprises five Atlantic volcanic archipelagoes (the Azores, Madeira, Savages, Canary and Cape Verde Islands) as well as the Macaronesian enclave on the African mainland (Báez & Sánchez-Pinto, 1983; Jaén-Molina et al., 2009), and it is characterized by high levels of endemicity in a broad variety of taxa (Juan et al., 2000). The most striking examples are species restricted to a single island, the so-called single-island endemics or SIEs (Whittaker & Fernández-Palacios, 2007). In the case of angiosperms, SIEs dominate the endemic flora of many oceanic

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Figs. 1–6. *Lotus* species of the *Rhyncholotus* group from the Canary Islands (**1**–**5**) and their closest relative (**6**): *L. berthelotii* (**1**), *L. maculatus* (**2**), *L. gomerythus* (**3**), *L. pyranthus* (**4**), *L. eremiticus* (**5**) and *L. sessilifolius* (**6**). (Color figure online).

islands. For example, in the Canary Archipelago and Madeira Island most endemics are confined to a single island, with very few endemic taxa widespread across the archipelago (Carine & Schaefer, 2009).

In the Canary Islands, the genus *Lotus* comprises 24 species (Acebes Ginovés et al., 2010; Portero-Álvarez et al., 2019). Sixteen of them are endemic, mostly SIEs with each having a very restricted distribution and known from very few localities. Most of them are currently considered vulnerable or critically endangered, and have been included in the Red List of Spanish Vascular Flora and in the IUCN Red List of Threatened Species (IUCN, 2019; Moreno, 2008). Three sections recognised within *Lotus* are present in the Canary Islands, namely *Chamaelotus*, *Pedrosia* and *Rhyncholotus*, the last one endemic to the Canarian archipelago. Different studies have shown that *Rhyncholotus* group is nested among members of section *Pedrosia* (Degtjareva et al. 2008; Kramina et al., 2016; Ojeda et al., 2012) and therefore should not be segregated as a distinct taxon. However, these Canarian endemic species have been treated as a distinct group in many papers (Allan et al., 2004; Ojeda et al., 2012, 2014; Sandral et al., 2006) mainly due to its morphological distinctiveness and geographic distribution and following to these authors, we have also referred to them as *Rhyncholotus* group.

Until recently, the *Rhyncholotus* had only 4 species (Ojeda et al., 2014; Sandral et al., 2006). Recently, a new species of *Lotus* (*L. gomerythus*) was described from a small volcanic plug in La Gomera Island (Portero-Álvarez et al., 2019). Morphologically this new species belongs to the *Rhyncholotus* group (Figs 1–6), however, molecular and phylogenetic information about this new taxon is not available. The occurrence of hybrids within the genus *Lotus* is not a rare event (Liston et al., 1990; Kramina et al., 2012; Somaroo & Grant, 1971). There are currently several ornamental cultivars and man-made hybrids obtained from the *Rhyncholotus* group species, especially between *L. maculatus* and *L. berthelotii* cultivated not only in the Canary Islands but also in different parts of Europe (Hind, 2008; Ojeda & Santos-Guerra, 2011). This could lead to the hypothesis of a possible case of accidental (or

intentional) introduction of this taxon into the wild, which has not been proven so far.

Molecular techniques have been widely used in recent decades to reveal the genetic variation at intra- and inter-species levels. This development of molecular tools has brought a more accurate species delimitation and a better understanding of the evolution of organisms, not least in an oceanic island context (Baldwin et al., 1998; Francisco-Ortega et al., 2000; Meudt et al., 2009). However, each genetic marker systems has its own properties that must be taken into account. If the speciation event has taken place a sufficiently long time ago, marker such as the Internal Transcribed Spacer (ITS) and chloroplast DNA sequences should be informative for species delimitation; however, in the case of recent divergence, they might not provide any clues and more polymorphic markers or high genome coverage markers have to be used (Duminil & Di Michele, 2009). The ITS region has been the most commonly molecular marker used for phylogenetic studies in the genus *Lotus* (Allan et al., 2003, 2004; Degtjareva et al., 2006, 2008; Kramina et al., 2016; Ojeda et al., 2012, 2014; Sandral et al., 2010). Nevertheless, attempts to resolve evolutionary relationships within the *Rhyncholotus* group based on ITS or several loci from the chloroplast genome (Ojeda et al., 2012, 2014) have been unsuccessful due to the very high sequence similarity. Although increased resolution was achieved by the combined analysis of chloroplast and nuclear regions, including three homologous *CYCLOIDEA* genes, a better resolution of genetic affinities between species of the *Rhyncholotus* group requires faster evolving genomic regions (Ojeda et al., 2012).

Another issue to take into account for genetic analyses is the fact that many species of the genus *Lotus* (and in our case, all those belonging to the *Rhyncholotus* group) are polyploids, resulting in problems related with the use of co-dominant markers such as SNP or microsatellites because in this case, the genotyping of particular loci are compulsory.Therefore, in the context of genetic analysis of polyploids, the use of dominant markers such as AFLP (Amplified Fragment Length Polymorphism) or iPBS (inter-Primer Binding Site) could be advantageous since they avoid limitations of allele dosage uncertainty. Consequently, and in keeping with several authors, (Gailite & Rungis, 2012; Kalendar et al., 2019; Monden et al., 2014; Ozër et al., 2016), we suggest that genetic approaches that rely on large sets of dominant informative markers, like genetics polymorphisms generated by retrotransposons activity and detected by iPBS analysis, provide an alternative framework for delimiting polyploid species, and could be useful in very recently derived taxa.

Retrotransposons are mobile genetic elements that play a significant role in genome evolution and are very useful for the development of new genetic markers because of their high prevalence, structures, transposition mechanism, and genome distribution (Galindo-González et al., 2017; Kalendar et al., 2019; Lisch, 2013). Retrotransposons are found in all genomes and are particularly widespread in plant genomes (Gailite & Rungis, 2012). The DNA fingerprinting method known as inter-Primer Binding Site (iPBS), based on the genetic variability generated by the insertional activity of retrotransposons with long terminal repeats (LTR), was developed by Kalendar et al. (2010) as a universal markers system for the molecular characterization of plants and animals, which does not require previous knowledge about the DNA sequence of LTR. The iPBS approach consists of the use of primers that anneal to the primer binding sites of two proximal LTR-retrotransposons with head to head orientation, amplifying the two complete LTRs and the genomic DNA region between them (Kalendar et al., 2010). This molecular marker fulfils the necessary requisites for genetic diversity analysis because it is quick to perform, easy to handle, has relatively low analyses cost, and does not require previous knowledge about the DNA sequence of LTR (Kalendar et al., 2011; Kalendar & Schulman, 2014). Retrotransposons have not been extensively explored as phylogenetic markers (although see Andeden et al., 2013; Coutinho et al., 2018; Gailite & Rungis, 2012; Gedik et al., 2017; Kirdök and Çiftçi 2016; Ozër et al., 2016) although they are present in high copy and are diverse and widely distributed in eukaryotes (Kalendar et al., 2019).

To the best of our knowledge, iPBS- retrotransposons have not been previously used to study the relationship among *Lotus* species. The aims of the present study were to determine the utility of iPBS as a marker system for resolving taxon delimitation and relationships in this oceanic island species complex; to test the delimitation of taxa in the *Rhyncholotus* group and considerer the implications for conservation; and to test the possible hybrid origin for *L. gomerythus*.

Materials and methods

Plant material and DNA purification

Of the six *Rhycholotus* species, one is extinct in the wild (*L. berthelotii*), two have populations reduced to six or fewer individuals (*L. pyranthus* and *L. eremiticus*), and one is known from a single individual (*L. gomerythus*). Eleven samples in total were included for the six species in the complex (Table 1, Figs 1–6

Table 1. Origin of the eleven *Lotus* samples analysed in the present study.

Samples	Species	Locality	Source
Lm1	*L. maculatus*	El Puertito. El Sauzal. Tenerife (TC)	F
Lm2	*L. maculatus*	El Puertito. El Sauzal. Tenerife (TC)	F
Lb1	*L. berthelotii*	La Cueva. Guía de Isora. Tenerife (TC)	JAO
Lb2	*L. berthelotii*	La Cueva. Guía de Isora. Tenerife (TC)	CB
Lg	*L. gomerythus*	Roque del Sombrero. San Sebastián. La Gomera (TC)	F
Lp1	*L. pyranthus*	Marcos y Corderos. San Andrés y Sauces. La Palma (TC)	JAO
Lp2	*L. pyranthus*	Gallegos. Barlovento. La Palma	JAO
Le1	*L. eremiticus*	Don Pedro. Garafía. La Palma (TC)	JAO
Le2	*L. eremiticus*	Don Pedro. Garafía. La Palma (TC)	JAO
Ls1	*L. sessilifolius*	Abades. Arico. Tenerife	F
Ls2	*L. sessilifolius*	El Socorro. Güímar, Tenerife	F

TC: Type Locality. F: Field collected. CB: Cabildo de Tenerife. JAO: Jardín de Aclimatación de la Orotava (Tenerife).

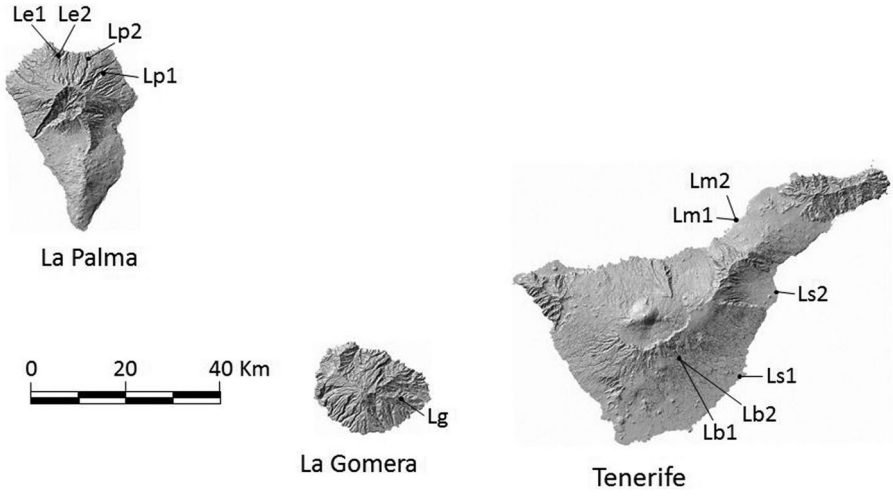

Fig. 7. Geographical distribution of the different *Lotus* species and populations sampled in this study. The accession codes at each point correspond to those displayed in Table 1.

and 7). Where possible, we collected from the type locality but in several instances we used material from the original localities preserved in gardens of the Canarian Public Administration (Cabildo de Tenerife and Jardín de Aclimatación de la Orotava). *Lotus sessilifolius* was included in our analyses because it has been suggested that this *Lotus* species is the closest relative of the *Rhyncholotus* group (Ojeda et al., 2012).

Genomic DNA (gDNA) was purified from 100 mg of fresh leaves using the E.Z.N.A.® SP Plant DNA kit (omega BIO-TEK) and following manufacturer's instructions with two modifications: 1) Leaf samples were homogenized in the presence of SP1 buffer (700 µl) using 2-mL Lysing Matrix-A tubes in the FastPrep-24TM 5 G instrument (MP Biomedicals) and applying two cycles of agitation (speed 5) during 30 s; and 2) After addition of buffer SP2, a more efficient precipitation of impurities was achieved by incubation on ice for 10 min and centrifugation at 20,000 x g for 20 min at 4 °C. Concentration and purity of the gDNA preparations were determined spectrophotometrically. The final concentration of each sample was adjusted to 0.4 ng/µl using 10 mM Tris-HCl, pH 8.0.

Generation of iPBS markers and data analysis

DNA amplification reactions were performed in a final volume of 20 µl containing 2 ng of gDNA (genomic DNA), 0.2 mM of each dNTP, 0.2 µl of Phire Hot Start II DNA Polymerase (Thermo Fisher Scientific), 1X reaction buffer (provides 1.5 mM $MgCl_2$) and one PBS primer at 1 µM. The thermal profile of PCR was as follows: initial denaturation at 98 °C for 30 s; 30 amplification cycles with denaturation at 98 °C for 10 s, primer annealing at optimal temperature for 30 s and extension at 72 °C for 40 s; final extension at 72 °C for 2 min. The PBS primers tested (2232, 2251 and 2373) were used with the annealing temperature recommended by

Table 2. Parameters of genetic diversity estimated after iPBS analysis of the six *Lotus* species.

	Number of bands							
Primer	**Total**	**P**	**P%**	**h**	**I**	**PIC**	**Rp**	**D**
PBS2232	67	64	95.5	0.300	0.465	0.314	24.72	0.930
PBS2251	81	78	96.3	0.306	0.473	0.318	34.36	0.926
PBS2373	79	70	88.6	0.254	0.400	0.339	27.81	0.899
Average	73.3	68.7	93.9	0.288	0.448	0.324	28.96	0.918

P: polymorphic bands. P%: Polymorphism percentage. h: Neís gene diversity index. I: Shannoń s information index. PIC: Polymorphic Information Content. Rp: Resolving power. D: Discriminating power.

Kalendar et al. (2010). The iPBS amplicons contained in 10 µl of PCR were separated on 20-cm length 1.7% agarose gels prepared with 1X TBE buffer. After running at 120 V for 10–11 h, gels were stained during 2 h in a solution of 3X GelRed® (Biotium) and images of DNA fingerprints were capture with the ChemiDoc™ system (Bio-Rad).

The band patterns obtained with the three PBS primers in each analysed individual were manually transformed in a unique sequence of 0's and 1's (absence and presence of a specific band, respectively). Only clear, repetitive and well-separated bands were selected for scoring. Variation in band intensity was not considered as a criterion for genetic polymorphism detection. The resulting matrix of binary data was converted into a genetic distance matrix among all pairs of samples by using the Jaccard's dissimilarity index as implemented in the Darwin 6.0 software (Perrier & Jacquemoud-Collet, 2006). The number of amplified fragments obtained with this multilocus genotyping approach tends to increase with higher ploidy levels, and this will increase the frequency of homoplasy (Dufresne et al., 2014). In this sense, we have used Jaccard's dissimilarity index because the weight given to shared bands is lower than, for example, Dice/Lynch index.

Summary statistics related to the number and percentage of polymorphic bands, average Shannon's information index (I) and Nei's gene diversity index (h) were calculated using POPGENE v. 1.32 software. In order to further evaluate the performance of the PBS primers and assess the genetic diversities among the species, Polymorphic Information Content (PIC), Resolving power (Rp) and Discrimination power (D) parameters were calculated using iMEC (Amiryousefi et al., 2018).

Darwin 6.0 software (Perrier & Jacquemoud-Collet, 2006) was used for Principal Coordinate Analysis (PCoA) and phylogenetic tree construction using Neighbor-Joining method. Robustness of the tree nodes was evaluated by bootstrapping (1000 replicates). Additionally, genetic similarity and genetic distance between pairs of *Lotus* species were calculated based on the proportion of shared bands produced by the set of three PBS primers according to Nei (1972) employing POPGENE v. 1.32 software (Yeh et al., 2000).

Results

From the eleven *Lotus* specimens (Table 1) and using 3 PBS primers, a total of 227 iPBS bands were scored and among them 212 were variable. The polymorphism percentage per primer ranged from 88.6 (primer PBS2373) to 96.3 (primer PBS2251) with an average of 93.9 per primer (Table 2). The Nei's gene diversity (h) per primer ranged from 0.254 to 0.306 while the Shannon's information index (I) ranged from 0.400 to 0.473.

The suitability of selected PBS primers for revealing genetic differences and affinities among compared taxa was further checked through several analytical parameters: Polymorphic Information Content (PIC), Resolving power (Rp) and Discriminating power (D) (Table 2). In our study, the PIC for the three tested primers varied among 0.314 and 0.339, with an average of 0.324. The mean of Rp value generated by these three primers was 28.96; out of these, primer 2251 gave the highest Rp value (34.36), followed by primer 2373 with 27.81 and giving the lowest Rp value was primer 2232 (24.72). In our analysis D varied from 0.899 (primer PBS2373) to 0.930 (primer PBS2232) with an average of 0.918.

In order to unravel the evolutionary relationships among *Lotus* species of the *Rhyncholotus* group and speciation patterns within it, three analyses were performed: 1) Quantification of genetic identity and genetic distance between pairs of species (Table 3); 2) Construction of a phylogenetic tree based on the Neighbor-Joining method and rooted using *L. sessilifolius* as outgroup; and 3) Principal Coordinate Analysis (PCoA). For these tasks, the iPBS binary data obtained with the three PBS primers were first refined by eliminating redundant information, i.e., shared bands generated from the same genomic loci by primers PBS2232 and PBS2373, and then combined so the whole dataset was reduced from 227 to 182 bands, most of them being polymorphic (95.6%).

Neighbour joining analysis (Fig. 8) showed a clear genetic differentiation among the six *Lotus* species and revealed the existence of two highly supported subclades (bootstrap values of 86% and 93%) within the *Rhyncholotus* group: one subclade includes the three

Table 3. Nei's unbiased measures of genetic identity (above diagonal) and genetic distance (below diagonal) among the six species of *Lotus*.

Species	*L. maculatus*	*L. berthelotii*	*L. gomerythus*	*L. pyranthus*	*L. eremiticus*	*L. sessilifolius*
L. maculatus	–	0.812	0.679	0.657	0.728	0.783
L. berthelotii	0.208	–	0.710	0.700	0.734	0.743
L. gomerythus	0.393	0.351	–	0.551	0.628	0.632
L. pyranthus	0.426	0.362	0.598	–	0.719	0.669
L. eremiticus	0.318	0.312	0.469	0.339	–	0.727
L. sessilifolius	0.245	0.296	0.473	0.408	0.321	–

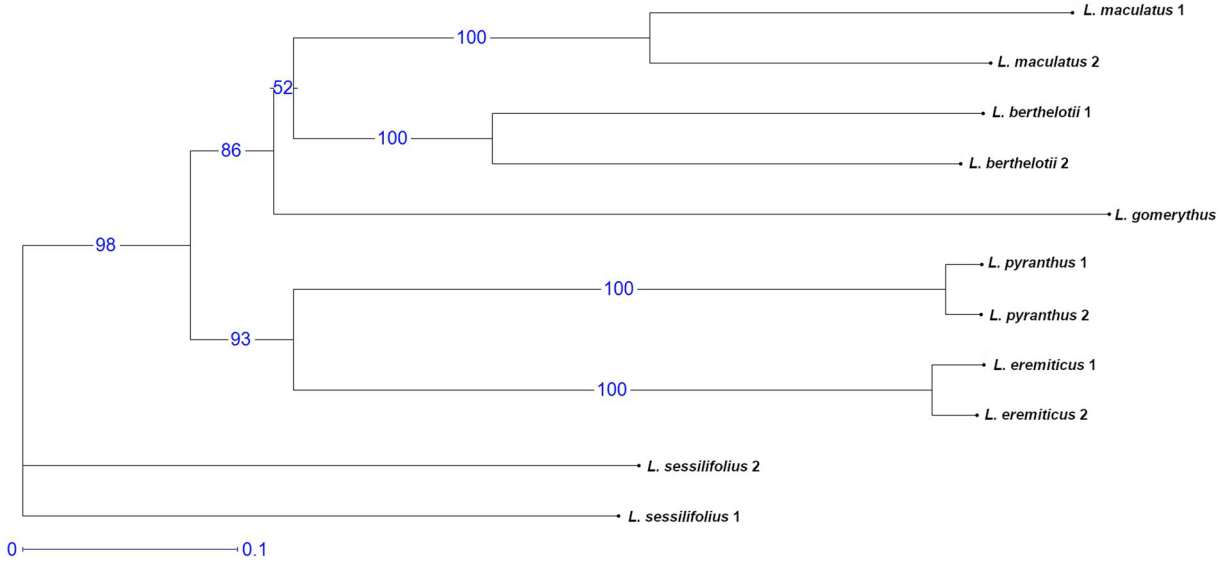

Fig. 8. Phylogenetic relationships among *Lotus* species of the *Rhyncholotus* group inferred through iPBS fingerprints. Information about dominant genetic markers was processed by a neighbor-joining approach. Numbers indicate bootstrap values of the different tree nodes.

species from Tenerife and La Gomera (*L. maculatus*/*L. berthelotii*/*L. gomerythus*), and the other consists of the two species from La Palma (*L. pyranthus*/*L. eremiticus*).

Three main groups were depicted in the PCoA diagram (Fig. 9). The first three PCoA axes accounted for the 61.29% of the total variation (first axis = 27.5%, second = 17.6% and third = 16.2%).

Discussion

Among *Lotus* species that occur in the Canary Islands, *L. gomerythus* is the most recent described species (Portero-Álvarez et al., 2019). It was discovered in a small volcanic plug in La Gomera island where only one individual grows. Singletons (species only known from a single specimen) together with unique or orphaned species (species that have only been collected once or very few times) are very common in biodiversity surveys (Feuerer & Hawksworth, 2007; Lim et al., 2012). Although this phenomenon is more common in animals (especially arthropods), it also occurs in plants (Coddington et al., 2009; Lim et al., 2012). According to flower characters (red pedicelled zygomorphic flowers with villous chalices, presence of a ventral tooth on the style and keel long ended with a beak longer than the wings), Portero-Álvarez et al. (2019) placed *L. gomerythus* in the group *Rhyncholotus*.

The low genetic variation observed in some studies by means of ITS markers in *Lotus* from the Canaries (Ojeda et al., 2012, 2014; Sandral et al., 2010; Schmidt, 2011), could be considered as an evidence of the recent and rapid speciation of *Lotus* in this Archipelago, with not enough time for significant molecular divergence at nucleotide sequence level. This lack of informative genetic variation from conventional markers like ITS in insular species is generally known and has been described for other genera as well (Baldwin et al., 1998).

Many species of the genus *Lotus* (and in our case, all those belonging to the *Rhyncholotus* group) are polyploids. A major problem for genetic analyses of polyploids using co-dominant markers, such as SNP or

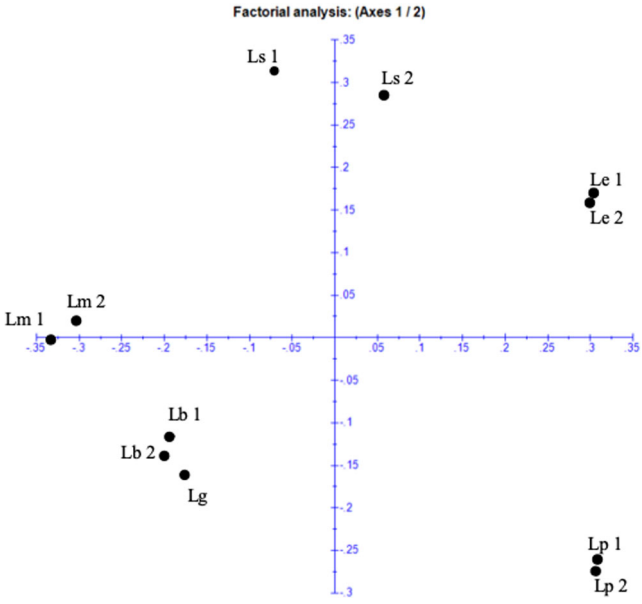

Fig. 9. Plot of the first two principal components of the principal coordinate analysis based on iPBS data. Symbols at each dot correspond to those displayed in Table 1.

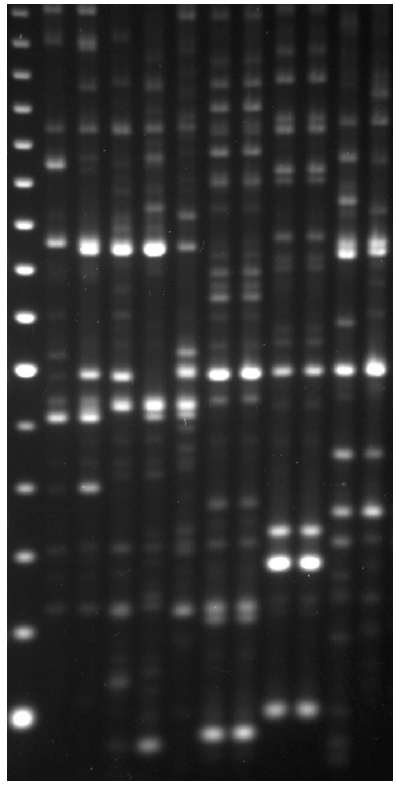

Fig. 10. Patterns of iPBS amplicons obtained with primer PBS2251. First lane: 100 bp DNA Step Ladder (Promega); DNA markers ranging from 500 bp (bottom) to 1.9 Kb (top) are shown. Remaining lanes are the iPBS fingerprints generated from the eleven *Lotus* specimens, which are displayed in the same order as they appear in Table 1.

microsatellites, is the uncertainty in determining allele dosage of individual loci, which is especially challenging in the case of partial heterozygous genotypes (e.g. AAAB, AACC, ABCC) (Dufresne et al., 2014; Meirmans et al., 2018). This limitation precludes the use of most existing methods for population genetic analyses since they rely on inferences based on the observed heterozygosity or allele frequency distributions, or require the complete genotyping of individuals. Frequently, the inability to ascertain allele dosage in polyploids requires the conversion of co-dominant markers in dominant ones, i.e., for each locus all heterozygous genotypes are arbitrarily grouped with one of the homozygous genotypes, with the inherent loss of information and usefulness of these type of markers (Dufresne et al., 2014, Bourke et al., 2018). Therefore, in the context of genetic analysis of polyploids, the use of dominant markers such as AFLP (Amplified Fragment Length Polymorphism) or iPBS (inter-Primer Binding Site) could be advantageous since they avoid limitations of allele dosage uncertainty. Most population genetics models that work with dominant marker data are currently applied in polyploid analysis without corrections related to ploidy level. Dominant scoring reduces the information content due to the failure to detect heterozygous genotypes, and the degree of information loss increases with the ploidy level. The abundance of informative markers provide by multilocus genotyping approaches like AFLP or iPBS analyses could outweigh this scoring issue and other as length homoplasy, i.e. the comigration of nonhomologous fragments during electrophoresis analysis of amplicons (Dufresne et al., 2014).

There are few studies in the *Fabaceae* family based on iPBS markers to compare with our results. For example, Andeden et al. (2013) used 10 PBS primers to analyse 71 accessions from 6 species of the genus *Cicer* (five wild and one cultivated) and obtained 130 bands, all of them polymorphic, with h and I values per PBS primer fluctuating between 0.15- 0.33 and 0.27-0.5, respectively. All these values are similar to those registered by us. However, the number of bands per primer was considerably higher in our study (73.3 vs. 13) probably due to a better band resolution in our electrophoresis conditions (Fig. 10).

In the case of dominant genetic markers, such as those generated by iPBS analysis, PIC can range from 0 to 0.5 (Amiryousefi et al., 2018). Our results are similar to those obtained by Kirdök and Çiftçi (2016) studying different *Pistacia* species, and higher than other investigations on intraspecific variation in some plants (Borna et al., 2017; Demirel et al., 2018). On the other hand,

Rp provided the basis for assessing the diagnostic effectiveness of primers used in this study. Previous analyses have found that Rp is more suitable than PIC for describing the discriminatory ability of primers in diverse group of plants (Mangini et al., 2010), by considering both the number of polymorphic bands in a pattern and the informative value of individual polymorphic bands (Prevost & Wilkinson, 1999). According to the equation of Prevost and Wilkinson, anyone of the three PBS primers used in the present study could differentiate more than 100 multilocus genotypes and, therefore, can unambiguously identify all the 6 *Lotus* species. Finally, the D parameter proposed by Tessier et al. (1999), which evaluates the efficiency of a primer for identification of accessions, was estimated for each PBS primer. The D parameter describes the probability of two randomly chosen individuals having different genetic patterns. A value of D close to 1 implies a very low probability of confusion between accessions (Amiryousefi et al., 2018). The values obtained for the D parameter, allow us to conclude that the obtained iPBS fingerprints can be considered variable enough to discriminate among the different specimens. In summary, all the analytical parameters in Table 2 are consistent, i.e., the usefulness of iPBS markers for evaluation of genetic diversity in the set of six *Lotus* species.

For the first time, our analysis with the iPBS, a polymorphic and high genome coverage marker, has yielded satisfactory results in the distinction of the *Rhyncholotus* species (Fig. 8). The phylogenetic tree based on the Neighbor-Joining method revealed the existence of two highly supported subclades. These two subclades were previously identified by Ojeda et al. (2012) analysing chloroplast and nuclear DNA sequences, before the discovery of *L. gomerythus*, but the scarcity of informative sites led to poor resolution of species within each subclade, especially those from La Palma, which were not clearly separated. However, our analysis took advantage of faster evolving molecular markers (LTR-retrotransposons) that allowed us to detect 46 genetic differences between *L. pyranthus* and *L. eremiticus*, including 13 iPBS bands exclusive of *L. pyranthus* and 5 exclusive of *L. eremiticus*, sustaining the notion that the two taxa are different species. From the perspective of the conservation biology, it is noteworthy the very low level of genetic variation detected in *L. pyranthus* and *L. eremiticus* populations (Fig. 8), which emphasizes their recognized status of critically endangered species (IUCN, 2019; Moreno, 2008). *Lotus gomerythus* appears to be a sister branch to the Tenerife clade (*L. maculatus* and *L. berthelotii*). It could be a clear example of allopatric speciation due to interisland colonization. It must be taken into account that La Gomera is very close to Tenerife (about 28 km) and also the age of the islands. According to Hoernle and Carracedo (2009), Tenerife and La Gomera are about 12 Ma and 9.4 Ma, respectively. Colonizations from the palaeo-islands of Tenerife have also been described for some groups including plants (Dlugosch & Parker, 2007; Puppo et al., 2015) and animals (Thorpe et al., 1994). Morphologically, *L. maculatus* is easy to distinguish from *L. gomerythus* due to its yellow flowers and coastal habitat. *L. berthelotii* bears a superficial similarity to *L. gomerythus*. However, the former has longer and wider leaves and narrower teeth in the calyx (Fig. 1) (Portero-Álvarez et al., 2019). *Lotus pyranthus* and *L. eremiticus* form a separate clade supported by a bootstrap value of 93%. Both species are endemic to La Palma, a younger island less than 2 Ma and more isolated in the North West of this archipelago (58 Km from La Gomera and 84 Km from Tenerife) (Fig. 7). *Lotus gomerythus* also resembles these two species but the former has short and wider leaves and small flowers, and the later species have longer and wider leaves and the flower is quite different with wider wings (Figs 4 and 5).

The 23 iPBS bands annotated for *L. gomerythus* not shared with the other *Rhyncholotus* species or with *L. sessilifolius*, strongly support at the molecular level the proposal of a new species (Portero-Álvarez et al., 2019), and dismiss a possible hybrid origin for this new taxon or the deliberate introduction into the wild of an artificial hybrid.

PCoA also resolved associations among studied *Lotus* species, and showing a similar clustering result than the phylogenetic tree. The closer relationship between *L. gomerythus* and *L. berthelotii* and *L. maculatus* could be easily observed. Species from La Gomera can be separated from those from Tenerife and La Palma according to axis 1. *Lotus sessilifolius* can also be distinguished from the rest of the species according to axis 1 but better according to axis 2 (Fig. 9).

All five species of the *Rhyncholotus* group have serious problems of conservation. *Lotus maculatus*, *L. berthelotii*, *L. pyranthus* and *L. eremiticus* are listed as Critically Endangered in the Red List of Spanish Vascular Flora and in the IUCN Red List of Threatened Species (IUCN, 2019; Moreno, 2008), whereas for the recently described *L. gomerythus* only one individual has been found. The genetic distinctiveness of *L. gomerythus* and the fact that only one specimen is known in nature, make urgent a recovery program for this threatened species. Preservation of endemic taxa, because of their fragility and narrow distribution range, is a major goal for the conservation efforts of the World's biodiversity (Conesa et al., 2010), specially on islands, which

host exceptionally high levels of endemism compared to mainland regions and are subject to disproportionately high rates of extinction and imperilment (Robertson et al., 2014). Conservation practitioners are given the challenging mandate of working to recover species at risk, often with extremely limited resources. The identification of taxa and assessing their evolutionary relationships is crucial for the design of efficient strategies for biodiversity management and conservation. Failing to recognize the existence of a distinct and threatened taxon can lead to insufficient protection and subsequent extinction. Conversely, identification of too many taxa (oversplitting) can waste limited conservation resources (Allendorf & Luikart, 2006). In this sense, all molecular markers used until now for the genetic characterization of the *Rhyncholotus* group have had a very limited success in resolving species boundaries (Degtjareva et al., 2006; Kramina et al., 2016; Ojeda et al., 2012, 2014). Based on the results of the present study, we concluded that the delimitation of the five species within the *Rynchotolus* group has been successfully clarified through iPBS markers in concordance with their morphological differences. The iPBS technique is a reliable molecular marker system that provides an alternative framework for delimiting recently derived species. This technique has provided valuable information to guide interventions for biodiversity conservation.

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Disclosure statement

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