Seventeen new microsatellites for *Tamarix gallica* and cross-amplification in *Tamarix* species

Alejandro Terrones1,2 and Ana Juan1

**PREMISE:** Microsatellite markers were developed for the western Mediterranean tree *Tamarix gallica* (Tamaricaceae) as part of a study of its genetic diversity and structure.

**METHODS AND RESULTS:** Seventeen microsatellite markers were developed for *T. gallica*, 14 of which were polymorphic. These microsatellites have di-, tri-, and tetrancleotide repeats with 1–13 alleles per locus and population. Levels of observed and expected heterozygosity ranged from 0.000 to 0.900 and from 0.000 to 0.863, respectively. Six microsatellites showed significant deviations from Hardy–Weinberg equilibrium in at least one population. Cross-amplification in 19 *Tamarix* species showed a wide transferability to other species of the genus.

**CONCLUSIONS:** The 14 new polymorphic microsatellite markers will be used to assess the genetic diversity and population genetic structure of *T. gallica*. Additionally, the successful cross-species amplification suggests their potential usefulness for investigating species delimitation and population genetics in the genus *Tamarix*.

**KEY WORDS** genetic diversity; saltcedar; simple sequence repeat (SSR) markers; species delimitation; Tamaricaceae; *Tamarix gallica*.

*Tamarix gallica* L. is a widespread tree that forms woodlands in the western Mediterranean Basin in saline habitats such as salt marshes, ravines, and rivers with brackish waters (Baum, 1978). This species is closely related to and commonly confused with *T. canariensis* Willd. because of their similar morphology, anatomy, and phenology (Villar et al., 2019). Hybridization is common in the genus *Tamarix*, making the species delimitation of *T. gallica* not well resolved (Villar et al., 2019). In addition, this and various other species of *Tamarix* have been reported as widespread invasives in North America (Villar et al., 2019).

Simple sequence repeat (SSR) markers (also referred to as microsatellites) are useful tools to help resolve species delimitation. Some microsatellite markers have already been described in the genus *Tamarix* (Gaskin et al., 2006; Terzoli et al., 2010, 2013; Zhang et al., 2019), but no study has focused on describing genomic SSR markers for *T. gallica*. Consequently, as part of a study of the genetic diversity and structure of *T. gallica* in the western Mediterranean Basin, the aim of this work is to characterize new polymorphic microsatellite markers for *T. gallica*. Cross-species amplification was also tested in 19 species of *Tamarix* to aid with future taxon delimitation studies and population genetic studies of the genus both in native and invaded areas, particularly with respect to hybridization.

**METHODS AND RESULTS**

DNA extraction was carried out from silica gel–dried leaves by a modified cetyltrimethylammonium bromide (CTAB) method (Csiba and Powell, 2006). For the microsatellite library, 12 individuals of *T. gallica* and *T. boveana* Bunge were selected from two different populations. A microsatellite library enriched with TG, TC, AAC, AAG, AGG, ACG, ACAT, and ACTC motifs was prepared from the pooled DNA by Genoscreen (Lille, France) using a 454 GS-FLX (Roche Diagnostics, Meylan, France) high-throughput DNA sequencer (Malausa et al., 2011). Sequencing provided 22,418 reads with an average length of 220 bp. Raw sequences were searched for microsatellites with QDD version 3.1.2 (Meglécz et al., 2014) with default settings, which produced primers for 248 loci. To identify and eliminate known transposable elements and contaminants, these sequences were queried with RepeatMasker version open-4.0.3 (Smit et al., 2015) in the database Repbase version 20140131 (Bao et al., 2015), and with BLAST+ version 2.2.28+ (https://blast.ncbi.nlm.nih.gov/Blast.cgi) in the National Center for Biotechnology Information (NCBI) nucleotide database. A total of 219 loci were developed for downstream testing.

The number of primer pairs was reduced according to the following criteria (based on Guichoux et al., 2011 and Meglèz et al.,...
2014): (1) high number of repeats, (2) pure repeats over compound repeats, (3) tri- and tetranucleotide repeats over dinucleotide repeats, (4) varying PCR product sizes and repeat motifs, (5) MIN_PRIMER_TARGET_DIST > 20, and (6) DESIGN A or B. Based on these criteria, primers for 52 loci were synthesized (Eurofins Genomics, Ebersberg, Germany). An M13 tail was attached to the 5′ end of the forward primers (Schuelke, 2000). Each locus was amplified for 12 individuals of T. gallica from four different populations (Appendix 1). PCRs were conducted in a final volume of 25 μL with DreamTaq PCR Master Mix (2×) with 20 ng of template DNA, and a final concentration of 0.2 μM of each primer and 20 ng/μL of bovine serum albumin (BSA) (Thermo Scientific). PCRs were conducted on a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, California, USA) with the following conditions: an initial denaturation of 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. PCR products were run on a 2.5% agarose gel stained with ethidium bromide. Loci with multiple bands or with non-successful amplification across all samples were discarded.

Fluorescent labeling of the 29 loci that amplified successfully was performed in simplex for the 12 samples with a three-primer protocol including a universal M13 primer fluorescently labeled with FAM, HEX, or TAMRA dyes (Schuelke, 2000). Fluorescent-labeled PCRs were conducted in a final volume of 10 μL with DreamTaq PCR Master Mix (2×) with 20 ng of template DNA, and a final concentration of 0.04 μM of the M13-tailed forward primer, 0.16 μM of the reverse primer, 0.16 μM of the fluorescent-labeled M13 primer, and 50 ng/μL of BSA. PCR conditions were as follows: an initial denaturation of 95°C for 5 min; followed by 30 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 45 s; followed by 10 cycles of 95°C for 30 s, 53°C for 45 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. PCR products were pooled in equimolar concentrations and run on an ABI Prism 310 Genetic Analyzer (Applied Biosystems) with GeneScan 500 Size Standard (Applied Biosystems) in the Research Technical Services of the University of Alicante (Alicante, Spain). Electropherograms were scored with Peak Scanner Software 2 (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Markers with excessive stuttering, with more than two alleles, or that were difficult to score were discarded, resulting in 17 microsatellite loci, 14 of which were polymorphic.

### TABLE 1. Characteristics of the 17 microsatellite loci developed in Tamarix gallica that successfully amplified.

| Locusa | Primer sequences (5′-3′) | Repeat motif | Allele size range (bp) | A | Mix | Fluorescent dye | Concentration (F/R) (μM)b | GenBank accession no. |
|--------|------------------------|-------------|-----------------------|---|-----|----------------|--------------------------|-----------------------|
| T125-4 | F: TGGAGGTTAAGAAGAGGATAAGAGA | (TGT)8 | 121–145 | 7 | 1 | FAM | 0.04/0.16 | MN497849 |
|        | R: AAAGCTCTCCCCACCCCTCCT |             |                     |   |     |                |                          |                       |
| T133-2 | F: AGCAGAAAGTTGATCTCTTGT | (TG)8 | 129–151 | 7 | 1 | HEX | 0.04/0.16 | MN497850 |
|        | R: TGGGTGCTATTCTGAGAGT |             |                     |   |     |                |                          |                       |
| T129-2 | F: CACCTAAGAAATAAGTGACACTGC | (CA)8 | 115–151 | 16 | 1 | TAMRA | 0.06/0.24 | MN497851 |
|        | R: CCATTCTAGGTTGATATTGTTG |             |                     |   |     |                |                          |                       |
| T163-3 | F: CGAAGGTAGGGCCAGTGGG | (CTC)8 | 186–198 | 5 | 1 | TAMRA | 0.04/0.16 | MN497852 |
|        | R: TGGAGATCTGTGACTCTGGA |             |                     |   |     |                |                          |                       |
| T140-3 | F: TGGTTGAAAGCTTACTGCTTGT | (TTT)8 | 137–152 | 7 | 2 | FAM | 0.04/0.16 | MN497853 |
|        | R: GGATATCTCTGAAATATACCAAGTCCA |             |                     |   |     |                |                          |                       |
| T113-3 | F: TGAGAAAGCATTCCAACCAA | (GAT)8 | 93–99 | 3 | 2 | HEX | 0.04/0.16 | MN497854 |
|        | R: GAGGACATATTGACCCATCCTGA |             |                     |   |     |                |                          |                       |
| T190-32 | F: CTCACACTCATGCTCCTCA | (CGA)8 | 128–135 | 4 | 2 | HEX | 0.04/0.16 | MN497855 |
|        | R: GGGGACAGCTTTGGTATAT |             |                     |   |     |                |                          |                       |
| T190-3 | F: GAAATAATCTAATCTGATTGGAAGCAG | (GAG)8 | 168–189 | 6 | 2 | TAMRA | 0.04/0.16 | MN497856 |
|        | R: GGACCTAAAGTTGAGAAGTTGGA |             |                     |   |     |                |                          |                       |
| T214-3 | F: TGGAGATGCTCTTTAGGAGTG | (ATT)8 | 104–107 | 2 | 2 | TAMRA | 0.04/0.16 | MN497857 |
|        | R: TCCATCTCCTATGGCTGCTCAAATCA |             |                     |   |     |                |                          |                       |
| T145-3 | F: ACTTCCTTCTCCTACGCCCAT | (TCT)10 | 90–117 | 10 | 3 | FAM | 0.04/0.16 | MN497858 |
|        | R: GGAGATGTGAGAAGTTGGA |             |                     |   |     |                |                          |                       |
| T134-31 | F: CCCCCACCTCCCTCTTTC | (TCT)11 | 141–168 | 7 | 3 | HEX | 0.04/0.16 | MN497859 |
|        | R: TCAGCTCTGCAGAAGAACCG |             |                     |   |     |                |                          |                       |
| T190-33 | F: TGCTTCTGGCTTGCTGATCTC | (CTT)8 | 107–113 | 3 | 3 | TAMRA | 0.04/0.16 | MN497860 |
|        | R: CTTGTACCTGAAGTATGATGGGA |             |                     |   |     |                |                          |                       |
| T140-32 | F: CCTTCACCTCCTCTCTTTCG | (CTT)8 | 123–132 | 4 | 3 | TAMRA | 0.04/0.16 | MN497861 |
|        | R: TGGTGGAATGCTGATATGTTG |             |                     |   |     |                |                          |                       |
| T230-2 | F: AACAAAGGAAATTTTGGACG | (TC)12 | 232–265 | 14 | 3 | TAMRA | 0.06/0.24 | MN497862 |
|        | R: CGTGTAAAATCTTCTGGGAGG |             |                     |   |     |                |                          |                       |
| T168-2 | F: TGGAGGTTGCTGCTGCTTC | (GA)8 | 169 | M | — | — | MN560186 |
|        | R: TAAGTGTGAGGCAAGAACCG |             |                     |   |     |                |                          |                       |
| T193-3 | F: TGGAGGTTGCTGCTGCTTAC | (TTC)14 | 188 | 1 | — | — | MN560187 |
|        | R: AAGAGAAGCATTTTGAAGG |             |                     |   |     |                |                          |                       |
| T300-2 | F: AAAGTAACCTCCCAAAACCTTTC | (AC)8 | 299 | M | — | — | MN560185 |
|        | R: TCGAGACAAATGCGCAAGTGA |             |                     |   |     |                |                          |                       |

Note: A = number of alleles; M = monomorphic.

aThe annealing temperature was 56°C for all loci.
bPCR primer concentration.
TABLE 2. Genetic properties of the 14 polymorphic microsatellites developed in *Tamarix gallica*.

| Locus   | Antas (n = 30) | Cagliari (n = 30) | Elche (n = 30) | Tablas de Daimiel (n = 32) |
|---------|----------------|------------------|----------------|--------------------------|
|         | A   | A   | H̅  | H̅  | Null alleles | A   | A   | H̅  | H̅  | Null alleles | A   | A   | H̅  | H̅  | Null alleles | A   | A   | H̅  | H̅  | Null alleles |
| T125-4  | 6   | 2.663 | 0.833 | 0.624 | — | 5 | 2.875 | 0.567 | 0.652 | — | 6 | 4.327 | 0.767 | 0.769 | — | 5 | 1.928 | 0.500 | 0.481 | — |
| T133-2  | 4   | 1.515 | 0.133* | 0.340 | 0.227 | 6 | 2.459 | 0.433* | 0.593 | 0.117 | 5 | 2.217 | 0.233* | 0.549 | 0.254 | 4 | 2.557 | 0.281 | 0.609 | 0.244 |
| T129-2  | 13  | 5.941 | 0.900 | 0.833 | — | 9 | 7.317 | 0.833 | 0.863 | — | 8 | 2.965 | 0.733 | 0.683 | — | 5 | 3.131 | 0.813 | 0.681 | — |
| T163-3  | 3   | 1.268 | 0.233 | 0.212 | — | 4 | 2.002 | 0.433 | 0.501 | — | 3 | 1.412 | 0.267 | 0.292 | — | 2 | 1.064 | 0.063 | 0.061 | — |
| T140-30 | 6   | 3.114 | 0.633 | 0.679 | — | 3 | 2.456 | 0.533 | 0.593 | — | 2 | 2.308 | 0.500 | 0.567 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T113-3  | 3   | 2.335 | 0.300* | 0.572 | 0.221 | 3 | 1.802 | 0.500 | 0.445 | — | 2 | 1.763 | 0.500 | 0.433 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T190-32 | 4   | 2.002 | 0.533* | 0.501 | 0.051 | 2 | 1.342 | 0.300 | 0.255 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T190-3  | 2   | 1.220 | 0.200 | 0.180 | — | 5 | 1.950 | 0.500 | 0.487 | — | 4 | 1.367 | 0.300 | 0.268 | — | 3 | 1.982 | 0.283 | 0.285 | — |
| T214-3  | 1   | 1.000 | 0.000 | 0.000 | — | 2 | 1.763 | 0.500 | 0.433 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T145-3* | 8   | 4.094 | 0.357* | 0.756 | 0.256 | 8 | 6.081 | 0.400* | 0.836 | 0.252 | 5 | 3.147 | 0.333* | 0.682 | 0.246 | 4 | 2.190 | 0.531 | 0.543 | — |
| T134-31 | 4   | 1.410 | 0.267 | 0.291 | — | 2 | 1.462 | 0.633 | 0.594 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T134-32 | 2   | 1.000 | 0.000 | 0.000 | — | 3 | 1.350 | 0.300 | 0.259 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T134-33 | 1   | 1.000 | 0.000 | 0.000 | — | 2 | 1.763 | 0.500 | 0.433 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T129-2  | 1   | 0.533 | 0.500 | 0.445 | — | 2 | 1.342 | 0.300 | 0.255 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T190-32 | 4   | 2.002 | 0.533* | 0.501 | 0.051 | 2 | 1.342 | 0.300 | 0.255 | — | 1 | 1.000 | 0.000 | 0.000 | — | 1 | 1.000 | 0.000 | 0.000 | — |
| T230-2  | 8   | 3.711 | 0.467* | 0.731 | 0.177 | 6 | 3.396 | 0.700 | 0.706 | — | 5 | 3.273 | 0.533* | 0.694 | 0.116 | 4 | 1.653 | 0.313 | 0.395 | — |

Note: A = number of alleles; A = effective number of alleles; H̅ = expected heterozygosity; H̅ = observed heterozygosity; n = number of individuals sampled.

For locus T145-3, n = 28.

*Significant deviation from Hardy-Weinberg equilibrium (P < 0.05).

CONCLUSIONS

The 14 polymorphic microsatellite markers described here showed high variability and will be used to assess the genetic diversity and population genetic structure of *T. gallica*. Additionally, the success of cross-species amplification suggests their potential usefulness to assess population genetic parameters and provide data on the role of interspecific hybridization in the genus.

ACKNOWLEDGMENTS

The authors thank the director and guard of Tablas de Daimiel National Park for permitting collection of material. This research was supported by a research grant from the Ministerio de Educación (FPU grant AP-2012-1954), the Ministerio de Medio Ambiente (Proyecto OAPN 354/2011), A.T. was supported by the grant BEST/2019/155 (Generalitat Valenciana). This study is part of the Ph.D. thesis of A.T. and to test for Hardy-Weinberg equilibrium (P < 0.05) (Table 2). Seven comparisons between pairs of markers showed significant linkage disequilibrium: T125-4 with T129-2, T125-4 with T190-33, T133-2 with T134-31, and T190-32 with T190-33. In addition, we performed cross-species amplification in posterior analyses. Seven comparisons between pairs of markers showed significant linkage disequilibrium: T125-4 with T129-2, T125-4 with T163-3, T125-4 with T190-33, T133-2 with T134-31, and T190-32 with T190-33. The number of alleles ranged from one to 13 (Table 2). Levels of observed and expected heterozygosity ranged from 0.000 to 0.900 and from 0.000 to 0.863, respectively. Almost all markers were polymorphic in four populations, except for T125-4 and T190-33, which were only polymorphic in the Cagliari and Elche populations (Table 3). In addition, we performed cross-species amplification in the remaining populations. Six microsatellites showed null alleles and significant population differentiation. Levels of observed and expected heterozygosity ranged from 0.000 to 0.900 and from 0.000 to 0.863, respectively.

MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004) was used to estimate null allele frequencies.
### TABLE 3. Size ranges (in base pairs) of the 14 polymorphic microsatellite loci developed in *Tamarix gallica* cross-amplified in 19 *Tamarix* species.

| Species            | T125-4 | T133-2 | T129-2 | T163-3 | T140-31 | T190-32 | T214-3 | T145-3 | T134-31 | T190-33 | T140-32 | T230-2 |
|--------------------|--------|--------|--------|--------|---------|---------|--------|--------|---------|---------|---------|-------|
| *T. africana*      | —      | 131–159| 116–137| 184–187| 134–163 | 87–99   | 129    | 165–171| 104     | 93–111  | 150–165 | 208–209| 229–247|
| (*n* = 16)         |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. amplexicaulis* | —      | 129–133| 118    | 186    | 160–172 | 96      | 129    | —      | 122–137 | 96–102  | 144     | 208    | 129   | 236–240|
| (*n* = 4)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. aphylla*       | —      | 128    | —      | 192    | 131–134 | 93 (1)  | —      | —      | 104     | —       | 156     | 209–210| 132  | —      |
| (*n* = 3)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. arceuthoides*  | —      | 131    | 115    | 183–189| 128–161 | 93      | 129–135| 171–183| 104     | 96–105  | 156–165 | 110    | 126   | 234–239|
| (*n* = 2)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. boveana*       | 113–129| 131–133| 119–129| 184–195| 137–157 | 96–99   | 129–132| 171–195| 104     | 96–114  | 150–162 | 110    | 114–129| 232–247|
| (*n* = 18)         |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. canariensis*   | 117–141| 131–159| 115–133| 187–189| 128–157 | 93–96   | 129    | 165–177| 104–107 | 93–117  | 150–162 | 110–209| 126–132| 229–261|
| (*n* = 12)         |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. chinensis*     | —      | 131–135| 101    | 180    | 128     | 96      | 126    | 168    | 104     | 99      | 156     | 183   | 123  | 238    |
| (*n* = 1)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. dalmatica*     | —      | 131–139| 123 (1)| 181–186| 137–157 | 96 (1)  | 129 (1)| 165 (1)| 104     | 96 (2)  | 150–159 | 198–208| 126–189| 229–243|
| (*n* = 4)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. hampeana*      | —      | 131–139| 104–125| 180–195| 128–135 | 93–96   | 129–135| 174–192 (2)| 104   | 93–99   | —      | 110–209| 123  | —      |
| (*n* = 3)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. hispida*       | —      | 131–135| 109    | 189    | 143–146 | 96      | 129–132| 167    | 104     | 96      | 150     | 107   | 123  | 239    |
| (*n* = 1)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. hohenackeri*   | 117–129(1)| 131   | 117–134| 183–195| 126–129 | —      | 129–132| 171–180| 104     | 99–126  | 150 (1) | 110–113| 123  | 232–241|
| (*n* = 2)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. leptostachya*  | —      | 131    | 123–127| 186    | 135–142 | —      | 129    | 168–174| 104     | 90–99   | 150     | —      | 123  | 234–245|
| (*n* = 1)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. minoa*         | —      | 131–139| 119–127| 186–189| 137–157 | 93–96   | 129–132| 192–195| 104     | —       | 150–153 | 110–208| 123–126| 235–260|
| (*n* = 3)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. nilotica*      | —      | 131    | 115    | 189    | 128     | 93      | 129    | 171    | 104     | 109     | 153–159 | 110    | 126–129| 240–267|
| (*n* = 6)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. parviflora*    | 160 (2)| 131–149| 123–133| 189    | 128–144 | 93      | 129    | 177    | 104     | 96      | 153–165 | 110–208| 123  | 232–236|
| (*n* = 3)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. ramossissima*  | —      | 131    | 124    | 180    | 128     | —      | 126    | 168    | 104     | 90      | —       | 208    | 123  | 238    |
| (*n* = 1)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. smyrnensis*    | —      | 131–149| 113–123| 180–189| 129     | —      | 129–132| 171–183| 104     | 99 (1)  | 150 (1) | 208    | 123 (1)| 234–236|
| (*n* = 2)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. tetragyna*     | —      | 129–133| 113–127| 183–195| 137     | 96      | 129–132| 174–177(2)| 104   | 91–99 (2)| 150–162 | 110    | 126  | 235–243|
| (*n* = 3)          |        |        |        |        |         |         |        |        |         |         |         |       |
| *T. usneoides*     | —      | 135–137| 183–189| 137–140 | 93      | —      | 153–165 (2)| 104   | —       | 162–168 | 208     | 238  | —      |
| (*n* = 3)          |        |        |        |        |         |         |         |        |         |         |         |       |

Note: Numbers in parentheses indicate the number of samples that successfully amplified. No number in parentheses indicates that all samples were successfully amplified. A dash indicates no successful amplification for any sample.
AUTHOR CONTRIBUTIONS

A.T. helped design the experiment, conducted the lab work, analyzed the results, and helped write the article. A.J. helped design the experiment and write the article.

DATA ACCESSIBILITY

Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1.

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APPENDIX 1. Voucher information for Tamarix species used in this study.

| Species                     | Voucher specimen accession no.* | Collection locality                  | Geographic coordinates (WGS84) | N     |
|-----------------------------|---------------------------------|--------------------------------------|--------------------------------|-------|
| T. africana Poir.           | ABH 73511                       | Portugal, Baixo Alentejo, Melides, Lagoa de Melides | 38.129, −8.789                | 2     |
|                            | ABH 70789                       | Spain, Castelão, Burnana, Clot de la Mare de Déu | 39.879, −0.055                 | 12    |
| T. amplexicaulis Ehrenb.     | ABH 70742                       | Spain, Murcia, Águilas, Rambla de Minglano de Caliarte | 37.433, −1.629                | 2     |
| T. aphylla (L.) H. Karst     | ABH 70064                       | Algeria, Biskra, N3 crossing with Oumache, Km 336 | 34.719, 5.739                 | 4     |
| T. arceuthoides Bunge        | ABH 71909                       | Morocco, Nador, Berkane, Oued Moulouya | 35.103, −3.360               | 1     |
|                            | ABH 54208                       | Morocco, Nador, Driouch              | 34.972, −3.360               | 1     |
| T. boeana Bunge              | MO 5568713                      | Iran, Esfahan, Road from Tehran to Nain, south of junction to Esfahan | 33.0152, 52.5238              | 1     |
|                            | MO 5568891                      | Iran, Qom, old rd. from Tehran to Qom | 35.1705, 50.9777              | 1     |
| T. boveana Bunge             | ABH 70782                       | Spain, Alicante, Santa Pola, Salinas de Santa Pola | 38.184, −0.602               | 6     |
|                            | ABH 68315                       | Spain, Almeria, Cabo de Gata        | 36.773, −2.238               | 12    |
| T. canariensis Willd.        | ABH 69066                       | Spain, Canary Islands, Gran Canaria, beach of Al Aida de San Nicolás | 27.996, −15.824              | 12    |
| T. chinensis Lour.           | Gaskin 202                      | South Korea                         | —                              | 1     |
| T. dalmatica B. R. Baum      | ABH 57833                       | Albania, Shkoder, next to rd. at south of Shkoder | 41.968, 19.547               | 1     |
|                            | ABH 57829                       | Albania, Vlore, Sarande, Borsh      | 40.047, 19.946               | 1     |
|                            | ABH 57830                       | Albania, Vlore, Sarande, Viron, rd. from Greece to Sarande | 39.904, 20.084              | 1     |
|                            | ABH 57843                       | Montenegro, Bar, south of Bar        | 42.093, 19.104               | 1     |
| T. gallica L                 | ABH 70037                       | Italy, Sardinia, Cagliari, Stani Simbririzi | 39.2631, 9.2086            | 30    |
|                            | ABH 69543                       | Spain, Alicante, Elche, Pantano de Elche | 38.3174, −0.718              | 30    |
|                            | ABH 67467                       | Spain, Almeria, Vera, rio Antas     | 37.2054, −1.829              | 30    |
|                            | ABH 73456                       | Spain, Ciudad Real, Daimiel, Tablas de Daimiel | 39.1521, −3.7106           | 32    |
| T. hampeana Boiss. & Heldr.  | ABH 59877                       | Greece, Central Greece, Molos-Agios Konstantinos, Neo Thronio | 38.834, 22.703               | 1     |

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### APPENDIX 1. (Continued)

| Species                     | Voucher specimen accession no.* | Collection locality                                      | Geographic coordinates (WGS84) | N  |
|-----------------------------|---------------------------------|----------------------------------------------------------|-------------------------------|----|
| *T. hispida* Willd.         | ABH 57891                       | Montenegro, Ulcin, Sveti Nikola, Bojana river            | 41.870, 19.352                | 1  |
|                             | Gaskin 10164                    | China                                                    | —                             | 1  |
| *T. hohenackeri* Bunge      | MO 5568893                      | Iran, Gilan, rd. from Rasht to Tehran, near Gangeh, south of Rasht | 36.8641, 49.4811              | 1  |
| *T. leptostachya* Bunge     | MO 5568696                      | Iran, Semnan, NE of Sharud toward Gorgan                 | 36.7252, 55.2975              | 1  |
| *T. minoa* J. L. Villar,    | ABH 51017                       | China                                                    | —                             | 1  |
| Turland, Juan, Gaskin,      |                                 |                                                          |                               |    |
| M. Á. Alonso & M. B. Crespo |                                 |                                                          |                               |    |
|                             | ABH 54194                       | Greece, Crete, Chania, Georgioupoli                      | 35.365, 24.248                | 1  |
| *T. nilotica* (Ehrenb.)     | ABH 54195                       | Greece, Crete, Chania, near Plataniai                    | 35.356, 24.260                | 1  |
| Bunge                       | MO 6207620                      | Greece, Crete, Nomos Chanion, Eparchia Apokoronou       | 35.359, 24.266                | 1  |
|                             |                                 | Georgioupoli beach                                      |                               |    |
| *T. parviflora* DC.         | ABH 54314                       | Greece, Crete, Heraklion, Aposelemis                    | 35.330, 25.327                | 1  |
|                             | ABH 54317                       | Greece, Crete, Heraklion, Kalo Nero                     | 35.014, 26.046                | 1  |
|                             | ABH 54326                       | Greece, Crete, Heraklion, near Dermatos                 | 34.979, 25.335                | 1  |
|                             | ABH 54323                       | Greece, Crete, Heraklion, near Dermatos                 | 34.979, 25.324                | 1  |
|                             | ABH 54316                       | Greece, Crete, Lassithi, Xerokambos                     | 35.051, 26.232                | 1  |
| *T. parviflora* DC.         | ABH 54197                       | Greece, Crete, Heraklion, near Aposelemis               | 35.321, 25.327                | 1  |
|                             | ABH 54321                       | Greece, Crete, Heraklion, near Dermatos                 | 34.979, 25.324                | 1  |
|                             | ABH 55398                       | Spain, Alicante, Bier, Santuario Mare de Dèu de Gràcia  | 38.629, −0.760                | 1  |
| *T. ramosissima* Ledeb.     | W 2009-19143                    | Argentina, San Juan, Ullum, at Termas de Talacasto      | −31.03, −68.75                | 1  |
| *T. smyrnensis* Bunge       | W 2003-14043                    | Armenia, Vayots’Dzor, Yeghegnadzor                      | 39.68, 45.22                  | 1  |
|                             | Gaskin 4690-06                   | Turkey                                                   | —                             | 1  |
| *T. tetragyna* Ehrenb.      | W 2007-14048                    | Egypt, New Valley, Western Desert Dakhleh Oasis         | 25.667, 28.870                | 1  |
|                             | W 2007-25728                    | Egypt, South Sinai, Dahab, Wadi Qnai, Oase, salzreicher Feuchtstandort | 28.4532, 34.4492              | 1  |
|                             | W 2007-07364                    | Jordan, Al Asimah, 11.5 km NE end of Dead Sea, 2 km N v. Tell Iktanu | 31.833, 35.676                | 1  |
| *T. usneoides* E. Mey.      | ABH 58684                       | Namibia, Erongo, Swerkoobmund                           | −22.708, 14.961               | 2  |
|                             | ABH 58683                       | South Africa, Western Cape, Prince Albert, betw. Lainsburg and Beaufort West | −33.085, 21.579               | 1  |

Note: N = number of individuals.

*Vouchers were deposited at the herbaria of Universidad de Alicante, Spain (ABH); research collection of John F. Gaskin, Sidney, Montana, USA (Gaskin); Missouri Botanical Garden, St. Louis, Missouri, USA (MO); and Naturhistorisches Museum Wien, Vienna, Austria (W).*