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PRIMER NOTE

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

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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 tetranucleotide 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* L., 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 Meglécz 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×) (Thermo Scientific, Vilnius, Lithuania) with 40 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 35 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.

Locus[a]	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: TGGAAGGTAAGAAGAGGATAAGAGA R: AAAGCCTCACCCAAACCTCT	$(TGTA)_7$	121–145	7	1	FAM	0.04/0.16	MN497849
T133-2	F: AGCAGAATGGTTGATCCTTG R: TGGGTGCTAATTTCTGGAGTG	$(TC)_{10}$	129–151	7	1	HEX	0.04/0.16	MN497850
T129-2	F: CACTATAGAAATAGGTGACACATGC R: CCATTTCTAGGGTGATTAGGTTG	$(CA)_7$	115–151	16	1	TAMRA	0.06/0.24	MN497851
T163-3	F: CGAAGGTAAGACCCAGTTGC R: TGGAGAGTGCTTGAACTTGA	$(CTC)_7$	186–198	5	1	TAMRA	0.04/0.16	MN497852
T140-31	F: TGGTTTGAAGCTTACTGGTTG R: GGATTACTTCAGAATATACAAGCTCA	$(TTC)_8$	137–152	7	2	FAM	0.04/0.16	MN497853
T113-3	F: TGAGAAGCATTCCAAACCAA R: GAGGACATTAATGCCACTGGA	$(GAT)_7$	93–99	3	2	HEX	0.04/0.16	MN497854
T190-32	F: CTCCAATCCATCGCTCTCA R: GGCGGACGACTTTGCTTAT	$(CGA)_8$	128–135	4	2	HEX	0.04/0.16	MN497855
T190-3	F: GAAATAATCTTAACTTGATGGCCAAG R: GGAGCTAAAGTTGAAAAAGAGTTGA	$(GAG)_7$	168–189	6	2	TAMRA	0.04/0.16	MN497856
T214-3	F: TTGACATGCCTCTTGAGGTG R: TCCATTCCTAGTTGCTACAATCA	$(ATT)_5$	104–107	2	2	TAMRA	0.04/0.16	MN497857
T145-3	F: ACTTGCTTTCTTCACCGCAT R: GGAGGATTTGAAGAATGTTGGA	$(TCT)_{13}$	90–117	10	3	FAM	0.04/0.16	MN497858
T134-31	F: CCCTTAGCCTCCCTTGTTTC R: TCATGCTTGCAGAGAAGACG	$(TCT)_{12}$	141–168	7	3	HEX	0.04/0.16	MN497859
T190-33	F: TTGTTGCTGATGGGTGATTC R: CCTTGTACTTGAAGTGTATGGCA	$(CTT)_6$	107–113	3	3	HEX	0.04/0.16	MN497860
T140-32	F: CCTTCACTCCTTCTGTTGCC R: TTGGTGGATGTGGTATGGTG	$(CTT)_7$	123–132	4	3	TAMRA	0.04/0.16	MN497861
T230-2	F: AACAAAGCAAATTTGGCAGC R: CGTGTTAAATTCTGGGACGG	$(TC)_{12}$	232–265	14	3	TAMRA	0.06/0.24	MN497862
T168-2	F: TGGACCGTCTTCTCGTCTTC R: TAAGTGATGGCACAGAACGC	$(GA)_7$	169	M	—	—	—	MN560186
T193-3	F: TGGGAGTTTAGTTGTCTGTAGCC R: AAGAGAAGCATCATTAGCAAGG	$(TTC)_{14}$	188	M	—	—	—	MN560187
T300-2	F: AAACTAATCCCCAACCCTTTC R: TCAGGAACAATGGCAAGTGA	$(AC)_6$	299	M	—	—	—	MN560185

Note: A = number of alleles; M = monomorphic.
[a]The annealing temperature was 56°C for all loci.
[b]PCR primer concentration.

TABLE 2. Genetic properties of the 14 polymorphic microsatellites developed in *Tamarix gallica*.

	Antas (n = 30)					Cagliari (n = 30)					Elche (n = 30)					Tablas de Daimiel (n = 32)				
Locus	A	A_e	H_o	H_e	Null alleles	A	A_e	H_o	H_e	Null alleles	A	A_e	H_o	H_e	Null alleles	A	A_e	H_o	H_e	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	0.832	—	9	7.317	0.833	0.863	—	8	2.965	0.733	0.663	—	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	—
T140-31	6	3.114	0.633	0.679	—	3	2.456	0.533	0.593	—	4	2.308	0.500	0.567	—	4	2.114	0.500	0.527	—
T113-3	3	2.335	0.300*	0.572	0.221	3	1.802	0.500	0.445	—	3	2.299	0.500*	0.565	—	3	1.575	0.375	0.365	—
T190-32	4	2.002	0.533*	0.501	—	2	1.342	0.300	0.255	—	2	1.763	0.500	0.433	—	2	1.882	0.313	0.469	0.152
T190-3	2	1.220	0.200	0.180	—	5	1.950	0.500	0.487	—	4	1.367	0.300	0.268	—	2	1.398	0.281	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[a]	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	7	4.830	0.469*	0.793	0.202
T134-31	4	1.410	0.267	0.291	—	4	2.462	0.633	0.594	—	4	1.468	0.333	0.319	—	4	2.190	0.531	0.543	—
T190-33	1	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	—
T140-32	4	2.799	0.400*	0.643	0.183	2	1.471	0.200*	0.320	0.153	4	1.978	0.533	0.494	—	3	2.118	0.344	0.528	0.165
T230-2	8	3.711	0.467*	0.731	0.177	6	3.396	0.700	0.706	—	10	3.273	0.533	0.694	0.116	4	1.653	0.313	0.395	—

Note: A = number of alleles; A_e = effective number of alleles; H_e = expected heterozygosity; H_o = observed heterozygosity; n = number of individuals sampled.
[a]For locus T145-3 in Antas population, n = 28.
*Significant deviation from Hardy–Weinberg equilibrium (P < 0.05).

(Table 1). These 14 loci were analyzed across 122 individuals from four populations of *T. gallica* in subsequent analyses (Appendix 1). To reduce the number of PCR reactions, some loci were multiplexed. Markers were combined to avoid size overlap, resulting in nine reactions, four in simplex and five in 2-plex, that were pooled and run in three different mixes (Table 1). For the simplex reactions, the PCR conditions were the same as described above. In the 2-plex reactions, PCR conditions were the same as described for fluorescent-labeled simplex reactions except for the final primer concentrations (Table 1) and the double concentration of the fluorescent-labeled M13 primer (0.32 μM). Allele calling was done with Peak Scanner Software 2, and allelic binning was done manually with the use of cumulative frequency plots of size distribution (Guichoux et al., 2011).

GenAlEx version 6.503 (Peakall and Smouse, 2006) was used to calculate the number of alleles, effective number of alleles, and levels of observed and expected heterozygosities for each population, and to test for Hardy–Weinberg equilibrium (P < 0.05) (Table 2). Evidence of linkage disequilibrium was assessed by GENEPOP version 4.7.2 (Rousset, 2008) based on 10,000 permutations (P < 0.05). MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004) was used to estimate null allele frequencies.

The number of alleles per population 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 the four populations, except for T214-3 and T190-33, which were only polymorphic in the Cagliari population. Six microsatellites showed null alleles and significant deviations from Hardy–Weinberg equilibrium in at least one population (Table 2), so these markers should be treated with caution 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, T129-2 with T190-33, T163-3 with T134-31, and T190-32 with T190-3. In addition, we performed cross-species amplification in 88 individuals from 19 species of the genus *Tamarix* with the same simplex and 2-plex PCR reactions used in *T. gallica* (Appendix 1), demonstrating wide transferability to other species of the genus such as *T. boveana*, *T. africana* Poir., and *T. canariensis* (Table 3).

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 successful rates of cross-species amplification suggest their potential usefulness to assess population genetic parameters and provide data on the role of interspecific hybridization in the genus.

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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	T113-3	T190-32	T190-3	T214-3	T145-3	T134-31	T190-33	T140-32	T230-2
T. africana (*n* = 16)	—	131–159	116–137	184–187	134–163	87–99	129	165–171	104	93–111	150–165	208–209	—	229–247
T. amplexicaulis (*n* = 4)	—	129–133	118	186	160–172	96	129	—	122–137	96–102	144	208	129	236–240
T. aphylla (*n* = 3)	—	128	—	192	131–134	93 (1)	—	—	104	—	156	209–210	132	—
T. arceuthoides (*n* = 2)	—	131	115	183–189	128–161	93	129–135	171–183	104	96–105	156–165	110	126	234–239
T. boveana (*n* = 18)	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
T. canariensis (*n* = 12)	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 (5)	229–261
T. chinensis (*n* = 1)	—	131–135	101	180	128	96	126	168	104	99	156	183	123	238
T. dalmatica (*n* = 4)	—	131–139	123 (1)	181–186	137–157	96 (1)	129 (1)	165 (1)	104	96 (2)	150–159 (2)	198–208	126–189	229–243 (3)
T. hampeana (*n* = 3)	—	131–139	104–125	180–195	128–135	93–96	129–135	174–192 (2)	104	93–99	—	110–209	123	—
T. hispida (*n* = 1)	—	131–135	109	189	143–146	96	129–132	167	104	96	150	107	123	239
T. hohenackeri (*n* = 2)	117–129 (1)	131	117–134	183–195	126–129	—	129–132	171–180	104	99–126	150 (1)	110–113	123	232–241
T. leptostachya (*n* = 1)	—	131	123–127	186	135–142	—	129	168–174	104	90–99	150	—	123	234–245
T. minoa (*n* = 3)	—	131–139	119–127	186–189	137–157	93–96	129–132	192–195	104	—	150–153	110–208	123–126	235–260
T. nilotica (*n* = 6)	—	131	115	189	128	93	129	171	104	109	153–159	110	126–129	240–267
T. parviflora (*n* = 3)	160 (2)	131–149	123–133	189	128–144	93	129	177	104	96	153–165	110–208	123	232–236
T. ramosissima (*n* = 1)	—	131	124	180	128	—	126	168	104	90	—	208	123	238
T. smyrnensis (*n* = 2)	—	131–149	113–123	180–189	129	—	129–132	171–183	104	99 (1)	150 (1)	208	123 (1)	234–236
T. tetragyna (*n* = 3)	—	129–133	113–127	183–195	137	96	129–132	174–177 (2)	104	91–99 (2)	150–162	110	126	235–243
T. usneoides (*n* = 3)	—	135–137	—	183–189	137–140	93	—	153–165 (2)	104	—	162–168	208	141	—

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.

LITERATURE CITED

Bao, W., K. K. Kojima, and O. Kohany. 2015. Repbase Update, a database of repetitive elements in eukaryotic genomes. *Mobile DNA* 6(1): 11.

Baum, B. R. 1978. The genus *Tamarix*. The Israel Academy of Sciences and Humanities, Jerusalem, Israel.

Csiba, L., and M. P. Powell. 2006. Appendix 1: Isolation of total plant cellular DNA for long-term storage: CTAB procedure. *In* V. Savolainen, M. P. Powell, K. Davis, G. Reeves, and A. Corthals [eds.], DNA and tissue banking for biodiversity and conservation: Theory, practice and uses, 114–117. Royal Botanic Gardens Kew, Richmond, Surrey, United Kingdom.

Gaskin, J. F., A. E. Pepper, and J. R. Manhart. 2006. Isolation and characterization of 10 polymorphic microsatellites in saltcedars (*Tamarix chinensis* and *Tamarix ramosissima*). *Molecular Ecology Notes* 6(4): 1147–1149.

Guichoux, E., L. Lagache, S. Wagner, P. Chaumeil, P. Léger, O. Lepais, C. Lepoittevin, et al. 2011. Current trends in microsatellite genotyping. *Molecular Ecology Resources* 11(4): 591–611.

Malausa, T., A. Gilles, E. Meglécz, H. Blanquart, S. Duthoy, C. Costedoat, V. Dubut, et al. 2011. High-throughput microsatellite isolation through 454 GS-FLX Titanium pyrosequencing of enriched DNA libraries. *Molecular Ecology Resources* 11(4): 638–644.

Meglécz, E., N. Pech, A. Gilles, V. Dubut, P. Hingamp, A. Trilles, R. Grenier, and J. F. Martin. 2014. QDD version 3.1: A user-friendly computer program for microsatellite selection and primer design revisited: experimental validation of variables determining genotyping success rate. *Molecular Ecology Resources* 14(6): 1302–1313.

van Oosterhout, C., W. F. Hutchinson, D. P. Wills, and P. Shipley. 2004. MICRO-CHECKER: Software for identifying and correcting genotyping errors in microsatellite data. *Molecular Ecology Notes* 4(3): 535–538.

Peakall, R. O. D., and P. E. Smouse. 2006. GenAlEx 6: Genetic analysis in Excel. Population genetic software for teaching and research. *Molecular Ecology Notes* 6(1): 288–295.

Rousset, F. 2008. GENEPOP'007: A complete re-implementation of the GENEPOP software for Windows and Linux. *Molecular Ecology Resources* 8(1): 103–106.

Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology* 18(2): 233–234.

Smit, A. F. A., R. Hubley, and P. Green. 2015. RepeatMasker Open-4.0. Website http://www.repeatmasker.org [accessed 20 December 2019].

Terzoli, S., I. Beritognolo, M. Sabatti, and E. Kuzminsky. 2010. Development of a novel set of EST-SSR markers and cross-species amplification in *Tamarix africana* (Tamaricaceae). *American Journal of Botany* 97(6): e45–e47.

Terzoli, S., E. Cattan, M. Sabatti, R. Valentini, A. Zilberstain, and E. Kuzminsky. 2013. Primer Note: A novel set of EST-SSR markers in *Tamarix*: A resource to characterize this genus. *Silvae Genetica* 62(3): 104–109.

Villar, J. L., M. Á. Alonso, A. Juan, J. F. Gaskin, and M. B. Crespo. 2019. Out of the Middle East: New phylogenetic insights in the genus *Tamarix* (Tamaricaceae). *Journal of Systematics and Evolution* 57(5): 488–507.

Zhang, R., Q. Wen, and L. Xu. 2019. Development and characterization of genomic SSR markers for *Tamarix chinensis* (Tamaricaceae). *Applications in Plant Sciences* 7(2): e1219.

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

Species	Voucher specimen accession no.[a]	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, Castellón, Burriana, Clot de la Mare de Déu	39.879, −0.055	12
	ABH 70742	Spain, Murcia, Águilas, Rambla de Minglano de Cañarete	37.433, −1.629	2
T. amplexicaulis Ehrenb.	ABH 70685	Algeria, Biskra, N3 crossing with Oumache, Km 336	34.719, 5.739	4
T. aphylla (L.) H. Karst	ABH 70064	Italy, Sardinia, Oristano, Cabras, Is Aruttas	39.954, 8.403	1
	ABH 71909	Morocco, Nador, Berkane, Oued Moulouya	35.103, −2.360	1
	ABH 54208	Morocco, Nador, Driouch	34.972, −3.360	1
T. arceuthoides Bunge	MO 5568719	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, Almería, Cabo de Gata	36.773, −2.238	12
T. canariensis Willd.	ABH 69606	Spain, Canary Islands, Gran Canaria, beach of La Aldea 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, 19.846	1
	ABH 57830	Albania, Vlore, Sarande, Vrion, 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 Simbirizzi	39.2631, 9.2086	30
	ABH 69543	Spain, Alicante, Elche, Pantano de Elche	38.3174, −0.718	30
	ABH 67467	Spain, Almería, Vera, río Antas	37.2054, −1.8291	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

(Continues)

APPENDIX 1. (Continued)

Species	Voucher specimen accession no.[a]	Collection locality	Geographic coordinates (WGS84)	*N*
	ABH 59025	Greece, Epirus, Igoumenitsa, Marshes at NW of Igoumenitsa	39.525, 20.198	1
	ABH 57891	Montenegro, Ulcinj, Sveti Nikola, Bojana river	41.870, 19.352	1
T. hispida Willd.	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
	MO 5568696	Iran, Semnan, NE of Sharud toward Gorgon	36.7252, 55.2975	1
T. leptostachya Bunge	Gaskin 10177	China	—	1
T. minoa J. L. Villar, Turland, Juan, Gaskin, M. Á. Alonso & M. B. Crespo	ABH 54194	Greece, Crete, Chania, Georgioupoli	35.365, 24.248	1
	ABH 54195	Greece, Crete, Chania, near Platanias	35.356, 24.260	1
	MO 6207620	Greece, Crete, Nomos Chanion, Eparchia Apokoronou Georgioupoli beach	35.359, 24.266	1
T. nilotica (Ehrenb.) Bunge	ABH 54320	Greece, Crete, Chania, Paleochora beach	35.223, 23.670	1
	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, Biar, Santuario Mare de Déu 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, Swerkobmund	−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.

[a]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).