Phylogeography of cliff-dwelling relicts with a highly narrow and disjunct distribution in the western Mediterranean

Jose L. Silva1,2, So-Yeon Lim3, Seung-Chul Kim3,5, and José A. Mejías2

PREMISE OF THE STUDY: The high biodiversity in the Baetic-Rifan hotspot of Mediterranean region is shaped by complex geological and climatic histories and has been a subject of recent intensive studies. However, very little is known about phylogenetic and biogeographic history of three rare and critically endangered cliff-dwelling species of Sonchus in section Pustulati in this region.

METHODS: We investigated the genetic variation and phylogenetic relationships of populations based on nuclear (ITS/ETS) and plastid (3′trnL–ndhJ/psaI–accD) DNA sequences, and amplified fragment length polymorphisms (AFLPs). We performed a Bayesian relaxed molecular clock analysis with ITS data to estimate divergence times for major lineages.

KEY RESULTS: ITS/ETS and AFLP phylogenies showed high concordance and contrasted with cpDNA data. The divergence between S. masguindalii and S. fragilis was dated at 5.48 Ma, between S. fragilis and S. pustulatus at 3.89 Ma, and between the Baetic and Rifan S. pustulatus at 1.18 Ma. Within each distribution area, AFLP data showed a relatively high genetic structuring and moderate genetic diversity, the latter being impoverished in the Baetic populations.

CONCLUSIONS: Our results further confirm the hybrid origin of S. pustulatus, a critically endangered species. The origin and diversification of lineages appear to have occurred on the temporary land bridge that joined Iberian and North Africa during the Messinian Salinity Crisis (5.96–5.33 Ma) and the subsequent Zanclean flood that progressively refilled the Mediterranean Basin (5.33–3.60 Ma). The only Baetic populations of S. pustulatus most likely originated from the Rifan ones.

KEY WORDS Baetic-Rifan region; cliff ecology; disjunct distribution; Gibraltar arc; long-distance dispersal; Messinian-Zanclean ages; relaxed Bayesian molecular clock dating; Sonchus section Pustulati; vicariance
Middle Miocene (15 Ma), probably joined Africa and Iberia in a position in the middle of the present-day Alboran Sea (Rosenbaum et al., 2002). As the eastward-dipping subduction zone migrated to the west and the Alboran domain underwent progressive back collapse, this land bridge/stretch migrated westward until it reached its current position in the Gibraltar Arc (Late Miocene, 10 Ma). Closure of the Rif and Betic corridors accompanied the process, finally leading to the separation of the Mediterranean and Atlantic seas during the Messinian (6 Ma). This promoted the desiccation of the Mediterranean Sea (Messinian Salinity Crisis, 5.96–5.33 Ma; Krijgsman et al., 1999; Fauquette et al., 2006) and facilitated intercontinental expansion of plant distributions until the opening of the Strait of Gibraltar (5.33 Ma; e.g., Caujapé-Castells and Jansen, 2003; Cano-Maqueda et al., 2008) and the Zanclean flood that progressively refilled the Mediterranean basin (5.33–3.60 Ma). Thereafter, the sea barrier likely made intercontinental migration of plants highly dependent on long-distance seed dispersal and favored vicariance processes in those plants present in both sides before the total refilling of the Mediterranean, i.e., leading to differentiation in pairs of closely related species.

Most phylogeographical studies in the Baetic-Rifan region focus upon the role of the Strait of Gibraltar as a bridge or a barrier for species migration and gene flow between the northern (European) and southern (African) ranges (see review of Rodriguez-Sánchez et al., 2008). Apart from addressing the disjunct populations around the Strait of Gibraltar, phylogeographic/phylogeographical investigation of additional Baetic-Rifan plant taxa that do not occur on both sides of such strait provides us with insights into the role of sea barriers as a limiting factor for range expansion. Incorporating time-calibrated phylogeographical analyses of taxa with disjunct distributions is crucial with regard to fully understanding dispersal, colonization, and isolation processes in the region (e.g., Caujapé-Castells and Jansen, 2003; Casimiro-Soriguer et al., 2010; Fernández-Mazuecos and Vargas, 2011). In addition, analyses of population genetic structure and diversity enable us to determine the degree of connection between them and the regions that potentially acted as the centers of diversification (e.g., Casimiro-Soriguer et al., 2010). Furthermore, it is expected that the genetic landscape in species with high habitat specificity and low dispersal ability, as in many cliff-dwelling species (e.g., García et al., 2002; Picó and Riba, 2002), is highly structured among their populations.

Sonchus pustulatus Willk., S. fragilis Ball, and S. masguindali Pau and Font Quer constitute a group of rare, long-lived, cliff-dwelling species (Asteraceae), endemic to narrow areas within the Baetic-Rifan hotspot (Fig. 1a; Boulos, 1973; Silva et al., 2015). They belong to the well-supported section Rifan hotspot (Fig. 1a; Boulos, 1973; Silva et al., 2015). All three species are distributed in the Rifan range, where they have been considered the most important past geologic and climatic events occurred in the Baetic-Rifan region. We conducted phylogenetic and phylogeographic studies in Sonchus section Pustulati based on noncoding cpDNA (3′trnL–ndhJ/psaI–accD) and nrDNA ITS/ETS sequences, and amplified fragment length polymorphism ( AFLP). The following specific questions were addressed: (1) How consistent are phylogenetic analyses with the current taxonomic treatment? (2) Could the origin and diversification of the species be related to the Messinian Salinity Crisis, the subsequent Zanclean flood that progressively refilled the Mediterranean basin and/or the onset of the Mediterranean climate? (3) Could the current disjunct distribution of S. pustulatus have resulted from historical vicariance events rather than recent long-distance dispersal events between both sides of the Baetic-Rifan region? (4) What evidence about recent and current biogeographic trends of these species within each distribution area can be inferred from their current population genetic structuring and known biological/habitat traits? And finally, (5) is the narrow distribution range of these rare species associated with low genetic diversity?

MATERIAL AND METHODS

The species of Sonchus section Pustulati—The 19 extant populations of Sonchus section Pustulati are distinguished in five small distribution areas restricted to the Baetic-Rifan Internal Zone (Silva et al., 2015; Fig. 1a). The rare Baetic populations of S. pustulatus are located in the environs of Almeria city (Fig. 1a, area A, southeastern Spain), whereas the Rifan ones colonize cliffs in the Oued Laou valley and some neighboring valleys (Fig. 1a, area B, western Rif). Sonchus fragilis is exclusive to the mountains surrounding the city of Tetouan (Fig. 1a, area C, northwestern Rif). Finally, S. masguindali is distributed along the coast in the Bokkoya Mountains and some sea cliffs in the environs of Al-Hoceima city (Fig. 1a, area D, central Rif). Some herbarium specimens of S. pustulatus from northwestern Algeria collected in the 19th century (e.g., herbarium COI), with standard morphological characters of the species, indicate that this species was also present in rocky places on the oceanfront near Ghazauet (formerly Nemours; Fig. 1a, area E, northwestern Algeria), but its persistence in the area has not been determined.

These endemic species are found in a highly fragmented habitat and are specifically associated with north-facing cliffs, which are at a low to medium altitude, have an alkaline, mainly limestone substrate, and are located on the coast or less than 20 km from the sea (Silva, 2014; Silva et al., 2015). These plants are polycarpic perennials, mostly self-incompatible (except for S. fragilis that has a high incidence of self-compatibility), with generalist entomophilous pollination (Silva, 2014). Achenes have a short-lasting pappus and are heavier than other more widespread Sonchus species (Silva, 2014).

Populations sampled and DNA extraction—Leaf tissue samples were collected from 15 to 20 georeferenced individuals per population (using GPS). To obtain representative samples of whole populations and to avoid collecting siblings, we made a linear transect along the cliffs and collected one sample every 10–15 m. The leaves were immediately dried and stored in silica gel at room temperature until further processing. Voucher specimens were deposited in the University of Seville herbarium (SEV; Appendix S1, see Supplemental Data with the online version of this article). A total of 316 individuals were used for the molecular analyses. Total genomic
DNA was isolated from ca. 1 cm² of leaf tissue with the NucleoSpin Plant II Kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany) according to the manufacturer’s protocol. DNA concentration and quality of each sample were checked on 0.9% agarose gels and quantified in a NanoDrop1000 spectrophotometer (ThermoScientific, Wilmington, USA). DNA was diluted to a final concentration of 30 ng/μL.

**DNA sequencing**—For cpDNA sequencing, 92 individuals were selected (3–5 per population; Table 1), plus three individuals of the

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**FIGURE 1** (a) Distribution range of the species of *Sonchus* section *Pustulati*, locations of all known populations, and area of occupancy (Silva et al., 2015). Area A (blue), Baetic populations of *S. pustulatus*; Rifan populations: area B (red), of *S. pustulatus*; area C (orange), of *S. fragilis*; area D (green), of *S. masguindalii*; and area E, unconfirmed current presence of *S. pustulatus* (dark gray). (b) Statistical parsimony network of the six plastid haplotypes found in the section. Circle: haplotype, line: nucleotide substitution, small black dot: inferred intermediate unsampled haplotype.
related species *Sonchus pustulatus*, which was used as the outgroup. The previous phylogenetic study of Sonchinae (Kim et al., 2007) strongly suggested that *S. pustulatus* is the closest sister lineage of section *Pustulati*, and thus it was used as a sole outgroup. To sequence the ITS/ETS region of nrDNA, we selected three individuals from each population, for a total of 60 individuals including three from each of the three outgroup species. For cpDNA, of the 34 noncoding regions that were initially surveyed (Shaw et al., 2007), the two regions with the highest potentially informative character value were selected, i.e., the regions with the highest potentially informative character value were selected, i.e., the *trnL–ndhJ* (UAA) [=Tab E] and *accD–psal*. PCR amplification of nrDNA ITS and ETS was performed as described by Lee et al. (2005), except that the ETS region was amplified with the same PCR conditions for two cpDNA regions were applied. PCR products were purified with GENELAB EXPIN PCR SV (GeneAll Biotechnology, Seoul, Korea). Sequencing was conducted at the GenoTech Corp. (Seoul, Korea). Base calling and sequence editing were performed with SEQUENCER v4.2.2 (Gene Codes, Ann Arbor, Michigan, USA). Sequences were aligned manually with MacClade (v.4.06, Maddison and Maddison, 2003).

**AFLP reaction**—For the AFLPs, we analyzed a total of 167 individuals, ranging from 4 to 12 per population (Table 1). We followed the protocol of Vos et al. (1995) with some modifications (Lauterbach et al., 2011). A total of 18 AFLP primer combinations were tested for their capacity to detect polymorphisms and their reproducibility of banding patterns in all the *Sonchus* taxa studied. The three most polymorphic primer combinations were used for selective PCR: EcoRI ACA–TruII CTG; EcoRI ACA–TruII CAG; EcoRI ACC–TruII CAA. One sample from each distribution area (3% of the total) was repeated on all different plates to calculate the average reproducibility of the method (Bonin et al., 2004). The AFLP fragments were separated on a polyacrylamide gel with an internal size standard (GenomeLab DNA Size Standard Kit 400, Beckman Coulter, Krefeld, Germany) by means of an automated sequencer (CEQ 8000, Beckman Coulter). Data files were imported into Genographer software (v1.6.0, J. J. Benham, Montana State University, Bozeman, Montana, USA) and fragments from 75 to 450 bp were identified and scored automatically for their presence and absence. A negative control (no DNA) was included in each plate. Finally, fragments were checked individually, without prior information on their origin, by means of the “thumbnail” function, and a presence/absence matrix was exported for further analyses.

**Analyses of cpDNA and nrDNA ITS/ETS sequences**—Three different phylogenetic analyses were conducted for both cpDNA (two combined noncoding regions) and nrDNA (combined ITS and ETS regions) data sets. We first conducted an equally weighted unordered maximum parsimony (MP) approach (Fitch, 1971) implemented in the program PAUP* v4.0b10 (Swoford, 2002). The MP analyses included a default heuristic search option with tree-bisection-reconnection (TBR) branch swapping and MULPARS on. Bootstrap support (BS) was calculated by bootstrap analysis from 1000 replicates with the same heuristic options. Secondly, we conducted

### TABLE 1. Populations of *Sonchus* section *Pustulati* and AFLP results.

<table>
<thead>
<tr>
<th>Species and location, m.a.s.l.</th>
<th>Code</th>
<th>Coordinates (N / W)</th>
<th>N</th>
<th>n₁</th>
<th>n₂</th>
<th>n₃</th>
<th>%P</th>
<th>Frₚₑ</th>
<th>Hₑ ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A)</strong> Spain, SE Baetic range</td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sonchus pustulatus</em></td>
<td></td>
<td></td>
<td>898</td>
<td>15</td>
<td>9</td>
<td>34</td>
<td>0.109±0.056</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bco. San Antonio, Aguadulce, 300 m</td>
<td>ANT</td>
<td>36°49.8'/2°34.4&quot;</td>
<td>484</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>41.9</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Bco. San Telmo, Almeria, 22 m</td>
<td>TEL</td>
<td>36°49.7'/2°29.0&quot;</td>
<td>112</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>45.1</td>
<td>1</td>
<td>2.6</td>
</tr>
<tr>
<td>Bco. Caballar, Almeria, 100 m</td>
<td>CAB</td>
<td>36°50.6'/2°28.7&quot;</td>
<td>292</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>47.8</td>
<td>2</td>
<td>1.9</td>
</tr>
<tr>
<td><strong>B)</strong> Morocco, W Rif</td>
<td></td>
<td></td>
<td>999</td>
<td>24</td>
<td>15</td>
<td>48</td>
<td>0.167±0.082</td>
<td></td>
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<tr>
<td><em>Sonchus pustulatus</em></td>
<td></td>
<td></td>
<td>1062</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>50.6</td>
<td>2.3⁰</td>
<td>3.6⁰</td>
</tr>
<tr>
<td>Oued Laou valley, 120 m</td>
<td>LAUF</td>
<td>35°23.3'/5°12.6&quot;</td>
<td>540</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>52.6</td>
<td>2.0</td>
<td>2.9</td>
</tr>
<tr>
<td>Oued Laou valley, 153 m</td>
<td>LAUL</td>
<td>35°20.8'/5°11.0&quot;</td>
<td>221</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>44.3</td>
<td>2.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Oued Laou valley, 150 m</td>
<td>LAO</td>
<td>35°17.4'/5°13.8&quot;</td>
<td>1980</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>48.0</td>
<td>2</td>
<td>—</td>
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<tr>
<td>Oued Al-Kannar defile, 244 m</td>
<td>KAN</td>
<td>35°13.0'/5°01.2&quot;</td>
<td>6188</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>52.2</td>
<td>1.8</td>
<td>2.7</td>
</tr>
<tr>
<td><strong>C)</strong> Morocco, NW Rif</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td><em>Sonchus fragilis</em></td>
<td></td>
<td></td>
<td>33927</td>
<td>18</td>
<td>12</td>
<td>11</td>
<td>0.138±0.074</td>
<td></td>
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<tr>
<td>Montes Ghoghziz, Tetouan, 550 m</td>
<td>GHO1</td>
<td>35°32.1'/5°23.1&quot;</td>
<td>26985</td>
<td>5</td>
<td>3</td>
<td>6</td>
<td>45.8</td>
<td>1</td>
<td>2.6⁰</td>
</tr>
<tr>
<td>Montes Ghoghziz, Tetouan, 550 m</td>
<td>GHO2</td>
<td>35°32.8'/5°22.6&quot;</td>
<td>661</td>
<td>5</td>
<td>3</td>
<td></td>
<td>41.9</td>
<td>2.5⁰</td>
<td>4.6⁰</td>
</tr>
<tr>
<td>Montes Ghoghziz, Tetouan, 550 m</td>
<td>GHO3</td>
<td>35°32.3'/5°22.3&quot;</td>
<td>6188</td>
<td>5</td>
<td>3</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Montes Dersa, Tetouan, 275 m</td>
<td>DER</td>
<td>35°35.9'/5°24.6&quot;</td>
<td>6282</td>
<td>5</td>
<td>3</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td><strong>D)</strong> Morocco, central Rif</td>
<td></td>
<td></td>
<td>42269</td>
<td>35</td>
<td>21</td>
<td>74</td>
<td>0.177±0.086</td>
<td></td>
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<tr>
<td>Torres de Alcalá beach, s.l.</td>
<td>TOR</td>
<td>35°09.4'/4°19.7&quot;</td>
<td>6244</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>44.3</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Bades beach—Peñón Veléz, s.l.</td>
<td>BAD1</td>
<td>35°10.2'/4°17.9&quot;</td>
<td>6444</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>50.6</td>
<td>2</td>
<td>2.1</td>
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<tr>
<td>Valley to Bades beach, 60 m</td>
<td>BAD2</td>
<td>35°09.6'/4°16.9&quot;</td>
<td>638</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>43.5</td>
<td>1</td>
<td>1.8</td>
</tr>
<tr>
<td>Boumahi beach, s.l.</td>
<td>BOU</td>
<td>35°14.0'/4°00.7&quot;</td>
<td>19513</td>
<td>5</td>
<td>3</td>
<td>12</td>
<td>45.1</td>
<td>1.6</td>
<td>0.117±0.018</td>
</tr>
<tr>
<td>Cebadilla beach, Al-Hoceima, s.l.</td>
<td>ALH</td>
<td>35°14.6'/3°58.0&quot;</td>
<td>688</td>
<td>5</td>
<td>3</td>
<td>9</td>
<td>42.3</td>
<td>1.5</td>
<td>0.096±0.018</td>
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<tr>
<td>Quenmedo beach, Al-Hoceima, s.l.</td>
<td>QUE</td>
<td>35°14.5'/3°55.5&quot;</td>
<td>6708</td>
<td>5</td>
<td>3</td>
<td>11</td>
<td>52.2</td>
<td>3</td>
<td>2.2⁰</td>
</tr>
<tr>
<td>Stilba beach, Al-Hoceima, s.l.</td>
<td>SFI</td>
<td>35°12.8'/3°54.3&quot;</td>
<td>2035</td>
<td>5</td>
<td>3</td>
<td>10</td>
<td>50.2</td>
<td>2.0</td>
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maximum likelihood (ML) analyses, in which optimal models of molecular evolution were chosen with the likelihood ratio test (Whelan and Goldman, 1999) implemented in the program MODELLTEST v3.7 (Posada and Crandall, 1998). Model parameters were then imported into PAUP*, and a heuristic search (asis sequence addition, TBR branch swapping, and MULPARS option on) was executed. We also conducted ML bootstrap analyses with 1000 replicates to determine the support for each clade. Thirdly, we performed Bayesian analyses (BI) using the program MRBAYES v3.1.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). We calculated the likelihood parameters for the Bayesian analysis using the program MRMODELTEST v2.2 (Nylander, 2004). We ran the Bayesian Markov chain Monte Carlo (MCMC) algorithm for 4000000 generations, with four simultaneous chains (three “cold” and one “heated”), starting from random trees and sampling every 100 generations. The program TRACER v1.5 (Rambaut and Drummond, 2009) was used to evaluate the burn-in and to examine log likelihoods, ensuring that the run was in the stationary phase and that adequate effective sample sizes (ESS) were attained. A conservative 20% burn-in was removed from the sampled set of trees and a 50% majority-rule consensus tree was generated from the remaining trees. For estimates of Bayesian clade support, we considered strong support for values of posterior probability (PP) ≥ 0.95, moderate support for 0.90 ≤ PP ≥ 0.94, and weak support, we considered strong support for values of posterior probability (PP) ≥ 0.95, moderate support for 0.90 ≤ PP ≥ 0.94, and weak

to no support for PP ≤ 0.89.

A haplotype network was also constructed from the cpDNA data set. Due to the absence of homoplasy in this data set, based on the consistency index (CI) of the MP analysis (see Results), haplotypes were networked manually using the principle of maximum parsimony. The manual network of haplotype was confirmed by the statistical parsimony implemented in the program TCS version 1.21 (Clement et al., 2000). Gaps were treated as missing data, and the connection limit was set to 95% in accordance with Hart and Sunday (2007).

To calibrate the phylogeny, we performed a Bayesian relaxed-clock approach using the uncorrelated-rates model implemented in the program BEAST v1.7.3 (Drummond et al., 2006; Drummond and Rambaut, 2007). Our newly generated sequences from Sonchus section Pustulati were combined with the ITS data set of the subtribe Sonchinea available in GenBank, which were generated by Kim et al. (2007). We identified the best-fit substitution model for the ITS data (GTR+I+G) with MRMODELTEST v2.2 (Nylander, 2004). The input data were compiled in the program BEAUTI v1.7.3 (Drummond et al., 2006; Drummond and Rambaut, 2007) with the two prior sets as follows: (1) the age for the most recent common ancestor of genus Dendrosperis in the Juan Fernandez Islands; normal prior distribution with a mean of 3.3 Ma and standard deviation of 0.4 (giving a 95% confidence interval ranging from 2.6–4.0 Ma; Sang et al., 1994); (2) the age for the monophyletic clade of Reichardia, early-diverged genus within the Sonchinea; uniform prior distribution with a mean of 8.1 Ma and standard deviation of 2.8 (giving a 95% confidence interval of 3.6–13.6 Ma; S.-C. Kim, unpublished data). The clock model was set to relaxed uncorrelated lognormal, and the Yule process was chosen as the speciation process. Posterior distributions for each parameter were estimated by means of a MCMC run for 4000000 generations with parameters logged every 5000 generations. The output log files were analyzed with TRACER v1.5 (Rambaut and Drummond, 2009) to assess convergence and to confirm that the effective sample sizes (ESS) for all parameters were larger than 200, ensuring that the MCMC had run long enough to produce a valid estimate of the parameters. We discarded 10% of burn-in (i.e., 800 trees) based on the TRACER results and produced a maximum credibility tree using TreeAnnotator v1.7.3 (Drummond et al., 2006; Drummond and Rambaut, 2007).

AFLP analyses—To assess population genetic diversity from the presence/absence matrix, we calculated the percentage of polymorphic fragments (%P) with the program AFLP-SURV v1.0 (Vekemans, 2002) and the number of private fragments (Fr) with the program FAMD v1.25 (Schlüter and Harris, 2006). Average gene diversity (Hₑ) at the population level was computed with the program Arlequin v3.11 (Excoffier et al., 2005). We determined an additional measure of isolation degree, the Rarity 1 index (equivalent to the frequency of down-weighted marker values; i.e., DW sensu Schönswetter and Tritsch, 2005) with R v2.14.1 (R Development Core Team, 2011) and AFLPDAT functions (Ehrich, 2006). The values were calculated from 100000 repetitions by means of the R function Rarity.permut, which implements a permutation approach to assess whether rarity values for populations are higher or lower than what is to be expected by chance, assuming that all markers are distributed in all populations at random. To estimate among-populations relationships, we computed Net’s pairwise genetic distance data and 10000 bootstrap distance matrices using AFLP-SURV v1.0 (Vekemans, 2002). We used these genetic distances to build a neighbor-joining tree (PHYLIP software; Felsenstein, 1993).

We employed two Bayesian approaches to infer the number of distinct genetic clusters (K) in our data using STRUCTURE v2.3.1 (Pritchard et al., 2000) and the R package Geneland v4.0.2 (Guillot et al., 2005; Guillot and Santos, 2010). For the structure analysis, we used the admixture and the correlated allele frequency models. This combination most accurately assigns individuals to closely related groups (Falush et al., 2007). Twenty independent runs for each K = 1–18 were performed by means of 750000 iterations of burn-in followed by 500000 iterations to ensure convergence of the MCMC. The best estimate for the number of clusters (K̂, as described by Evanno et al., 2005) was computed with STRUCTURE HARVESTER (Earl and vonHoldt, 2011). The software CLUMPP, v1.1.2 (Jakobsson and Rosenberg, 2007) was used with the Greedy algorithm and 10000 random input orders of 20 independent runs to determine the optimal alignment of clusters across individual runs for each K. The results from CLUMPP were imported into the program DISTRUCT v1.1 (Rosenberg, 2004) for viewing. For the Geneland analysis, we ran 100000 MCMC iterations and 20 replications for each value of K, setting the thinning to 100, allowing K to vary from 1 to 18, and with all spatial individual coordinates added. We employed the correlated frequency model, which enables even subtle genetic structuring to be detected (Guillot, 2008). A burn-in period of 200 was computed in the postprocessing. We then calculated the mean PP distribution of the data for each of the 20 runs and selected only the 10 runs with the highest posterior distribution to be considered in the analysis. The results of computations were visually checked but were not consistent across runs (10 independent MCMC simulations) in terms of estimated number of populations K, so the conclusion was based on the run giving the highest average PP, as suggested by the Geneland software manual.

We determined the extent of genetic differentiation, measured as Fst (the fixation index), both for pairs of populations (pairwise Fst) and for geographically based sets of populations (AMOVA; Arlequin v3.1.1; Excoffier et al., 2005). The confidence intervals of
the $F_{ST}$ values were determined through bootstrapping (20,000 replicates), as implemented in Arlequin. Finally, to detect possible patterns of isolation by distance, we performed Mantel tests with 10,000 permutations using XLSTAT software (Addinsoft, 2010). To this end, we tested the correlation between population pairwise-$F_{ST}$ values and geographic distances for each distribution area.

RESULTS

**cpDNA sequences**—The aligned sequences of accD–psaI and 3′trnL(UAA)–ndhJ intergenic spacers were 775 and 723 bp long, respectively. Of 1498 bp of aligned sequences from the two combined intergenic regions, 1475 characters were constant, and 23 characters were parsimony informative. For the accD–psaI intergenic spacer, three sets of indels were found: (1) a 7-bp insertion and a 17-bp direct repeat insertion shared by *S. fragilis* and *S. masguindalii*; (2) a 4-bp direct repeat insertion and an 8-bp direct repeat deletion in all ingroup taxa; and (3) a 7-bp insertion shared by *S. pustulatus* and *S. masguindalii*. As for the 3′trnL(UAA)–ndhJ intergenic spacer, 23-bp and 40-bp deletions were found in *S. fragilis* and *S. pustulatus*, respectively.

The MP analysis for cpDNA data provided more than 100,000 equally parsimonious trees with a tree length (TL) of 23, a consistency index (CI) of 1.0 and a retention index (RI) of 1.0. The topology of the resulting MP and ML trees was identical and also consistent with the Bayesian analysis results (Fig. 2). These trees showed early divergence of *S. pustulatus* within the section *Pustulati*, followed by a clade containing the two other remaining species of *S. masguindalii* and *S. fragilis* (MP: 100%BS / ML: 100%BS / BI: 1.00PP). Both *S. pustulatus* and *S. masguindalii* turned out to be not monophyletic perhaps due in part to slow mutation rate of two short chloroplast noncoding regions.

In the haplotype network (Fig. 1b), two haplotypes were found in *S. pustulatus*, H1 for Rifan populations (LAUF, LAUL, LAUO, TAL, and KAN) and H2 for Baetic populations (ANT, TEL, and CAB). *Sonchus fragilis* had only one haplotype (H3) and *S. masguindalii* three: H4 (TOR, BAD1, and BAD2), H5 (BOU, ALH, QUE, SFI, and one individual from BAD2), and H6 (two individuals from SFI).

**ITS and ETS nuclear ribosomal DNA sequences**—The aligned ITS and ETS regions were 646 and 393 bp, respectively. Of 1039 aligned sites, 908 were constant, 2 were variable but parsimony uninformative, and 129 were variable and parsimony informative. In the Rifan populations of *S. pustulatus*, we detected few polymorphic sites in the ITS and ETS sequences, with only four of 393 aligned sites in the ETS (1%) and 12 of 495 aligned sites in the ITS 1 and 2 regions (2.4%). The populations of Baetic *S. pustulatus*, *S. fragilis*, and *S. masguindalii* showed no polymorphisms in the ITS and ETS sequences.

The MP search found more than 100,000 equally parsimonious trees, with a TL of 144, a CI of 0.9583, and an RI of 0.9946 (trees not shown). The model test analysis found GTR+G as the best model for nucleotide evolution, and the ML search found a single best tree (Fig. 3). The BI tree (not shown) presented the same tree topology as that of the MP and ML. All three different analyses based on
combined ITS/ETS showed that the section *Pustulati* is monophyletic (100%BS/100%BS/1.00PP) and is quite divergent from the chosen outgroup species *S. palustris*. The monophyly of section *Pustulati* was further confirmed based on the broader phylogenetic analysis of subtribe Sonchinae (Kim et al., 2007; Appendix S2, see online Supplemental Data). They also clearly demonstrated early divergence of *S. masguindalii* within the section *Pustulati* (100%BS/100%BS/1.00PP) and a sister relationship between *S. pustulatus* and *S. fragilis* (90%BS/93%BS/1.00PP). Within *S. masguindalii*, populations TOR, BAD1, and BAD2 turned out to be sisters to populations BOU and ALH (86%BS/99%BS/0.99PP), and in turn, this group of five populations was sister to SFI and QUE (100%BS/100%BS/1.00PP). There was no ribotype variation within *S. fragilis*, whereas three ribotypes were found in each of the *S. masguindalii* and *S. pustulatus* species. In *S. pustulatus*, two and one ribotypes were found in the Rifan and Baetic populations, respectively.

The most recent common ancestor age of *Sonchus* section *Pustulati* was estimated to be approximately 5.48 Ma (3.171–8.766 Ma 95% CI; Fig. 4a; Appendix S2). Within section *Pustulati* (Fig. 4), the divergence time between *S. pustulatus* and *S. fragilis* was approximately 3.89 Ma (2.04–6.48 Ma 95% CI) and between Baetic and Rifan populations of *S. pustulatus*, approximately 1.18 Ma (0.42–2.41 Ma 95% CI). The latter time was similar to the observed divergence between *S. masguindalii* populations (1.39 Ma; 0.46–2.83 Ma 95% CI).

**AFLP marker diversity**—The negative controls showed no amplification. The average reproducibility of the AFLP makers, i.e., the average proportion of consistently reproduced bands over all replicates, was 98.4 ± 1.17% (mean ± SE). All individuals (167) exhibited different multilocus genotypes. The percentage of polymorphism (%) varied from 41.9 to 52.6%, and Frag PRI was zero in most populations (Table 1). The DW values, either taking into account all studied plants or only those plants within populations of *S. pustulatus* and *S. fragilis*, were significantly higher in the *S. fragilis* populations and, in general, were significantly lower in the Baetic populations (Table 1). The genetic diversity values of the Baetic populations of *S. pustulatus* were low (total $H_E = 0.109$) in comparison with the remaining distribution areas (total $H_E = 0.138–0.177$), and with Rifan *S. pustulatus* (total $H_E = 0.167$; Table 1).

**Genetic distances among populations**—Two main groups within section *Pustulati* were recognized in the neighbor-joining phylogram based on Nei’s genetic distance (Fig. 5a). One of them comprised the *S. pustulatus* and *S. fragilis* species, and the other comprised *S. masguindalii*. The first group was clearly divided into three clusters. The populations of *S. fragilis* were separated from the whole set of *S. pustulatus* (82% BS), and within this latter set, the Baetic and Rifan populations were also separated (82% BS). The Rifan populations of *S. pustulatus* from the Oued Laou valley were grouped together excluding LAUO, which was clustered apart with KAN (78%), the most isolated population located in the Oued Al-Kannar valley (20 km away, Fig. 1a). Within the Baetic cluster, the first node separated population ANT from the others (100%). A notable feature within the cluster of *S. masguindalii* was the presence of an independent clade (100% BS) that included the two easternmost populations, SFI and QUE.

**STRUCTURE and Geneland results**—The Bayesian analysis conducted with STRUCTURE showed that $K = 2$ was the most likely number of clusters in our data set, followed by $K = 7$ (online Appendix S3); $K = 2$ clearly separated all individuals of *S. masguindalii* from the rest (Fig. 5b), and $K = 7$ separated five groups in which most individuals

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**FIGURE 3** One maximum likelihood (ML) tree from the analysis of nuclear ITS/ETS sequences. Numbers at branches are maximum parsimony bootstrap values /ML bootstrap values/posterior probability.
FIGURE 4  (a) Chronograms of the evolution of *Sonchus* section *Pustulati* based on molecular dating from the nuclear ITS/ETS sequences. Capital letters (A–D) represent the distribution areas of taxa as in Fig. 1a. Node bars represent the 95% highest posterior density intervals for the divergence time estimates of clades. (b–g) Phylogeographic reconstruction of the lineages across the western Mediterranean basin since the Middle Miocene. Capital letters on maps represent the estimated occupied region for the ancestor (Pre-D) and taxa (A–D). Black arrows indicate events of diversification. Hypotheses suggested for the origin of the current disjunction of *S. pustulatus*: (1) recent long distance seed-dispersal events over the Alboran Sea (gray arrows in g), and (2) short-distance dispersal events across the land bridge that connected Iberia and North Africa (gray arrows in f) followed by historical geographical isolation (g). Paleogeographic information was taken from Krijgsman et al. (1999), Martín et al. (2001), Rosenbaum et al. (2002), Fauquette et al. (2006), and Garcia-Castellanos et al. (2009).
exhibited very high assignment probabilities (Fig. 5b). All individuals of *S. fragilis* were grouped in the same cluster, and individuals of *S. pustulatus* were separated into two clusters: a Rifan one and a Baetic one. The individuals from the populations QUE and SFI were also separated from the remaining individuals of *S. masguindalii*, although with a higher level of genetic admixture. The two remaining clusters appear in many individuals of diverse origins and may result from noise produced by the sensitivity of the substructuring of population nuclei that Geneland generates in cases of isolation by distance (Kalinowski, 2011).

The Bayesian analysis performed with Geneland found *K* = 6 as the most likely number of clusters in our data set (Appendix S3). This analysis also separated individuals according to distribution areas, but giving three (rather than two) clusters for individuals of *S. masguindalii*: those from the western (populations TOR, BAD1 and BAD2), intermediate (ALH and BOU), and eastern ends (SFI and QUE). The splitting between western and intermediate populations could be a consequence of the over substructuring of population nuclei that Geneland generates in cases of isolation by distance (Cushman et al., 2006), although it was not detected by the Mantel test for this species (see below).

**FIGURE 5** (a) Neighbor-joining analysis of 17 populations of *Sonchus* section *Pustulati* based on Nei’s genetic distance data. Numbers by nodes are bootstrap values >50. Colored circles denote main genetic groups inferred from Bayesian clustering for *K* = 7, and colored squares for *K* = 2. (b) Genetic structure of populations inferred using the model-based Bayesian algorithm implemented in the software STRUCTURE for *K* = 7 and *K* = 2. Each vertical line represents an individual that is divided into *K* colored segments depicting their membership in each of the *K* clusters. Populations are separated by vertical black bars.

**DISCUSSION**

**Phylogenetic congruence with current taxonomic treatment in *Sonchus* section *Pustulati***—Present phylogenetic results support current taxonomic treatment of the three species that confirm section *Pustulati* Boulos of genus *Sonchus*: *S. masguindalii*, *S. fragilis*, and *S. pustulatus*. These were clearly separated with each of the used molecular markers. The high congruence identified between the ITS/ETS and AFLP analyses supports the monophyletic origin of *Sonchus* section *Pustulati* (Kim et al., 2007, 2008). Furthermore, the topological incongruence detected between phylogenies based on maternally inherited chloroplast sequences (cpDNA) and biparentally inherited nuclear sequences (ITS/ETS and AFLP) led to the hypothesis that *S. pustulatus* originated by interspecific hybridization from *S. fragilis*, as a likely paternal contributor, and a still unknown maternal donor from the root lineage of sections *Sonchus*/*Asperi* (Kim et al., 2008). However, alternative hypotheses, such as incomplete lineage sorting of ancient polymorphisms or *S. pustulatus* simply being the carrier of a captured plastid lineage to explain the incongruence between nuclear and chloroplast data, cannot be completely ruled out. Additional study based on several independent single- or low-copy nuclear loci may sort out those conflicting patterns between chloroplast and nuclear DNA.

**A pre-Mediterranean clade: Late Miocene-Pliocene origin of the species**—In this study, we also found that *Sonchus* section *Pustulati*
shows a clear taxonomic and geographical pattern across the western Mediterranean basin. Our dating of the molecular divergence indicates an old origin for the group which suggests a paleoendemic (Thomson, 2005) and, likely, a relic condition of the species. Within the genus Sonchus sensu lato (Kim et al., 2007), the section Pustulati appears to be quite old, more ancient than the Macaronesian woody approximately 5.48 Ma (8.77–3.17 Ma 95% CI), suggesting that section divergence of Sang et al., 1994). Based on our molecular dating, the early divergence of S. masguindali (sensu lato), the section S. pustulatus sensu lato (Kim et al., 2007), the section Pustulati (Thompson, 2005) and, likely, a relict condition of the species. Within our knowledge, only the Colchicaceae Androcymbium gramineum

Nonetheless, we cannot determine whether the ancestors of these species (Fig. 4b–d) were found in North Africa and/or the Baetic-Rifan microplate. Based on our molecular dating, the origin and diversification of the species of Sonchus section Pustulati occurred when Iberia and North Africa were continuously or near-continuously connected by a land bridge (Fig. 4e, f). Moreover, the current clear predominance of the species in the Rifan range and the narrow distribution of the oldest species in the central Rif suggest that they diversified in southern areas of the land bridge (Fig. 4e, f).

**Divergence of the Baetic and Rifan populations of S. pustulatus—**

The highly disjunct distribution of the populations of Sonchus pustulatus is one of the most striking aspects of the phylogeography of the section. Both Baetic and Rifan ranges of this species (areas A and B; Fig. 1a) are included in different floristic units (Valdés, 1991) or ecoregions (Molina-Venegas et al., 2013), present significant climatic differences and are located approximately 280 km from each other. Mean annual rainfall in the Baetic (A) and Rifan (B) areas is 250 and 850 mm, respectively (Hijmans et al., 2005). The disjunction is reflected by the clear genetic differentiation detected by the AFLP (Fig. 5) and ITS/ETS (Fig. 3) analyses, as well as the presence of different cpDNA haplotypes in both ranges (Fig. 1b). However, the hypothesis that cryptic speciation might have occurred can be discarded due to the high interfertility detected between plants from both areas and because they have some small morphological, ecological, and physiological differences (Silva, 2014).

The distribution of S. pustulatus is not common among Baetic-Rifan plants. Most of the studied plant taxa exhibiting disjunct distributions in the region are located close to the Strait of Gibraltar in both Baetic and Rifan ranges (e.g., Burban and Petit, 2003; Ortiz et al., 2007; Arroyo et al., 2008; Escudero et al., 2008) which present similar climates and a transmarine separation of only 14 km. To our knowledge, only the Colchicaceae Androcymbium gramineum

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**TABLE 2.** Results of analyses of molecular variance (AMOVA) based on AFLP analysis of the populations of Sonchus section Pustulati. Statistics included degrees of freedom (df), variance component (VC), percentage of variation (%), and fixation index ($F_{st}$).

<table>
<thead>
<tr>
<th>Groupings</th>
<th>N</th>
<th>Source of variation</th>
<th>df</th>
<th>VC</th>
<th>%</th>
<th>$F_{st}$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Among distribution areas</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[Rifan S. pustulatus]; [Baetic S. pustulatus]; [S. fragilis]; [S. masguindali]</td>
<td>4</td>
<td>Among groups</td>
<td>3</td>
<td>13.96</td>
<td>62.27</td>
<td>0.70* (0.675–0.748)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Among populations within groups</td>
<td>13</td>
<td>1.68</td>
<td>7.50</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Within populations</td>
<td>150</td>
<td>6.77</td>
<td>30.23</td>
<td></td>
</tr>
<tr>
<td>S. pustulatus</td>
<td>2</td>
<td>Among groups</td>
<td>1</td>
<td>8.51</td>
<td>40.29</td>
<td>0.51* (0.438–0.568)</td>
</tr>
<tr>
<td>[Baetic range]; [Rifan range]</td>
<td></td>
<td>Among populations within groups</td>
<td>6</td>
<td>2.23</td>
<td>10.54</td>
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<td></td>
<td>Within populations</td>
<td>74</td>
<td>10.39</td>
<td>49.17</td>
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<tr>
<td>Area B–Area C</td>
<td>2</td>
<td>Among groups</td>
<td>1</td>
<td>7.55</td>
<td>46.97</td>
<td>0.57* (0.498–0.631)</td>
</tr>
<tr>
<td>[Rifan S. pustulatus]; [S. fragilis]</td>
<td></td>
<td>Among populations within groups</td>
<td>5</td>
<td>1.07</td>
<td>6.64</td>
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<td></td>
<td></td>
<td>Within populations</td>
<td>52</td>
<td>7.46</td>
<td>46.39</td>
<td></td>
</tr>
<tr>
<td>Baetic S. pustulatus</td>
<td>1</td>
<td>Among groups</td>
<td>2</td>
<td>0.84</td>
<td>13.58</td>
<td>0.14* (0.116–0.199)</td>
</tr>
<tr>
<td>[ANT, TEL, CAB]</td>
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<td>Within populations</td>
<td>31</td>
<td>5.33</td>
<td>86.42</td>
<td></td>
</tr>
<tr>
<td>Rifan S. pustulatus</td>
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<td>Among groups</td>
<td>4</td>
<td>2.53</td>
<td>17.17</td>
<td>0.17* (0.128–0.220)</td>
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<tr>
<td>[LAUF, LAUL, LAUO, TAL, KAN]</td>
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<td>Within populations</td>
<td>43</td>
<td>12.21</td>
<td>82.83</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Within populations</td>
<td>1</td>
<td>1.97</td>
<td>13.63</td>
<td>0.14* (0.091–0.189)</td>
</tr>
<tr>
<td>S. masguindali</td>
<td>1</td>
<td>Among groups</td>
<td>3</td>
<td>1.97</td>
<td>13.63</td>
<td>0.14* (0.091–0.189)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Within populations</td>
<td>12.50</td>
<td>86.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Among groups</td>
<td>6</td>
<td>4.31</td>
<td>25.04</td>
<td>0.25* (0.219–0.312)</td>
</tr>
<tr>
<td>[TOR, BAD1, BAD2, BOU, ALH, QUE, SFI]</td>
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<td>Within populations</td>
<td>67</td>
<td>12.90</td>
<td>74.96</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Among groups</td>
<td>1</td>
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<td>13.78</td>
<td>0.30* (0.251–0.393)</td>
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<td></td>
<td>Within populations</td>
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<td>3.09</td>
<td>16.68</td>
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<td></td>
<td></td>
<td>Within populations</td>
<td>67</td>
<td>12.90</td>
<td>69.54</td>
<td></td>
</tr>
</tbody>
</table>

Note: * $P < 0.001.$
(Caujapé-Castells and Jansen, 2003), which colonizes the Atlantic Rifen coast and southeastern Spain, has the most similar geographic disjunction, at least in the northern range.

We propose two alternative hypotheses to explain the current disjunction of *S. pustulatus*: long-distance dispersal (LDD) vs. vicariance (Fig. 4f, g). The first hypothesis suggests that recent LDD over the Mediterranean Sea during the Quaternary is responsible for this current disjunction (hypothesis 1). This hypothesis suggests that a long-dispersal event from North Africa to the southeastern Iberian Peninsula occurred after the Mediterranean basin was completely reflooded, via an unknown vector (e.g., wind, marine currents), followed by effective colonization. The second hypothesis, a vicariance process, is that a former semicontinuous distribution of the species across the temporary land bridge that joined Iberian and North Africa was split into two allopatric units (Baetic and Rifen) following the reflooding of the Mediterranean basin (hypothesis 2). Given the typical fragmented nature of cliff habitats, this hypothesis might involve a south to north range expansion following a stepping-stone process (Kimura, 1953) across the land bridge during late Messinian or early Zanclean times. At the end of the Mediterranean reflooding (5.33–3.60 Ma), a restriction of distribution range may have taken place by direct progressive flooding of their habitats, resulting in the current disjunct distribution, and therefore leading to a vicariance process through historical isolation. The differences in the rainfall regimen between the two ranges, probably established during the late Pliocene (3–2.8 Ma; Suc, 1984), could have contributed further to observed genetic differentiation.

The molecular dating of the divergence between the Rifen and Baetic populations of *S. pustulatus* (1.18 Ma, Fig. 4) seems to fit better with hypothesis 1. Further supports for hypothesis 1 are given by the low DW values of the Baetic populations (Table 1), as expected in relatively recently established populations (Schönswetter and Tribsch, 2005) and also by their impoverished genetic diversities, which seem to be associated with neoendemisms rather than with paleoendemisms (Fernández-Mazuecos et al., 2014). However, assuming that hypothesis 1 explains the phylogeography of *S. pustulatus* contradicts the ecological and biological traits of *Pustulati* species (heavy seeds with a short-lasting pappus, relatively strong self-incompatibility systems, high ecological specificity, low capacity for recruitment, long-life cycle, etc.; Silva, 2014; Silva et al., 2015), generally typical of cliff-dwelling species (Larson et al., 2005), and that are associated with limited dispersal and colonization abilities. But LDD events have recently been proposed to explain the disjunct distributions of several other taxa without special LDD mechanisms (e.g., Guzmán and Vargas, 2009; Fernández-Mazuecos and Vargas, 2011; Vargas et al., 2012; Lavergne et al., 2013), but probably with different demographical structures and dynamics, and wider ecological amplitude than *Pustulati* species.

Hypothesis 2, invoking a vicariance process, has been suggested to be responsible for the similar disjunction seen in *Androecymbium gramineum*, a species also lacking adaptations to LDD of seeds. This disjunction is argued to have originated through range expansion in the region during the Messinian desiccation of the Mediterranean Sea followed by posterior historical isolation with the end of the Messinian Salinity Crisis (Caujapé-Castells and Jansen, 2003). This biogeographical scenario has also been proposed for several species disjunctions that are clearly closer to the Strait of Gibraltar, have a current wider distribution and a high ecological range, and/or show adaptations to LDD of seeds (e.g., *Saxifraga*, Vargas et al., 1999; *Frangula*, Hampe et al., 2003; *Campanula broussonetiana/C. transtagana* clade, Cano-Maqueda et al., 2008).

This continuing uncertainty clearly highlights the need for further study on the phylogeographic relationships between plants from both sides of the western Mediterranean basin. The incorporation of molecular data of *S. pustulatus* samples from northern Algeria (area D, Fig. 1), assuming that the species still occurs there, will be crucial with regard to discerning which of the two proposed hypotheses is preferred, or for revealing new possibilities.

**Population genetic structure and recent biogeographical trends**

As expected for cliff-dwelling species, a relatively high genetic structuring was the rule within each distribution area, especially in *S. masguindali*. Gene flow generally appears to be virtually restricted to neighboring populations (Appendix S4). This scenario has also been detected in other cliff-dwelling taxa, e.g., in *Centaurea horrida* (Mameli et al., 2008) or in *C. borjae* (Lopez and Barreiro, 2013). As a strictly entomophilous species, pollen-mediated gene flow in *Pustulati* species is probably spatially limited from a few meters to several kilometers (e.g., Ishihama et al., 2005) and rarely over 10 km (e.g., Fénart et al., 2007). Moreover, seed dispersal is surely limited to short displacements on the same cliff or to nearby ones, and most seeds end up at the cliff base where they do not successfully develop (Silva et al., 2015). The high genetic structuring detected among the populations of *S. masguindali* may be also favored by a restricted pollination environment, typical of sea cliff habitats (e.g., Gemmill et al., 1998; Cureton et al., 2006).

Because we have detected moderate gene flow between adjacent or nearby populations (Appendix S4) and a relatively high genetic structuring within each distribution area, we might propose that each area, excluding that of *S. masguindali*, constitutes a system of metapopulations. However, neither recurrent population extinctions nor colonization events are expected to be frequent due to the low individual recruitment and high population resilience (Silva, 2014; Silva et al., 2015). In Baetic *S. pustulatus* (area A), the current narrow distribution range with only three small and relatively genetically impoverished populations might be the result from a restriction process from a previous larger set of populations, including intermediate locations. The clear tendency toward decline detected in two populations and the registered extinction of another population (Silva et al., 2015) would support such a restriction in range. In the case of the populations of *S. fragilis*, a restriction process similar to area A might have taken place. In this case, the very high levels of self-compatibility detected (Silva, 2014), which ensure seed production when conditions for cross-pollination become limited (Kalisz et al., 2004), may help to maintain greater population sizes, consequently enhancing their resilience.

At present, *Sonchus masguindali* occupies a narrow range (11 km²) with a shared continuously distributed coastal geologic sub-strate (Triassic and Jurassic limestone and dolostone; Déil and Hammoumi, 1997). Although we do not have data relating to changes in their distribution throughout geologic times, the observation of high population genetic structuring indicates a long presence in the area, likely during most of the Pleistocene. The Al-Hoceima Cape, located between the easternmost populations (QUE and SFI) and the remaining ones, surely acted as a semipermeable but effective physical barrier to gene flow among populations from both sides and is the responsible for the detected genetic divergence between them (1.39 Ma, 0.46–2.83 Ma 95% CI). This has led to long-term isolation resulting in the substantial genetic differentiation detected by most AFLP and ITS analyses.
Genetic diversity and conservation considerations—In general, genetic diversity in the taxa of Sonchus section Pustulati is not depleted, with the exception of the Baetic populations of S. pustulatus. The genetic diversities of these taxa are only moderate in comparison with those estimated in other rare endemic cliff-dwelling species with the same AFLP technique; e.g., in the phylogenetically close Sonchus gandoger (H = 0.38; Kim et al., 2005), or in Centaurea borjae (H = 0.26; Lopez and Barreiro, 2013). However, these moderately high levels of genetic variation within Sonchus section Pustulati are consistent with studies of other narrow endemics (Jiménez-Mejias et al., 2015) and even of other Mediterranean palaeoendemics (Fernández-Mazuecos et al., 2014). Gene exchange among neighboring populations and long life-span (Silva et al., 2015) most likely account for the diversity detected within populations of section Pustulati (Jiménez-Mejias et al., 2015).

Although all populations of Sonchus section Pustulati should be preserved because of the rarity of the group, conservation efforts should concentrate on those populations with the most restricted geographical distribution and that are not located within protected areas, i.e., those of the Baetic S. pustulatus, S. fragilis and the easternmost populations of S. masquinulii (SFI and QUE). These conservation actions might consist of creating microreserves, which have become an essential tool for effective protection of diverse flora in the western Mediterranean region (Laguna et al., 2004). In addition, the extant genetic diversity in the populations should be preserved by ex situ conservation programs that allow future reintroductions or population reinforcements (Fernández-Mazuecos et al., 2014). To this end, it would be necessary to take genetic samples from each of the different evolutionary significant units, i.e., units of organisms that are sufficiently differentiated to require separate management on conservation actions (Frankham, 2010), that we have defined based on our genetic analyses: two for S. pustulatus (corresponding to the Baetic and Rifan populations), two for S. masquinulii (the TOR-BAD1-BAD2-BOU-ALH and QUE-SFI populations), and a single unit for S. fragilis.

CONCLUSIONS

Our results support the suggested monophyly of Sonchus section Pustulati and the hybrid origin of S. pustulatus from S. fragilis and an unknown maternal donor species and show that the section has a clear taxonomic and geographical pattern across the western Mediterranean basin. The dating of the molecular divergence of the Pustulati lineages indicates that its origin and diversification appear to have occurred in the Gibraltar arc during the Messinian Salinity Crisis and the subsequent Zanclean flood. This old origin, with the current highly narrow ecological amplitude (Silva, 2014) and restricted distribution of the species (Silva et al., 2015), suggests a relict condition. Although genetic data more consistently support that the only Baetic populations, of S. pustulatus, most likely originated from the Rifan ones via recent (Quaternary) LDD, the known biological traits of this species (Silva, 2014) and the most similar case studied (Caujapé-Castells and Jansen, 2003) also led us to propose that Baetic populations may have originated through ancient (Late Tertiary) vicariance. On the other hand, the observed genetic variation and diversity of populations in these cliff-dwelling species suggest that seed dispersal and gene flow are virtually restricted to neighboring populations and that conservation efforts may well be crucial for the future viability of those populations with the most restricted geographical distribution.

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