**STRAIT OF GIBRALTAR: AN EFFECTIVE GENE-FLOW BARRIER FOR WIND-POLLINATED CAREX HELODES (CYPERACEAE) AS REVEALED BY DNA SEQUENCES, AFLP, AND CYTOGENETIC VARIATION**

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The Strait of Gibraltar is the most important barrier disconnecting the landmasses of Europe and Africa on the western Mediterranean extreme. *Carex helodes* is a wind-pollinated species endemic to the western Mediterranean. Because molecular and cytogenetic data allow the inference of its evolutionary history, we analyzed variations in chromosome number, including meiotic chromosome behavior, amplified fragment length polymorphism (AFLP) fingerprints, and nucleotide substitutions in plastid and nuclear DNA sequences. Cytogeographic results showed that the African populations have stabilized at a single chromosome number of 2n = 74, whereas the most frequent cytotype in Iberia is 2n = 72. Phylogenetic reconstructions of 17 sequences from nine closely related species revealed that *C. helodes* is monophyletic and that the Moroccan populations are embedded in the Iberian lineages. The haplotype network is also consistent with a European origin of the northern African haplotype. AFLP analysis also revealed hierarchical levels of genetic variation compatible with a founder effect process responsible for the African populations. All sources of evidence support the hypothesis that the Strait of Gibraltar has been an effective gene-flow barrier, generating two isolated evolutionary lineages after their dispersal. Recent connections between the two lineages appear unlikely, whereas active gene flow occurs among populations within the two lineages.

**Key words:** AFLP; cytogeography; founder effect; genetic diversity patterns; ITS; long-distance dispersal; rps16 intron; western Mediterranean Basin.

The Mediterranean areas of the Iberian Peninsula and Morocco are considered one of the two primary centers of biodiversity in the Mediterranean basin (Médail and Quézel, 1997). In fact, 5000 species form the Mediterranean flora of the Iberian Peninsula and 3800 species are present in the Mediterranean flora of northern Morocco. Interestingly, 75% of the Mediterranean flora of Iberia is shared with northern Morocco (Valdés, 1991). These floristic figures may be the result of land bridges between the Iberian Peninsula and northwestern Africa in different Tertiary periods. During the last land connection between Iberia and northwestern Africa, the Mediterranean Sea underwent intense desiccation (the Messinian crisis 4.5–5.5 million years ago [Ma]; Duggen et al., 2003), potentially allowing contacts between the two floras and increasing the salinity levels in the region. Therefore, the historical and present characteristics of the Strait of Gibraltar provide an ideal scenario in which to analyze the patterns of evolution of disjunct taxa. The last opening of the Strait of Gibraltar occurred c. 4.5 Ma, and the water that refilled the Mediterranean basin fragmented the distributions of the extant land species. Vicariant species represent part of the current Ibero–North African disjunct endemics (*Androcybium gramineum*; Caujapé-Castells and Jansen, 2003) together with other disjunct taxa that originated by dispersal (*Hypochaeris salzmanniana*; Ortiz et al., 2007). It is unclear which of the many plant disjunctions across the strait is the result of vicariance or dispersal and whether genetic isolation has been maintained over time. This question has already been addressed for some southern Iberian–northern African plants, yielding different results. On the one hand, the 14 km of the Strait of Gibraltar has been shown to be sufficient to cause the isolation of two independent lineages of different plant groups (*Quercus suber*, Toumi and Lumaret, 1998; *Saxifraga globulifera*, Vargas et al., 1999; *Bellis annua*, Fiz et al., 2002; *Quercus ilex*, Lumaret et al., 2002), although neither of the two biogeographic hypotheses (vicariance vs. dispersal) has yet been confirmed. On the other hand, active gene flow between populations has also been documented (*Pinus pinaster*, Burban and Petit, 2003; *Olea europaea*, Rubio de Casas et al., 2006; *Hypochaeris salzmanniana*, Ortiz et al., 2007; *Cistus*, Guzmán and Vargas, in press; Rodríguez-Sánchez et al., in press). Although the gene flow of wind-pollinated trees (*Quercus, Pinus, Olea*) has been analyzed in the geographic context of the Strait of Gibraltar, no phyleogeographic study has been undertaken for herbs displaying anemophily, such as the Gramineae and Cyperaceae.

DNA sequencing at the haploid level is the tool most frequently used in phyleogeography (Schaal et al., 1998; Avise, 2000). Plastid DNA sequencing has been particularly widely used to reconstruct phyleogeographic patterns (Taberlet et al., 1998; Newton et al., 1999; Abbott et al., 2000). Sequencing the

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nuclear genome has also been undertaken in phylogeographic studies, using either low-copy genes (Olsen and Schaal, 1999; Olsen, 2002; Batish et al., 2006) or in the internal transcribed spacer (ITS) regions of multicyclop patterns (for drawbacks, see Alves and Wendel, 2003). However, the low nucleotide variation in mitochondrial and plastid sequences and the inference difficulties caused by the biparental inheritance of nuclear genes hinder phylogeographic reconstruction in many plant species. The amplified fragment length polymorphism (AFLP) technique has been widely used as an alternative in the past few years because of its reliable reproducibility, high levels of genetic variation, its capacity to screen total DNA, and its extended applicability to disparate plant groups (Nyborn, 2004; Meudt and Clarke, 2007).

The use of cytogenetic variation to reconstruct organismal histories at the species level is not widespread in angiosperms because of the low variability in chromosome numbers and features. In contrast, nucleotide substitutions in highly variable regions of DNA (i.e., introns and spacers) generally occur at a higher rate than cytogenetic changes. However, some species display certain chromosomal peculiarities that promote the formation and persistence of different chromosome numbers, providing a potentially valuable source of data for evolutionary studies. This is the case for the species of the genus Carex, which display certain cytogenetic characteristics of interest in phylogeography: the chromosomes contain diffuse centromeres (holocentric chromosomes) that promote the formation of aneuploid series with haploid numbers ranging from \( n = 6 \) to \( n = 66 \) (Tanaka, 1949) via fission (agmatoploidy; Malheiros Gardê and Gardê, 1950; Davies, 1956), fusion (symplody; Luceño and Guerra, 1996), and chromosomal rearrangement (dysplody sensu stricto, mainly reciprocal translocations; Greilhuber, 1995). In fact, fusion, fission, and chromosomal rearrangement processes are so frequent in Carex species that they have even been observed within the same individual (Schmid, 1982; Luceño, 1992a). As a result, cytology has been widely used in evolutionary studies of the Cyperaceae, not only to infer general trends at the species level (Faulkner, 1972; Cayouette and Morisset, 1985), but also to analyze microevolutionary processes among closely related populations (C. laevigata, Luceño and Castroviejo, 1991). Chromosome numbers have also been suggested to vary at a higher rate than one of the most variable nuclear regions, the nuclear ribosomal ITS (nrITS) (Escudero et al., 2008).

The main objective of this study was to infer the evolutionary history of \( C. helodes \) using molecular and cytogenetic data. The specific aims were to (1) clarify the origin of the current species disjunction and (2) analyze the role of the Strait of Gibraltar as a potential barrier to gene flow.

**MATERIALS AND METHODS**

**Study species**—Carex helodes Link (sect. Spirostachyae) is a diploid, wind-pollinated, perennial herb (minimum generation time is two years). This species is well characterized by its caespitose habit (without creeping rhizomes), rough upper leaf surface, high number of male spikes (1–4–7), and androgy nous spikes (Luceño, 1992b). Endemic to the western Mediterranean (locally distributed in southern Portugal and southwestern Spain, rare in northern Morocco) (Luceño and Escudero, 2006; Luceño et al., 2007), this sedge occurs in temporarily inundated acidic soils in open, cork oak woodlands. Despite its well-characterized morphology, \( C. helodes \) has been misidentified as \( C. laevigata \) Sm. by some authors (Kükenthall, 1909; Vicitoso, 1959; Maire, 1957), whereas others (Sampaio, 1921) supported its taxonomic independence. Recent cytotaxonomic and nuclear-based phylogenetic studies have revealed the monophyly of \( C. helodes \) populations and its taxonomic independence within sect. Spirostachyae (Luceño, 1992b; Escudero et al., 2008).

**Sampling strategy**—We sampled nine populations (84 individuals) of \( C. helodes \) representing its distribution: six populations from the southwestern Iberian Peninsula (P1–P5, S) and three from northwestern Africa (M1–M3, Fig. 1). A cytogenetic study was conducted on the same individuals included in the molecular studies (Table 1). We also sequenced the Spanish population (Seville) rediscovered in 2006 (Luceño et al., 2007).

The 13 individuals in which the ITS region was sequenced were chosen to represent the whole distribution of the species and included the different meiotic configurations observed in the cytogenetic study. These \( C. helodes \) sequences were analyzed together with eight ITS sequences of closely related species of \( C. helodes \) and eight individuals from eight species of sect. Spirostachyae. The 83 alleles were sequenced in the \( rps16 \) intron to be the most variable. Therefore, the \( rps16 \) intron was sequenced in nine individuals from the nine populations of \( C. helodes \) and eight individuals from eight species of sect. Spirostachyae. Eighty-three individuals of eight \( C. helodes \) populations were included in the AFLP study: five populations from southern Portugal (51 individuals) and three populations from northern Morocco (52 individuals) (Table 1).

**Chromosome counting, AFLPs, and sequencing**—Meiotic plates were prepared with the method of Luceño (1988). The direct and reverse primers used for the amplifications included ITS-A and ITS-4 for the ITS regions and \( rps16 \) and \( rps2R2 \) for the \( rps16 \) intron (White et al., 1990; Oxelman et al., 1997; Blattner, 1999). For the sequencing conditions for the ITS region and \( rps16 \), see Escudero et al. (2008) and Schönswetter et al. (2006), respectively. The limits of the ITS region and the \( rps16 \) intron were determined following Star et al. (1999) and Schönswetter et al. (2006), respectively. The AFLP procedure followed that of Gaudeul et al. (2000), but the polymerase chain reaction volume was reduced by 50%. A pilot study was performed on six samples consisting of five Moorcan and Iberian individuals plus one replicate. This pilot study included EcoRI AGA (6-FAM) and EcoRI AGG (VIC), and the samples were screened with a set of eight MseI primers with three selective nucleotides. The chosen primer combinations were those that retrieved the greatest number of potentially informative characters after they were tested for reproducibility. As a result, EcoRI AGA (6-FAM)–MseI CAC and EcoRI AGG (VIC)–MseI CAA were chosen. For each individual, 0.5 \( \mu \)L of 6-FAM-labeled and 0.5 \( \mu \)L of VIC-labeled selective PCR products were combined with 0.5 \( \mu \)L of GeneScan 500 LIZ (Applied Biosystems, Foster City, California, USA) and 13.5 \( \mu \)L of formamide and run on a capillary sequencer (ABI 3730, Applied Biosystems). One replicate per population was used to test for contamination and reproducibility.

**Data analysis—Cytogenetic variation**—Diploid numbers were deduced from the meiotic configurations in metaphase I of pollen mother cells. When different configurations were observed in the same individual, 50 pollen mother cells were studied to determine the percentage configuration.

**Sequence variation**—Matrices were compiled with an unambiguous manual alignment. The ITS matrix included 13 sequences of \( C. helodes \) plus eight sequences of sect. Spirostachyae. The \( rps16 \) matrix included nine sequences of \( C. helodes \) plus eight sequences of sect. Spirostachyae. The resulting plastid haplotypes were analyzed under statistical parsimony following the algorithm described by Templeton et al. (1992), as implemented in the program TCS 1.20 (Clement et al., 2000). Gaps were coded as missing data, because they do not affect the ingroup. To gain a better insight into the monophyly of \( C. helodes \) and its sequence ancestry, the three matrices were analyzed with PAUP* version 4.0b10 (Swofford, 2002).

**Maximum parsimony (MP)** analysis using a heuristic search strategy with 100 random sequence addition replicates, branch swapping tree bifurcation reconfiguration (TBR), and the MULTREES option, was conducted on each matrix. Branch supports were obtained by full bootstrapping (100 replicates). The hierarchical likelihood ratio test (hLRT) and the Akaike information criterion (AIC), as implemented in MrModeltest 1.1b (Nylander, 2002), were applied to the ITS, \( rps16 \), and combined matrices. The best models of sequence evolution for \( rps16 \) and ITS were chosen and the three matrices were analyzed using MrBayes 3.0 (Ronquist and Huelsenbeck, 2003). Four Markov chain Monte Carlo simulations
were run simultaneously in each Bayesian analysis for 5000000 generations with an interval of 100 generations. Burn-in was evaluated over generations. The trees generated before likelihood stationarity was achieved were discarded, and the remaining trees were compiled into a majority-rule consensus tree, using posterior probability as the measure of clade support (Alfaro et al., 2003).

**AFLP scoring**—GeneMapper Software v3.7 (Applied Biosystems) was used to visualize and score the DNA fragments. A presence/absence (i.e., 1/0) matrix was constructed based on the contamination and reproducibility tests (Meudt and Clarke, 2007). The potential correlation between fragment size and locus frequency was evaluated with the program AFLP-SURV 1.0 (Vekemans, 2002) to avoid possible size homoplasy (Vekemans et al., 2002). Gene diversity indices \( H_s, H_c, \) and \( H_t; \) Nei, 1972) were calculated using the program POPGENE version 1.31 (Yeh et al., 1999), assuming dominant diploid markers at Hardy–Weinberg equilibrium. An alternative index \( H_{sh}; \) Shannon index), assuming no Hardy–Weinberg equilibrium, is additionally provided. The genetic diversity of the populations was corrected with \( n/(n-1) \) (Nei, 1987). The pairwise genetic distances were calculated using the Nei–Li similarity coefficient (Nei and Li, 1979), implemented with the neighbor-joining algorithm (NJ; Saitou and Nei, 1987) using PAUP* (Swofford, 2002). Branch reliability was assessed by bootstrapping (10000 replicates). The tree obtained was midpoint rooted. Genealogical relationships were indirectly inferred from the analysis of genetic diversity and genetic differentiation patterns. To estimate the genetic population structure, we used a Bayesian clustering approach with the program Structure version 2.0 (Pritchard et al., 2000), assuming the following postulates: (1) Hardy–Weinberg equilibrium and linkage equilibrium within groups; (2) no admixture model, as recommended for dominant data (Pritchard and Wen, 2003); and (3) correlated allele frequencies, recommended when the frequencies in different groups are likely to be similar (Falush et al., 2003). We ran 10 simulations for each value of \( K \) from \( K = 1 \) to \( K = 11 \), using a burn-in period of 10⁶ Markov chain Monte Carlo iterations and a run length of 10⁶ iterations. The modal value of \( \Delta K \) (the second-order rate of change of the log likelihood function with respect to \( K \)) was also calculated (Evanno et al., 2005). The number of clusters \( (K) \) was chosen based on two criteria: (1) the stability of the assignment, and (2) the second-order rate of change of the log likelihood function with respect to \( K \) (\( \Delta K \)). An alternative Bayesian approximation in the program Bayesian Analysis of Population Structure (BAPS version 3.1; Corander et al., 2003), was used to estimate the population structure by clustering individuals (mixture clustering) into panmictic groups. We ran 10 simulations from \( K = 2 \) to \( K = 11 \). Analysis of the molecular variance (AMOVA) was performed with the program Arlequin 3.01 (Excoffier et al., 2005) for (1) the whole data set with two (without groups) and three (with two groups: Moroccan and Portuguese) hierarchical levels, and (2) two subsets of data (independent Moroccan and Portuguese populations) with two hierarchical levels. The correlation between the genetic differentiation among pairs
of populations (pairwise FST values calculated with Arlequin 3.01) and the geographic distances among pairs of populations was estimated with a Mantel test implemented in Arlequin 3.01.

RESULTS

Cytogenetic study—Four different chromosome numbers were identified. The most frequent diploid number, 2n = 72, was found in 25 of 27 individuals from Portugal. Two other chromosome numbers were observed in two individuals from Portugal: 2n = 71 (1P5) and 2n = 75 (2P4). All the individuals counted in the Portuguese populations had the same configuration of 2n = 36K, except for four individuals that displayed irregular configurations (Table 2, Fig. 2B-I). The chromosome number 2n = 74 was exclusive to all (eight) Moroccan individuals, which displayed the same regular meiotic configuration of 2n = 37I (Table 2, Fig. 2A).

Sequencing analyses—The characteristics of the ITS, rps16, and combined sequences are shown in Table 3. Two different ITS copies (ribotypes) were identified across the populations of C. helodes, based on a one-nucleotide substitution (A → G) at position 563 of the aligned matrix. Statistical parsimony analysis of the rps16 intron yielded one single network including 10 plastid haplotypes, with no loop (Fig. 3A). Two haplotypes were detected within C. helodes based on a single-nucleotide substitution (G → A) at position 126 of the rps16 intron. The northern African haplotype of C. helodes was only connected to the Iberian haplotype of C. helodes (Fig. 3A), which, in turn, was connected to the rest of the haplotype network. The characteristics of the MP analysis are also in Table 3. The topologies and branch support of the MP strict consensus trees based on ITS, rps16, and the combined matrix are similar.

A Bayesian inference (BI) analysis was conducted by partitioning the matrix and implementing both GTR+G (hLRT) and HKY (hLRT, AIC) models of sequence evolution for ITS, and HKY (hLRT, AIC) for rps16. The stationarity of the likelihood scores was reached before 6000 generations. Trees generated by different ITS models of evolution were identical in terms of both their resolution and branch support. The topologies and branch support of the BI majority-rule consensus trees based on ITS, rps16, and the combined matrix were similar. The topologies of BI majority-rule consensus trees were identical to those of the MP strict consensus trees.

All phylogenetic reconstructions confirmed the monophyly of C. helodes and the independence of the Moroccan populations (Fig. 3B).

AFLP analysis—Contamination and reproducibility tests gave values of 96% when all the markers scored in the samples and replicates were compared. Contamination was rejected, and unreproducible markers were excluded for the final analyses. As a result, 114 AFLP phenotypes were included in the complete analysis (52 from EcoR I AGG [VIC]–MseI CAA and 62 from EcoRI AGA [6-FAM]–MseI CAC). The potential correlation between fragment size and locus frequency was rejected for EcoR I AGG (VIC)–MseI CAA (R = 0.0244, P = 0.86351), whereas the primer combination EcoRI AGA (6-FAM)–MseI CAC indicated a high level of correlation (R = −0.4366, P = 0.00039). To evaluate the degree of size homoplasy for the EcoRI AGA (6-FAM)–MseI CAC primer combination, the resulting matrix, including all (62) fragments, was compared with
a reduced matrix from which fragments of 50–150 bp were excluded (Coart et al., 2003). For this reduced data set (39 markers), a negative correlation was apparent between fragment size and frequency ($R = -0.5005$, $P = 0.00117$). Potential size homoplasy was rejected because the degree of differentiation for all hierarchical levels considered between both matrices was not statistically significant (results not shown). Therefore, 114 AFLP fragments of 59–474 bp were finally scored for the 83

Fig. 2. Meiotic configurations in Carex helodes (see Table 1 for population coding). Moroccan populations: (A) Individual 8M2 ($2n = 37^{III} = 74$). Portuguese populations: (B) Individual 7P1 ($2n = 36^{II} = 72$); (C) Individual 3P3 ($2n = 34^{IV} + 1^{IV} = 72$); (D) Individual 3P3 ($2n = 34^{II} + 1^{III} + 1^{IV} = 72$); (E) Individual 5P3 ($2n = 34^{II} + 1^{IV} = 72$); (F) Individual 2P4 ($2n = 33^{II} + 5^{I} + 1^{IV} = 75$); (G) Individual 2P4 ($2n = 35^{II} + 1^{V} = 75$); (H) Individual 2P4 ($2n = 36^{II} + 1^{III} = 75$); (I) Individual 1P5 ($2n = 34^{II} + 1^{III} = 71$). All cells were observed in metaphase I. Arrows indicate chromosome pairing irregularities. Scale bar = 10 μm.
was $K = 2$ (Portuguese vs Moroccan individuals with a probability of 100%). The four clusters found for $K = 4$ were (with a probability >70% except for individuals 4P1, 2P5, 5P5, and 11P5, which had probabilities of 50%–70%): (1) all Moroccan individuals (with 100% probability), (2) individuals from six to 10 of the Portuguese population P5, (3) an admixture of individuals from different Portuguese populations (4P1, 6P4–11P4, 2P5, 5P5, 11P5), and (4) all the individuals from Portuguese populations P2 and P3, with the remaining individuals of populations P1, P5, and P4 (Fig. 4). In the BAPS, the best partition was $K = 4$ and the clusters identified were similar to those retrieved by the Structure analysis for $K = 4$, except for individuals 4P1 and 2P5, which fell into cluster 4 (Fig. 4). A Mantel test detected very strong isolation by distance when the whole data set was analyzed ($R = 0.89$, $P = 0.013$), strong isolation with marginal significance when the Portuguese subset was analyzed ($R = 0.59$, $P = 0.093$), and nonsignificant results when the Moroccan subset was tested ($R = 0.71$, $P = 0.158$). The AMOVA for the whole data set with two hierarchical levels (Table 5) assigned 49.96% of the total genetic variance to variation among the eight populations. However, AMOVA with three hierarchical levels attributed 14.12% of the overall variation to differences among the populations within the groups and 46.49% to variation among the groups. When we applied AMOVA to each data set, the percentage of overall variation attributed to variation among populations was similar within Portugal and within Morocco (24.54% and 25.48%, respectively).

**DISCUSSION**

Cytogeography: A powerful tool for inferring phylogeographic patterns in Carex—Although the use of cytogenetics has dropped substantially in the last decade, our results demonstrate the utility of cytogenetics in a geographic framework, as already stated by Favarger (1984). The reduced area of distribution of *C. helodes*, confined to a few populations in the southwestern Iberian Peninsula and northwestern Africa, revealed four different chromosome numbers ($2n = 71, 72, 74, 75$) and 12 different meiotic configurations (Table 2).

The two different euploid numbers of *C. helodes* are geographically distributed: $2n = 72$ in Iberian populations (Luceño, 1992b; Table 2) and the newly reported number $2n = 74$ in all individuals. Eighty-nine fragments were polymorphic (78.07%): 77 in the Portuguese populations (67.54%) and 28 in the Moroccan populations (24.56%). Seventy-five different AFLP phenotypes were detected among the 83 individuals analyzed. Three different AFLP fragments were exclusive and fixed to the 51 Portuguese individuals, whereas no exclusive and fixed AFLP fragments was detected in all the Moroccan samples. The gene diversity ($H_s$) index varied from 0.0263 (in the Moroccan M1) to 0.1778 (in the Portuguese P5) (Table 1). The calculated Shannon index ($H_s$) varied from 0.0378 (in the Moroccan M1) to 0.2423 (in the Portuguese P5) (Table 1). The expected total heterozygosity ($H_s$) was 0.1453 ± 0.0305 for *C. helodes* and the average expected within-population heterozygosity ($H_s$) was 0.0966 ± 0.0305 for *C. helodes* (Table 4) and the newly reported number $2n = 74$ in all populations.

### Table 2. Results from meiotic chromosome observations of the 35 studied individuals of *Carex helodes* including population name (Pop.), individual coding, meiotic configurations in metaphase I (with the proportion of each configuration) and deduced diploid number.

<table>
<thead>
<tr>
<th>Pop. no.</th>
<th>Individuals</th>
<th>Meiotic configurations</th>
<th>Deduced 2n no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>1M1, 2M1, 4M1, 10M1</td>
<td>$2n = 37^{II}$</td>
<td>74</td>
</tr>
<tr>
<td>M2</td>
<td>4M2, 10M2</td>
<td>$2n = 37^{II}$</td>
<td>74</td>
</tr>
<tr>
<td>M3</td>
<td>1M3, 2M3</td>
<td>$2n = 37^{II}$</td>
<td>74</td>
</tr>
<tr>
<td>P1</td>
<td>1P1, 2P1, 3P1, 4P1, 5P1, 6P1, 7P1</td>
<td>$2n = 36^{II}$</td>
<td>72</td>
</tr>
<tr>
<td>P2</td>
<td>1P2, 2P2, 3P2, 4P2, 5P2</td>
<td>$2n = 36^{II}$</td>
<td>72</td>
</tr>
<tr>
<td>P3</td>
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<td>$2n = 36^{II}$</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>3P3</td>
<td>~5% $2n = 34^{III} + 1^{IV}$</td>
<td>72</td>
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<tr>
<td></td>
<td></td>
<td>~5% $2n = 34^{III} + 1^{IV}$</td>
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<td></td>
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<td>~5% $2n = 34^{III} + 1^{IV}$</td>
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</tr>
<tr>
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### Table 3. Summary of nuclear ITS and plastid *rps16* sequences characteristic and major features obtained from phylogenetic reconstructions.

<table>
<thead>
<tr>
<th>Data set</th>
<th>ITS region</th>
<th>ITS-1</th>
<th>5.8S</th>
<th>ITS-2</th>
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<td><em>Carex helodes</em></td>
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<td>222</td>
<td>166</td>
<td>225</td>
<td>753</td>
<td>—</td>
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<tr>
<td>Number of variable vs. informative characters</td>
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<td>0/0</td>
<td>1/1</td>
<td>1/1</td>
<td>2/2</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Sect. Spirostachya</strong>e</td>
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<td>220–221</td>
<td>166</td>
<td>224–227</td>
<td>753–786</td>
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</tr>
<tr>
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<td>166</td>
<td>227</td>
<td>791</td>
<td>1405</td>
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<tr>
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<td>33/24</td>
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<td>23/16</td>
<td>24/17</td>
<td>81/53</td>
</tr>
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<td>0</td>
<td>3</td>
<td>6</td>
<td>10</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>0.87</td>
</tr>
<tr>
<td>RI</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>0.93</td>
</tr>
<tr>
<td>Number of steps</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>24</td>
<td>95</td>
</tr>
<tr>
<td>Number of most parsimonious trees</td>
<td>1</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>1</td>
<td>3</td>
</tr>
</tbody>
</table>
northwestern African individuals, which suggests cytogenetic isolation caused by the Strait of Gibraltar. The directionality of the chromosome number shifts in Carex has been analyzed in a molecular study (Roalson et al., 2001) and suggests that increases and reductions in the chromosome number are equally likely at the genus level. Chromosome number reduction was reported as the primary evolutionary pattern in species of Carex sect. *Ovales* (Crins and Ball, 1988) and in sect. Spirostachyae, which includes *C. helodes* (Lucento and Castroviejo, 1991, 1993). This hypothesis is supported by our molecular data (Fig. 3A), at least for *C. helodes*. The northwestern African cytotype 2n = 74 appears to be derived from the southwestern Iberian 2n = 72. The existence of high chromosome variability in the Portuguese populations but great homogeneity in the Moroccan populations (Table 2) is expected, indicating that the Iberian populations are more ancient than the northwestern African populations. The cytogenetic peculiarities of the genus Carex support this proposition (Greilhuber, 1995) and are consistent with genetic diversity inferences (Kropf et al., 2006; discussed later). We argue that cytogeography is still a powerful tool with which to reconstruct the evolution of species at the population level (Favarger, 1984; Suda et al., 2007).

**Colonization history of African populations**—The monophyly and relatedness of the populations of *C. helodes* were inferred from the MP and BI trees of the nuclear and plastid regions separately (results not shown) and combined (Fig. 3B). In this way, *C. helodes* split into two groups of populations, those from southern Iberia and those from northern Africa. Dating the origin of the disjunction of each particular species is not an easy task, and identifying whether dispersal or vicariance was the process involved is usually difficult (Kropf et al., 2006). The evidence from the four sources studied (plastid and nuclear sequences, AFLPs, and cytogenetics) is congruent with dispersal as the

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**Table 4.** Results from the analyses of AFLP fragments of *Carex helodes* indicating gene diversity indices (*Ht*, *Hc*, and *Hs*) for three different data sets: the whole data set (8 populations) and two subsets (Morocco, 3 populations; Portugal, 5 populations). SD = standard deviation.

<table>
<thead>
<tr>
<th>Data set</th>
<th><em>Ht</em> ± SD</th>
<th><em>Hc</em> ± SD</th>
<th><em>Hs</em> ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. helodes</em></td>
<td>0.1453 ± 0.0305</td>
<td>0.0720 ± 0.0072</td>
<td>0.0966 ± 0.0120</td>
</tr>
<tr>
<td>Morocco</td>
<td>0.0498 ± 0.0138</td>
<td>—</td>
<td>0.0370 ± 0.0076</td>
</tr>
<tr>
<td>Portugal</td>
<td>0.1247 ± 0.0240</td>
<td>—</td>
<td>0.0930 ± 0.0127</td>
</tr>
</tbody>
</table>

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**Fig. 3.** (A) Statistical parsimony network of 10 cpDNA haplotypes, indicating the 10 haplotypes, black circles haplotypes extinct or not found, and each line between haplotypes sequence mutation steps. (B) Strict consensus of the three most parsimonious trees found in the analysis of the combined ITS and rps16 matrix (95 steps, CI = 0.8737 and RI = 0.9264). Numbers above branches represent bootstrap percentages >50% (100 replicates) and below branches indicate posterior probabilities (criterion AIC, HKY+G and HKY for ITS and rps16 intron).
Fig. 4. Neighbor-joining tree constructed using the 83 AFLP phenotypes from the eight populations of *Carex helodes* and the genetic distance coefficient of Nei and Li (1979). Numbers above branches indicate bootstrap values >50% (10,000 replicates). Individual coding as in Table 1. Vertical bars indicate clusters retrieved by the analyses with the programs BAPS ($K = 4$) and Structure ($K = 2$ and $K = 4$).
mechanism underlying the current North African–South Iberian *Carex helodes* disjunction. The low number of ITS (1) and *rps16* (1) substitutions in an angiosperm context (Richardson et al., 2001) led us to rule out a vicariance process, given that the last opening of the Strait of Gibraltar occurred between 4.5 and 5.5 Ma (Duggen et al., 2003). The estimated time of the disjunction divergence is congruent with the plastid and ITS nucleotide substitution rates of angiosperms (Richardson et al., 2001) although dating from a single-nucleotide substitution could be unreliable.

The analysis of sequence variation in *C. helodes* and its relatives allows us to determine the direction of dispersal from southwestern Iberia to northwestern Africa. The African haplotypes are tips in the minimum-length spanning network and are solely connected to the Iberian haplotype, which occupies the central position (Fig. 3A). The coalescent theory predicts a derived condition for tip haplotypes and an ancestral condition for central haplotypes, suggesting that the Moroccan haplotypes are derived. Because chromosome increase is the main evolutionary mechanism in sect. Spirostachyaeeae (Luceño and Cabroviejo, 1991, 1993), these cytogenetic results constitute an additional source of evidence for the dispersal of *C. helodes* to northern Africa because the Moroccan individuals have a higher diploid number (as described before). A founder effect pattern after dispersal is also consistent with the lower overall genetic diversity in the new populations relative to that in the source populations (Kropf et al., 2006). The low values for the *H_1* and *H_e* indices observed in Morocco relative to those observed in Portugal (Table 4), together with the lack of private fixed alleles in the African populations, is also interpreted as strong evidence of a founder effect in the Moroccan populations.

In the evolution of this study suggest that the Strait of Gibraltar constitutes an effective geographic barrier to gene flow for *C. helodes*. Further contact between the Iberian and northwestern African populations may not have occurred after the current disjunction, as inferred from the phylogenetic reconstructions (Fig. 3B) and from the AFLP NJ tree (Fig. 4). This isolation is also supported by the strong genetic differentiation found between Iberian and northwestern African populations (Table 5) and the clustering in the Bayesian analysis (Fig. 4). Furthermore, AMOVA with three hierarchical levels indicated that a large proportion of the variation is attributable to differences among groups rather than among populations. The results of the Mantel test (*R = 0.89, P = 0.013*) are a consequence of the high correlation identified between genetic and geographic distances between the populations of Morocco and Portugal. This geographic isolation is also supported by the existence of different euploid numbers in Morocco (*2n = 74*) and the Iberian Peninsula (*2n = 72*). The cytogenetic homogeneity of the Morocco populations also supports the pattern of spatial isolation, with no subsequent connections. The isolation between European and African populations is not surprising given that similar results have been already been obtained by others for similar Iberian Peninsula–northern African disjunctions. For instance, genetic isolation caused by the Strait of Gibraltar has been suggested in *Quercus suber* (Toumi and Lumaret, 1998), *Saxifraga globulifera* (Vargas et al., 1999), *Q. ilex* (Lumaret et al., 2002), and *Androcymbium gramineum* (Caujapé-Castells and Jansen, 2003), regardless their pollen dispersal syndromes. In contrast, there are well-documented disjunct species for which current gene flow across the Strait of Gibraltar has been demonstrated (*Hypochaeris salzmanniana*, Ortiz et al., 2007), including anemophilous plants (*Pinus pinaster*, Burban and Petit, 2003; *Olea europaea* subsp. *europaea*, Rubio de Casas et al., 2006). Moreover, no ITS sequence divergence has been detected between populations on both sides of the Strait of Gibraltar in two relatives of *C. helodes* (*C. distans* and *C. punctata*; Escudero et al., 2008).

Gene flow at a regional scale—In contrast to the strong geographic isolation of southwestern Africa and the southwestern Iberian Peninsula, no segregation was detected among Iberian populations and African populations. No populations were clustered in well-supported groups in the AFLP NJ analysis (Fig. 4), and in none of the populations were all the individuals assigned to the same cluster in the BI analysis (Fig. 4, see Results, *AFLP analysis*). However, gene flow among the Iberian populations and among the African populations may be frequent but not extensive, as relatively moderate differentiation within the groups was identified by AMOVA (Table 5). These data are not surprising given the patchy distribution and the wind-pollination syndrome of this species. In fact, the Mantel test revealed significant isolation by distance in the Portuguese populations. This pattern of limited population structure within areas, but predominant isolation between groups of populations on both sides of the Strait of Gibraltar has already been documented in some groups of animals (Castella et al., 2000; Broderick et al., 2003).

Conclusions—Endemic *C. helodes* colonized northern Africa by relatively recent, long-distance dispersal from the Iberian Peninsula after the last opening of the Strait of Gibraltar (c. 5 Ma). No further connection appears to have occurred, driven by either seed or pollen dispersal. The colonization history of *C. helodes* is somewhat paradoxical in that migrants succeeded in colonizing northern Africa at least once, despite an apparently limited seed dispersal mechanism.
LITERATURE CITED


GUZMAN, B., and P. VARGAS. In press. Long distance colonisation by the Mediterranean Cistus ladanifer (Cistaceae) despite the absence of special dispersal mechanisms. Journal of Biogeography.


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