# Isolation of 91 Polymorphic Microsatellite Loci in the Western Mediterranean Endemic Carex helodes (Cyperaceae)

Authors: Arroyo, Juan M., Escudero, Marcial, and Jordano, Pedro

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PRIMER NOTE

# ISOLATION OF 91 POLYMORPHIC MICROSATELLITE LOCI IN THE WESTERN MEDITERRANEAN ENDEMIC *CAREX HELODES* (CYPERACEAE)[1]

JUAN M. ARROYO[2], MARCIAL ESCUDERO[2,3,4], AND PEDRO JORDANO[2]

[2]Department of Integrative Ecology, Estación Biológica de Doñana, Consejo Superior de Investigaciones Científicas (CSIC), Calle Américo Vespucio s/n, E-41092 Seville, Spain; and [3]Department of Plant Biology and Ecology, Universidad de Sevilla, Apartado 1095, E41080 Seville, Spain

- *Premise of the study:* Microsatellite primers were developed for *Carex helodes* (Cyperaceae), a western Mediterranean endemic that is locally distributed in southern Portugal and southwestern Spain and rare in northern Morocco.
- *Methods and Results:* One hundred nine nuclear microsatellite markers were developed using a shotgun pyrosequencing method, resulting in 91 polymorphic and 18 monomorphic loci when tested using 19 individuals sampled from five populations from Portugal, Spain, and Morocco. Loci averaged 3.23 alleles per locus (SD = 1.15). In a single population (Cortelha population, Portugal), the 34 most polymorphic loci showed a mean observed heterozygosity of 0.357 (SD = 0.292) and mean expected heterozygosity of 0.384 (SD = 0.255).
- *Conclusions:* Next-generation sequencing allowed us to develop a high number of genetic markers with levels of polymorphism adequate to study gene flow among populations. However, when genotyping the individuals within a population, we found low levels of variation.

**Key words:** *Carex helodes*; Cyperaceae; endemism; sedge; shotgun sequencing; simple sequence repeat (SSR) marker.

*Carex helodes* Link (sect. *Spirostachyae* (Drejer) L. H. Bailey, Cyperaceae) is a diploid, wind-pollinated, perennial herb with a minimum generation time of two years. The species is endemic to the western Mediterranean, being locally distributed in southern Portugal and southwestern Spain, and rare in northern Morocco (Escudero et al., 2008a). 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 (see Luceño et al., 2009). Recent cytotaxonomic and nuclear- and plastid-based phylogenetic studies have revealed the monophyly of *C. helodes* populations and its taxonomic independence within sect. *Spirostachyae* (Escudero et al., 2008a, 2008b; Escudero and Luceño, 2009). *Carex helodes* is an endangered species in Spain because the extent of its severely fragmented occurrence is less than 100 km$^2$, with continued loss of area and habitat quality (Moreno, 2008; Bañares et al., 2010).

Our aim is to develop molecular markers for further studies of gene flow among and within populations. Nuclear microsatellites have been proven to be highly variable and very suitable to the study of recent gene flow between populations (Ouborg et al., 1999). To accomplish our task, we isolated and characterized 109 nuclear microsatellites.

## METHODS AND RESULTS

We extracted genomic DNA using a DNeasy Plant Mini Kit (QIAGEN, Valencia, California, USA). We used ~5 µg from one *C. helodes* individual collected in Madroñalejo (Aznalcóllar, Seville, Spain; see Appendix 1 for GPS coordinates and voucher specimens) to construct a shotgun genomic library that was sequenced on 1/4th of a plate using 454 GS FLX Titanium chemistry (Roche Applied Science, Indianapolis, Indiana, USA) at the University of Arizona Genetic Core (Tucson, Arizona, USA). We generated 108.3 Mb of quality-filtered data, distributed over 221,198 unique reads with an average length of 490 bp after quality filtering (quality score [Q] ≥ 20 using a 10-bp sliding window). We searched for all possible microsatellite loci containing at least six perfect repeats for hexa-, penta-, tetra-, and trinucleotides or 12 perfect repeats for dinucleotides and designed primers using the software QDD version 3 (Meglécz et al., 2014). We used the unique reads as input to detect microsatellite sequences. The reads were used to build contigs using QDD version 3 (default options were used: sequence set limit of 80 bp, 95% minimum identity between two sequences to make a consensus, and 66% as the proportion of sequences that must have the same base on the aligned site to accept it as a consensus). For primer design, the default options were also used but the minimum size of the PCR product was set to 100 bp and the maximum to 450 bp.

We found a total of 3985 microsatellite loci, including 51 hexa-, 58 penta-, 78 tetra-, 406 tri-, and 3392 dinucleotide loci. We selected 27 hexa-, 26 penta-, 23 tetra-, 206 tri-, and 152 dinucleotide loci that met our criteria (at least 12 repeats for dinucleotides and six for the rest) and tested a total of 132 loci. Specifically, we tested the eight hexa-, eight penta-, eight tetra-, 18 tri-, and 90 dinucleotide loci with the highest numbers of repeats (Table 1).

For primer testing, DNA was isolated from silica gel–dried leaves using a modified cetyltrimethylammonium bromide (CTAB) extraction method

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[4]Author for correspondence: amesclir@gmail.com

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TABLE 1. Characteristics of 109 microsatellite markers isolated from populations of *Carex helodes*.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | $A$[a] | $T_a$ (°C) | Fluorescent dye | Mix no. for capillary electrophoresis[b] |
|---|---|---|---|---|---|---|---|
| Cahe914 | F: ATCAAGAATTCAAGATTCAGGG<br>R: AAGTCCACGTTGAGGACGAA | $(TATTAC)_7$ | 112–130 | 3 | 53 | FAM | 1 |
| CahePM5[c] | F: TCGGAGCTTTATCAGATTGATGT<br>R: TCCAAACATTTGTTCTACGACTT | $(TATAGA)_8$ | 282–294 | 4 (5)[d] | 53 | FAM | 1 |
| Cahe587[c] | F: TGTCTAGTCCATGCCACGTT<br>R: GCAACCTAGTTATGCTGAAGCTG | $(ATACAT)_{14}$ | 320–420 | 6 | 55 | FAM | 1 |
| CaheYES[c] | F: AAGTGCCAAAGTTTATTATGAGTTGT<br>R: GTTTGCCATGTGGGAAGTTT | $(AGGCTG)_6$ | 110–134 | 5 | 54 | VIC | 1 |
| Cahe9PD | F: GGTTAATACCGTTATTCAGATGAAAC<br>R: CATTCCGGTTATTTAATGCGA | $(TGATGG)_7$ | 286–292 | 2 | 54 | VIC | 1 |
| Cahe837 | F: CACATACACTATGATCGAAACAAAT<br>R: CACTATACAATAGCACTGCACCA | $(TTTTAC)_7$ | 447–464 | 3 | 53 | VIC | 1 |
| Cahe130[c] | F: TCCAGTTCCTCCCTCTCCTC<br>R: CATTCACCATCAGTACCGGA | $(CCATCC)_7$ | 268–292 | 4 | 55 | NED | 1 |
| CaheR56 | F: GAGTTTGAGGAGCGAGGAGA<br>R: GCTATTGTTACAGGGTGCGA | $(CTACT)_8$ | 138–144 | 2 | 54 | NED | 1 |
| Cahe8IO | F: CCGTAAGGCCAGCTGTAAAT<br>R: TGTCACTGGACAGTGGGAAG | $(CATTT)_7$ | 130–152 | 2 | 55 | PET | 1 |
| CaheFOY | F: TTTCTTCTCTATGTTTCTTTCCGTT<br>R: CAGAGGGAGACTACATTATATGGAA | $(TTTTC)_6$ | 158–187 | 6[d] | 53 | NED | 2 |
| CaheC2B[c] | F: CTCTGACTGTACATCTGACCGA<br>R: GATACCTATCGAATATTCCTTCTTCC | $(TGCG)_7$ | 169–181 | 4 | 53 | FAM | 2 |
| CaheWE1[c] | F: AAGTGAACCACCTTTGGCAC<br>R: GATTTGGAGGAAATGTACGCA | $(TTAT)_8$ | 229–269 | 4 (7) | 55 | NED | 2 |
| CaheBI5[c] | F: GGTGTTTATCATAAGAATGAAATTGA<br>R: TTATCTGTGTATCTCATGATCCATTG | $(AGAT)_7$ | 215–231 | 3 | 53 | PET | 2 |
| CaheXAF[c] | F: TCATCAATTGGAGCAATTACAGTT<br>R: ATAAAGAGTGCTGCCGATGG | $(TCA)_{18}$ | 321–449 | 5 (6) | 55 | FAM | 3 |
| Cahe147[c] | F: TCGGCAAATACGTTGTCCAT<br>R: AATGATGACAATTTAGATAAGACCACT | $(TTA)_{16}$ | 147–154 | 4 (5)[d] | 53 | VIC | 3 |
| CaheJEU | F: TAATTGCAATCAAGCGACCA<br>R: TTGTGGATTGCCGAAGGTAT | $(TAA)_{10}$ | 235–241 | 2 | 56 | VIC | 3 |
| CaheFG7[c] | F: ATCCGCTTCTTCATTCGCT<br>R: TGATAGTCACGACTGTTCATGC | $(TAA)_{11}$ | 469–496 | 4 (6)[d] | 54 | VIC | 3 |
| CaheMIW[c] | F: GGATGATTCATAAGGCCTCTCA<br>R: TTCAGGTTTAATCACAAGACAGGA | $(ATC)_{12}(GTC)_4$ | 262–280 | 3 | 56 | NED | 3 |
| CaheQWB[c] | F: GTGTAAATGGCTACTGAAACATTG<br>R: CCACTTGCATAACAGTGAATTG | $(AAT)_{10}AG(TAA)_6$ | 122–147 | 3 | 53 | PET | 4 |
| Cahe121[c] | F: GATATAATCGTGGAAGTCATTTCA<br>R: TTGCAATTAGCAAGAGATCAATTAG | $(ATT)_{16}$ | 374–410 | 4 (6) | 53 | NED | 3 |
| CaheGVB[c] | F: CTACGAGCACTTTGGGCATT<br>R: TTGATTTGAATTTGACCGTTTG | $(TAT)_{14}$ | 112–135 | 4 | 55 | PET | 3 |
| Cahe4Q8 | F: AACACAAAGAAGAGGGCGAG<br>R: GGAGAATGACGACGCTGAG | $(GAG)_{10}$ | 219–234 | 3 | 55 | FAM | 4 |
| Cahe965 | F: GGAGGATACATAACAGAGATTGGG<br>R: TTGTAAGTTTGCAGAATCAATATGGT | $(AAT)_{10}$ | 275–278 | 2 | 56 | VIC | 4 |
| Cahe468[c] | F: GGCAAGAACAAAGAAAGGTCG<br>R: ATGGATCTAGTCACCGCTCC | $(AGA)_9$ | 461–475 | 3 (5) | 55 | VIC | 4 |
| CaheT2N[c] | F: TATTCACCAATAATTCCACAAACAA<br>R: TCTTCTACAGCCTCTCAAAGACTTG | $(CA)_{17}(TA)_6$ | 154–176 | 4 | 55 | FAM | 3 |
| CaheODD[c] | F: TCAATATTTCTACTTGATATGATGAGC<br>R: AACATTCCGCAAATAACAAATACG | $(TA)_{16}$ | 129–180 | 7 (10) | 53 | NED | 4 |
| Cahe7LF[c] | F: TTGAGGAGGTACAACATTATCCA<br>R: TTTGCCCATTAATCACCTATTT | $(AT)_{16}(AAT)_3$ | 225–258 | 7 (9)[d] | 53 | NED | 4 |
| Cahe408 | F: GATGCGCGAGATACACATTT<br>R: AAAGACCTGAGACTAGAGCAGGAA | $(AT)_{18}$ | 468–473 | 3 | 54 | NED | 4 |
| CaheTDK | F: TGGAGATTCTGTTTGTAACCACC<br>R: TTTAGAGTCATGGATAATTGGTCCT | $(TA)_7TG(TA)_3C(GT)_{16}$ | 238–242 | 3 | 55 | PET | 4 |
| Cahe829[c] | F: TTTGTTGGGCTGAGCCTG<br>R: ATCCATTGAAGAAATGGAAGGA | $(GT)_3GG(GT)_{10}(AT)_{12}$ | 342–402 | 4 (6) | 55 | FAM | 5 |
| Cahe971 | F: AATTAGCATTAATTTCCACAGGC<br>R: GGAGTTCGACAGCTCATTCA | $(CT)_9$ | 133–137 | 2 | 54 | PET | 5 |
| Cahe215 | F: CTCACAGAAGAGAAAGTCAGCG<br>R: TGGAATTTCTAAGTCTTGACTAGCG | $(GA)_8$ | 277–282 | 2 | 55 | PET | 5 |
| Cahe2121 | F: AGGTCAGAAGTCTTGCCGAG<br>R: CCGGGTAGATATACTCTCCTCTCA | $(GA)_9$ | 419–427 | 3 | 55 | PET | 5 |

TABLE 1. Continued.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | $A$[a] | $T_a$ (°C) | Fluorescent dye | Mix no. for capillary electrophoresis[b] |
|---|---|---|---|---|---|---|---|
| Cahe677[c] | F: TTAGTTGAACCGAACAGCCC | $(CT)_{15}$ | 131–139 | 3 | 54 | FAM | 6 |
| | R: CAGCCTAGGTTCAGCACTCTT | | | | | | |
| Cahe84P[c] | F: AATCAAGGTTGCTTTGAGCC | $(AT)_{13}$ | 245–247 | 2 | 55 | FAM | 6 |
| | R: CAATCTAAAGACATGATACGATCGAG | | | | | | |
| CahePUI[c] | F: TGTGGCTGATACTGACATTCTG | $(AT)_{15}$ | 318–337 | 4 | 54 | FAM | 6 |
| | R: TTGCTCGAGATCCTTTGCAG | | | | | | |
| CaheL4G | F: ATGTTGCGCTGGTACGGT | $(AG)_{13}$ | 467–475 | 3 | 56 | FAM | 6 |
| | R: TTTACGGGAGAAACAGTCGG | | | | | | |
| Cahe222[c] | F: ATCTCGATTGGAAAGAGGGC | $(TA)_{14}$ | 324–336 | 5 (6) | 55 | VIC | 6 |
| | R: TTGCTCTTCTCCTCACGGTT | | | | | | |
| CaheEZE[c] | F: TTTCCCTTTCTTGGGCTTTC | $(TG)_{14}$ | 153–164 | 4 | 54 | NED | 6 |
| | R: ACCGATAAATTAGTCAATTATGCTATG | | | | | | |
| Cahe1SD | F: TTACGTGTTTAGACCATCCCTCA | $(TA)_{15}$ | 218–226 | 3 | 55 | NED | 6 |
| | R: TCTAAACAGATTCATTTGACATTTCC | | | | | | |
| CaheVSD | F: CCCAGTTTCCCTCCCTCTTT | $(TC)_{13}$ | 306–308 | 2 | 56 | NED | 6 |
| | R: AAGCCCGGGAATACTTTACG | | | | | | |
| CaheSGU[c] | F: AAATCCTATCTTATACACCTACGGG | $(AT)_{15}$ | 114–128 | 4 | 54 | PET | 6 |
| | R: TTCAGTCCATATGCTTCCGA | | | | | | |
| Cahe423 | F: TCAACTTTGGCCTCCTATTG | $(AG)_{14}$ | 167–171 | 3 | 53 | NED | 8 |
| | R: CCATTTCGTACCCTTGACCT | | | | | | |
| CaheORP[c] | F: AGCTTTCTATGCAACCCATAACA | $(TA)_{13}$ | 177–185 | 5 | 55 | PET | 6 |
| | R: GCTTGGATTAACCAAATAATATTGAGA | | | | | | |
| CaheWMU[c] | F: CAGATGCATGTCTTCTGACACA | $(TA)_{15}$ | 398–402 | 3 | 55 | FAM | 7 |
| | R: GTCTGCCTCCTTAGGCACATA | | | | | | |
| Cahe192[c] | F: ACTTGGTGCGGAGCCTATAA | $(AC)_{14}$ | 295–305 | 3 | 55 | VIC | 7 |
| | R: GCTCGATTGCTTCCTGAGTC | | | | | | |
| CaheB4G | F: TTACCTTGAATGCTCGAGGAAG | $(AT)_{13}$ | 175–187 | 3 | 53 | NED | 7 |
| | R: GATATCATCTTTCATATTGTATTGGC | | | | | | |
| Cahe8RA[c] | F: TGAATGGAAGGCACTGCTTA | $(CT)_{13}$ | 154–158 | 3 | 54 | PET | 7 |
| | R: TCCAGTGGATTTCTAACATAGCTC | | | | | | |
| CaheSF0 | F: TTTGAAGTTCACTTTCATTTAGAAAC | $(AT)_7AC(AT)_6(AG)_5$ | 175–181 | 3 | 53 | VIC | 8 |
| | R: ATGTAACAAAGAAGACATATACATTGC | | | | | | |
| Cahe242 | F: GATCCAACGGTGGCTTAACA | $(CT)_{10}(AT)_7$ | 148–150 | 2 | 55 | PET | 8 |
| | R: AAGATACCAGGAAGAGAAGAGGG | | | | | | |
| CaheL7Z[c] | F: CAATGAATTGTTCATCAAGACTGG | $(CT)_9(CA)_{11}$ | 373–380 | 4[d] | 56 | FAM | 8 |
| | R: TGACTAGTGACTACAGCTGCCAA | | | | | | |
| Cahe488 | F: GGATCTGACCGGAGCTATTG | $(TC)_{11}(CA)_7$ | 238–246 | 2 | 54 | PET | 8 |
| | R: GCAAGGCAATGTGTAACTTGA | | | | | | |
| CaheDHT | F: TCCAAATAGCGAACCAAACC | $(AT)_8(AG)_9$ | 183–187 | 3[d] | 54 | VIC | 9 |
| | R: ATGGTGCATTTGAACCCTC | | | | | | |
| CaheUAX[c] | F: GATCGGAGGGTTGACATTC | $(AT)_6AG(TA)_8G(TA)_3$ | 182–229 | 3 | 53 | PET | 9 |
| | R: ATCAAATCCGACAGCTAGCTAAA | | | | | | |
| Cahe932[c] | F: AAACCACCGTCAAACTGTGATA | $(AG)_{12}$ | 158–160 | 2 | 55 | FAM | 10 |
| | R: AGAAGAGAAGGAAGGCAGGC | | | | | | |
| CaheQ9D[c] | F: AGCAATATTCATGTCGACTGTCA | $(AC)_{11}$ | 342–348 | 3 | 54 | FAM | 10 |
| | R: AACATTCAAATTACCTTAGAGCCA | | | | | | |
| Cahe553 | F: AGTGTTTGGTTGACACCCGT | $(AT)_{13}$ | 139–143 | 2 | 56 | NED | 10 |
| | R: AATGTTGTGGGAACCTTGGA | | | | | | |
| CaheFTM | F: ACCTCTTCCTGCCATCCCTA | $(TG)_{12}$ | 355–357 | 2 | 55 | NED | 10 |
| | R: CAGACTCAACCACAGCTTCG | | | | | | |
| Cahe2519 | F: TTGAGTCGTCATTTGTTGGC | $(AT)_{13}$ | 386–398 | 4 | 55 | VIC | 10 |
| | R: GTGAACGTGGCAAATCTTGA | | | | | | |
| Cahe002 | F: ACCCATAGCACCCTAGCCTT | $(TA)_{13}$ | 200–214 | 3 | 55 | FAM | 11 |
| | R: CCATGCGATAACCTTTCCTC | | | | | | |
| Cahe3IX | F: AATTGGAGCAAACATTTGGG | $(AG)_{12}$ | 281–287 | 3 | 55 | FAM | 11 |
| | R: CAGACGGTCTTGATCTAAACTCAG | | | | | | |
| CaheLJZ | F: AGGTAGACAGAATCAAATTCAGGG | $(AT)_{12}$ | 174–186 | 2 | 55 | VIC | 11 |
| | R: TTGACATATTAATGACAAGGTACCAAA | | | | | | |
| Cahe711 | F: GTTACAGTGGGCCATCGTG | $(CT)_{12}$ | 282–304 | 3 | 55 | NED | 11 |
| | R: GCGGATGCTTTAGTGTGCAT | | | | | | |
| CaheQWV[c] | F: GCGCTTCTAGATATACGTGCAA | $(TA)_{12}$ | 175–181 | 3 | 54 | PET | 11 |
| | R: AATTGTCTATACATTAACATGTCCTCG | | | | | | |
| Cahe3XU | F: CGACTTAGACGTTGTATGGGA | $(TA)_{12}$ | 279–285 | 3 | 53 | PET | 11 |
| | R: CTTGACTGCCCTTGTTTGTC | | | | | | |
| CaheD2S | F: CGCGAGAATAGATAGGCGAC | $(AAGGG)_6$ | 251–261 | 3 | 55 | PET | 2 |
| | R: ACGTCCAAACAAACCGTTCA | | | | | | |
| CaheUNE | F: TTTGTGGAAAGTTAAATCAGTTAATCA | $(ATT)_{10}AC(TAT)_{13}$ | 477–493 | 3 | 54 | FAM | 3 |
| | R: AAACCTTCACCTGTGGTTATAGAAT | | | | | | |

TABLE 1. Continued.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | $A$[a] | $T_a$ (°C) | Fluorescent dye | Mix no. for capillary electrophoresis[b] |
|---|---|---|---|---|---|---|---|
| CaheHGI | F: CTCTTGGCTTCCTTGTTTCTC<br>R: TTTCCTGCCATTCCACAAG | $(AG)_{16}A(TGTGT)_3$ | 159–185 | 3 | 54 | VIC | 4 |
| CaheTC7 | F: AGGTTAGGATAGTTGCATAATTTACGA<br>R: GAGCCTTATGCTCACCAACA | $(ATT)_{13}$ | 142–145 | 2 | 54 | FAM | 4 |
| Cahe522 | F: ACGAGATGATCTTCTGCCAT<br>R: TGAAAGTCGAATATTGAGCCG | $(AT)_6$ | 173–191 | 2 | 53 | FAM | 5 |
| Cahe86O | F: TTGAGTTGCCAGAACCATCA<br>R: TTCCTTGCCCTCTGTCTGTT | $(GA)_{14}$ | 447–453 | 3 | 55 | NED | 6 |
| Cahe68X | F: CATTTAGAATTGTTATGCCAGCG<br>R: GGAGTTGGAGATTGAGTCGG | $(AC)_{12}$ | 209–225 | 2 | 55 | NED | 10 |
| Cahe322 | F: GGTGTGCAAGCGATCATTTA<br>R: TTTCGCCTTCTTCTCCATTT | $(TC)_{12}$ | 386–408 | 2 | 54 | FAM | 11 |
| Cahe824 | F: CAGAGCCGATCTGTCCAATA<br>R: GACTCAGGTTAGCTGCCGAC | $(TA)_{12}$ | 128–141 | 3 | 54 | PET | 10 |
| Cahe818 | F: CGAGAAACATTGTTAGACTCCA<br>R: TTGCTTATTGGCAAGTTCTAGAGATA | $(AG)_{13}$ | 286–288 | 2 | 53 | VIC | 11 |
| CaheXN6 | F: TTTGGATTAGGGTTTGCAGC<br>R: TGGATTTACTATTATGAAATGAAGCA | $(TTCTGA)_7$ | 433–439 | 2 | 54 | FAM | 1 |
| CaheJSN | F: GGCAACTTGGCTGTTCCTAA<br>R: CTGAGGTCAGGTGGCAATTA | $(TTTCT)_7$ | 136–147 | 2 | 54 | NED | 3 |
| CahePYI | F: TTCGTTATTTCTGACTGACACG<br>R: CAAATGGTTGCAGCGGAA | $(TTTTC)_7$ | 440–444 | 2 | 53 | FAM | 2 |
| CaheASM | F: GGAAACTTCGCGGAGAAATA<br>R: AGATTCCATGTGGTTCTACTGTTC | $(TGTT)_7$ | 122–130 | 2 | 54 | VIC | 2 |
| Cahe5PU | F: CGCTTATCATGAAGTTTGTTCAC<br>R: GCCCAACCCTGACAGATAAA | $(CTAT)_7$ | 179–196 | 2 | 54 | VIC | 2 |
| CaheVDJ | F: CTGGCAGGAGTTCATTTGCT<br>R: GCTTACTAGAGGTTGTAGGCTTAACAG | $(ATA)_{16}$ | 256–262 | 2 | 55 | PET | 3 |
| CaheR17 | F: GTCTGAAAGCGACGATTGAA<br>R: TGGTTTCATTTCGAGAAGGG | $(ACC)_7$ | 428–434 | 2 | 54 | FAM | 5 |
| Cahe001 | F: CTTCCTGCAATGTAGGCTCC<br>R: AATCGGGCTGACATCCTAGA | $(TG)_4TC(AG)_8$ | 288–294 | 3 | 55 | NED | 5 |
| Cahe670 | F: TCCCGTATACCGGCTTCAG<br>R: CACATTGTGTCAAACACATGAAA | $(TA)_{14}$ | 189–191 | 2 | 55 | FAM | 7 |
| CaheGHR | F: TTACAACTTCCTTCAAATTTGTGAT<br>R: GAATAGGCACCGGACACG | $(CT)_{13}$ | 292–294 | 2 | 54 | FAM | 7 |
| Cahe7K7 | F: AAACTGAAGATATTGCGTGAAGT<br>R: AAAGTTTGAATGGGTACACTGAA | $(TC)_9(TA)_9$ | 155–157 | 2 | 53 | FAM | 8 |
| Cahe303 | F: GGCATTGTTTGGAGCGATATT<br>R: GAAATGGTAGTGCTTCTTCTGAAC | $(TC)_{11}N(TG)_6$ | 281–283 | 2 | 54 | NED | 8 |
| Cahe6A8 | F: TGGCCTTTGCTTCTATCTCA<br>R: GCCATGCCTCTCATGCTACT | $(GA)_5G(GA)_{12}$ | 168–174 | 2 | 54 | NED | 9 |
| CaheL2M | F: AAATGACCTGATCTGACCGA<br>R: ACGTGAATAGTATCACATCGAACA | $(GT)_7(GA)_9$ | 284–296 | 2 | 54 | FAM | 9 |
| Cahe647 | F: GGAGCATTCACCAATCCTCTA<br>R: CCCGTCGTAATCATATCTCTAATGA | $(TC)_{12}$ | 223–227 | 2 | 55 | PET | 10 |
| Cahe332 | F: AAGACAGCCAAAGTGAGCTTG<br>R: CGCCATGTTAACTTGATGAGG | $(CT)_{13}$ | 161–171 | 2 | 55 | NED | 11 |
| CaheBUE | F: TTCAATTATGATCCAGCTTCACA<br>R: TTCTTTCTTCCCTCCCTTCC | $(TATAT)_6$ | 443 | 1 | 55 | VIC | 2 |
| CaheOPJ | F: TCCACCTTACACTTTATTCACCC<br>R: GATGCATCCTATCCCTCCGT | $(CAAA)_7$ | 278 | 1 | 55 | FAM | 2 |
| CaheNT5 | F: TGAGCTGGCACACTCTATGG<br>R: AGAACTATAGCTTTGAAACAGCCC | $(GTAT)_6$ | 311 | 1 | 55 | VIC | 2 |
| CaheQQ1 | F: TAATGGTAAATTTGGATTTGCG<br>R: GCAATTGGTGTAACAGCACC | $(TAAT)_6$ | 145 | 1 | 54 | PET | 2 |
| Cahe06U | F: AGAACAAGACAAGACTATCTTTATGCC<br>R: TTCTCCCTTCGCATCTCC | $(GGA)_{10}$ | 423 | 1 | 54 | VIC | 3 |
| Cahe476 | F: ACCAGGAACCAGCTGGAATA<br>R: ATCACCAAGCTCATCAGGGT | $(TAG)_{10}$ | 477 | 1 | 55 | FAM | 4 |
| Cahe993 | F: ATGGGTGCTATATTTCACCTTG<br>R: ACACTAGAACATGACTCGTCGC | $(GAT)_6$ | 423 | 1 | 54 | NED | 5 |
| Cahe280 | F: CGGATTCGATTTCATTCACC<br>R: AAAGCAAGACAAATCAGCCAC | $(TC)_{12}$ | 184 | 1 | 55 | VIC | 5 |
| Cahe930 | F: AAGGCACATCCAAGTTTACCA<br>R: GGAAGGAGCATGCATCTGTA | $(AG)_5$ | 423 | 1 | 54 | VIC | 5 |

TABLE 1. Continued.

| Locus | Primer sequences (5′–3′) | Repeat motif | Allele size range (bp) | $A$[a] | $T_a$ (°C) | Fluorescent dye | Mix no. for capillary electrophoresis[b] |
|---|---|---|---|---|---|---|---|
| Cahe835 | F: TTTCGATCCATTACCCTCCC<br>R: TCACATCAACAACATCGCCT | $(CT)_7$ | 189 | 1 | 56 | NED | 5 |
| Cahe36X | F: AAGCTTGTCTTGAGTGACGGA<br>R: AAGAGTTGTTCGTTTCTGTTCCTT | $(AG)_{14}$ | 216 | 1 | 55 | VIC | 6 |
| CaheXTE | F: GGGACACCTTCCATTGATAAAG<br>R: TCACGATTTCATGCAACACA | $(AG)_{13}$ | 471 | 1 | 55 | VIC | 6 |
| CaheHL4 | F: CGGAGGTGAAATTAATTAAGCG<br>R: GTTTAAATTTGGGCTTGGGC | $(AG)_{13}$ | 241 | 1 | 55 | PET | 7 |
| Cahe2SB | F: ACTTAGCAGCTGCCACCAA<br>R: TGCATTGCAGCTTACTAGAACTT | $(AG)_5G(GA)_8$ | 229 | 1 | 54 | VIC | 8 |
| Cahe519 | F: ATCGATTATCCTTTAATCAACTAACAA<br>R: GGGTATCACTTGAAAGAAACAAA | $(GT)_4(AT)_4AC(AT)_8$ | 363 | 1 | 53 | VIC | 8 |
| Cahe0PF | F: CTTGCAGATCCAAAGGAGGT<br>R: ACTGCAATAACAGCCATAGGAAA | $(CT)_{12}$ | 147 | 1 | 55 | VIC | 10 |
| Cahe770 | F: TTGATCCACTTTCTATGACAAGGA<br>R: TCCCATAAGAATATCCTTCGATTC | $(TG)_{12}$ | 229 | 1 | 55 | VIC | 10 |
| CaheX4D | F: GGAAGTTTCAGATCAGTGACCA<br>R: AGAGGCTTGAGGAAGAAGCTC | $(CT)_{12}$ | 185 | 1 | 55 | NED | 1 |

*Note*: $A$ = number of alleles; $T_a$ = annealing temperature (given for nontailed primers).

[a] Total number of alleles, including seven additional individuals from the Cortelha population of Portugal, is shown in parentheses.

[b] Mix number indicates loci that were mixed in the same capillary electrophoresis run.

[c] One of 34 most polymorphic loci.

[d] Loci showing one to three alleles with a size difference of 1–2 bp relative to contiguous alleles.

(Milligan, 1998) that included tissue grinding in a Mixer Mill MM301 (Retsch GmbH, Haan, Germany) and resuspension in TLE buffer (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA). We sampled a total of 64 individuals from five different populations (Appendix 1). Initially, 19 individuals from the five populations were sampled and genotyped for 109 loci. Nine individuals from the Madroñalejo population, four individuals from the Barraçao-Caldas de Monchique population, one individual from the Cortelha population, four individuals from population 1 (Pop. 1) at Ksar el Kebir, and one individual from population 2 (Pop. 2) at Ksar el Kebir (Appendix 1). Finally, 38 additional individuals from the Madroñalejo population (Spain) and seven additional individuals from the Cortelha population (Portugal, Table 2) were genotyped for the 34 most polymorphic loci. The 34 most polymorphic loci were estimated based on the initial screening using 19 individuals from five populations.

PCR amplifications were performed in a 20-μL final volume containing 1× buffer (67 mM Tris-HCl [pH 8.8], 16 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.01% stabilizer), 2.5 mM $MgCl_2$, 0.01% bovine serum albumin (BSA; Roche Diagnostics, Mannheim, Germany), 0.25 mM dNTP, 0.40 μM dye-labeled M13 primer, 0.40 μM PIG-tailed reverse primer, 0.04 μM M13-tailed forward primer (see M13 and PIG-tail sequences in Table 1), 0.5 units *Taq* DNA polymerase (Bioline, London, United Kingdom), and approximately 50–70 ng genomic DNA. Reactions were undertaken in a touchdown PCR protocol in a Bio-Rad DNA Engine Peltier Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA), with an initial 2 min of denaturation at 94°C; 17 cycles at 92°C for 30 s, annealing at 60–44°C for 30 s (1°C decrease in each cycle), and extension at 72°C for 30 s; 25 cycles at 92°C for 30 s, 44°C for 30 s, and 72°C for 30 s; and a final extension of 5 min at 72°C. PCR products were labeled using FAM, VIC, NED, or PET dyes (Applied Biosystems, Foster City, California, USA) on an additional 19-bp M13 primer (5′-CACGACGTTGTAAAACGAC-3′) according to the methods of Boutin-Ganache et al. (2001). Moreover, a palindromic sequence tail (5′-GTGTCTT-3′) was added to the 5′ end of the reverse primer to improve adenylation and facilitate genotyping. Amplified fragments were analyzed on an ABI 3130xl Genetic Analyzer (Applied Biosystems) and sized using GeneMapper 4.0 and GeneScan 500 LIZ Size Standard (Applied Biosystems). No multiplexing was attempted at the PCR stage.

From a total of 132 loci tested, 18 were monomorphic, 14 showed complex or nonspecific amplification, and nine failed to amplify. The remaining 91 loci were polymorphic in Portugal and Morocco (Table 1). Sequences of 109 loci (18 monomorphic and 91 polymorphic) from a shotgun genomic library were submitted to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA; accession SRP062192). We observed a total of 275 alleles for our initial sampling of *C. helodes* (nine individuals from Madroñalejo, five from Portugal, and five from Morocco), averaging overall 3.23 alleles per locus (SD = 1.15). Gametic disequilibrium (GENEPOP 4.1.4; Rousset, 2008) and the presence of null alleles according to the Oosterhout method (MICRO-CHECKER 2.2.3; van Oosterhout et al., 2004) were checked using Bonferroni-corrected $P$ values ($P < 0.05 / 34 = 0.0015$) to assess the significance of the results obtained. No significant gametic disequilibrium was detected for any pair of loci ($P > 0.01$), and four loci (Cahe587, CaheWE1, CaheGVB, and CaheQWV) showed signs of the presence of null alleles suggested by an excess of homozygotes. Some loci showed one to three alleles with a size difference of one to two base pairs relative to contiguous alleles (see Table 1). Nevertheless, no variation outside of the microsatellite region was shown by these loci, and no scoring errors were detected by MICRO-CHECKER (van Oosterhout et al., 2004). These polymorphisms could result from nonstepwise mutations in the repeat array.

The 47 individuals from the Madroñalejo population (Spain) showed an identical genotype (nine individuals were genotyped using 109 loci, and 38 additional individuals were genotyped using the 34 most variable loci). This seems to indicate that this population has probably suffered a recent founder or bottleneck event.

We used Arlequin 3.5.1.3 (Excoffier and Lischer, 2010) to calculate number of alleles, observed ($H_o$) and expected heterozygosities ($H_e$), and to test for deviations from Hardy–Weinberg equilibrium (HWE; Table 2) for the 34 most polymorphic loci in the eight individuals from the Cortelha population (Portugal). The eight individuals from the Cortelha population had a mean $H_o$ of 0.357 (SD = 0.292) and a mean $H_e$ of 0.384 (SD = 0.255). Six loci were monomorphic in this population, and only one (CaheWE1, $P < 0.05$; Table 2) of the 28 remaining loci deviated from HWE (Table 2).

## CONCLUSIONS

This study provides 109 simple sequence repeat markers to quantify the degree of genetic diversity in the endangered *C. helodes*. These markers may also help disentangle the phylogeographic history of *C. helodes* as well as gene flow among populations. This next-generation sequencing approach may also be useful for linkage mapping studies of experimental crosses between populations in a nonmodel organism. However, the low observed levels of variation within populations

TABLE 2. Results of screening 34 loci in the Cortelha population of *Carex helodes*.[a]

| Locus | *N* | *A* | $H_o$ | $H_e$ | HWE |
|---|---|---|---|---|---|
| CaheC2B | 8 | 2 | 0.625 | 0.525 | 1 |
| Cahe587 | 8 | 2 | 0 | 0.233 | 0.0668 |
| CaheYES | 8 | 4 | 0.625 | 0.592 | 1 |
| CaheFG7 | 8 | 5 | 0.500 | 0.533 | 0.5924 |
| CaheWE1 | 8 | 6 | 0.500 | 0.825 | 0.0054 |
| Cahe121 | 7 | 1 | 0 | 0 | NA |
| CaheT2N | 8 | 2 | 0.625 | 0.525 | 1 |
| CaheXAF | 7 | 3 | 0.571 | 0.659 | 0.0975 |
| Cahe147 | 8 | 4 | 0.625 | 0.575 | 0.5498 |
| CaheODD | 8 | 4 | 1 | 0.642 | 0.0594 |
| CaheMIW | 8 | 1 | 0 | 0 | NA |
| CaheGVB | 8 | 2 | 0 | 0.233 | 0.0667 |
| CahePM5 | 8 | 3 | 0.250 | 0.433 | 0.1368 |
| CaheQ9D | 8 | 1 | 0 | 0 | NA |
| Cahe468 | 8 | 3 | 0.500 | 0.633 | 0.0969 |
| Cahe7LF | 8 | 3 | 0.875 | 0.625 | 0.3286 |
| CaheQWB | 8 | 2 | 0.625 | 0.525 | 1 |
| CaheQWV | 8 | 2 | 0.125 | 0.458 | 0.0768 |
| CahePUI | 8 | 2 | 0.125 | 0.125 | 1 |
| Cahe222 | 8 | 4 | 0.625 | 0.675 | 0.4806 |
| CaheEZE | 8 | 2 | 0.250 | 0.233 | 1 |
| CaheSGU | 8 | 1 | 0 | 0 | NA |
| Cahe8RA | 8 | 2 | 0.250 | 0.400 | 0.3843 |
| CaheORP | 8 | 2 | 0.250 | 0.233 | 1 |
| CaheBI5 | 8 | 2 | 0.125 | 0.125 | 1 |
| Cahe130 | 8 | 3 | 0.625 | 0.575 | 1 |
| Cahe829 | 8 | 5 | 0.750 | 0.767 | 0.0523 |
| Cahe677 | 8 | 1 | 0 | 0 | NA |
| Cahe84P | 6 | 2 | 0.333 | 0.485 | 1 |
| CaheWMU | 8 | 2 | 0.125 | 0.125 | 1 |
| CaheL7Z | 8 | 2 | 0.375 | 0.525 | 0.5299 |
| CaheUAX | 8 | 3 | 0.625 | 0.542 | 0.4024 |
| Cahe932 | 8 | 1 | 0 | 0 | NA |
| Cahe192 | 8 | 2 | 0.250 | 0.233 | 1 |

*Note*: *A* = number of alleles; $H_e$ = expected heterozygosity; $H_o$ = observed heterozygosity; HWE = nominal *P* values for tests of deviation from Hardy–Weinberg equilibrium; *N* = number of successfully genotyped individuals; NA = not applicable.

[a] GPS coordinates and voucher information are available in Appendix 1.

are not enough to genotype reproductive individuals and their seeds within populations and study gene interchange patterns within populations.

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APPENDIX 1. Voucher and locality information for *Carex helodes* populations sampled in this study. Vouchers are deposited at the Universidad Pablo de Olavide herbarium (UPOS), Seville, Spain.

| Locality | Population | Geographic coordinates | *N*[a] | Voucher no. |
|---|---|---|---|---|
| Spain, Seville, Aznalcóllar, Madroñalejo | Madroñalejo | 37°35′24.0″N, 6°21′30″W | 9 (38) | 24ME07, 1ME14 |
| Portugal, Algarve, betw. Barraçao Caldas de Monchique | Pop. 1 | 37°15′44.4″N, 8°45′47.4″W | 4 | 8101JMM |
| Portugal, Algarve, Cortelha | Pop. 2 | 37°14′56.4″N, 7°57′45.5″W | 1 (7) | 7901JMM |
| Morocco, Tanger-Tetuan, Chauen, Ksar el Kebir | Pop. 1 | 35°05′09.0″N, 5°22′07.0″W | 4 | 34JFA03, 27PJM04 |
| Morocco, Tanger-Tetuan, Chauen, Ksar el Kebir | Pop. 2 | 35°06′02″N, 5°20′37″W | 1 | 22PJM04 |

*Note*: *N* = number of sampled individuals.

[a] The number of additional individuals sampled to test the 34 most polymorphic loci is given in parentheses.