

Microcnemum coralloides (Chenopodiaceae-Salicornioideae): an example of intraspecific East-West disjunctions in the Mediterranean region

by

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Abstract

Kadereit, G. & A.E. Yaprak. 2008. *Microcnemum coralloides* (Chenopodiaceae-Salicornioideae): an example of intraspecific East-West disjunctions in the Mediterranean region. *Anales Jard. Bot. Madrid* 65(2): 415-426.

Microcnemum is a monotypic genus of Salicornioideae comprising rare, annual, hygrohalophytic herbs growing in hypersaline inland lagoons and salt pans. *Microcnemum coralloides* shows an East-West disjunction in the Mediterranean region: *M. coralloides* subsp. *coralloides* occurs in central and eastern Spain while *M. coralloides* subsp. *anatolicum* grows in Turkey, Syria, Armenia and Iran. We studied the phylogeny, biogeography and morphological differentiation of *M. coralloides*. Molecular analyses, using five western and eight eastern accessions of the species, were based on three different markers (nuclear ITS and plastid *atpB-rbCL* spacer and *trnT/F* region) analysed with Maximum Parsimony and Maximum Likelihood. Estimates of divergence times were calculated using a Likelihood Ratio Test (LRT) and the Penalized Likelihood (PL) method. The two subspecies can be clearly distinguished by their different seed testa surface. Other diagnostic characters were not found. The molecular data (ITS and ML analysis of the *trnT/F* region) indicate that *M. coralloides* subsp. *coralloides* originated from within *M. coralloides* subsp. *anatolicum* which implies an East Mediterranean origin and subsequent westward dispersal. Age estimates for the split of the two subspecies range from 2.8–0.5 million years ago. Considering the relatively low genetic differentiation and the low crown group age (0.7–0.1 mya) of *M. coralloides* subsp. *coralloides* in comparison to *M. coralloides* subsp. *anatolicum* we favour the hypothesis that the Iberian part of the species range was established during cold periods of the Early Pleistocene and that the range of the species was fragmented during a warmer period soon after its arrival in Iberia.

Keywords: Biogeography, disjunction, *Microcnemum*, molecular clock, East Mediterranean, Spain.

Resumen

Kadereit, G. & A.E. Yaprak. 2008. *Microcnemum coralloides* (Chenopodiaceae-Salicornioideae): un ejemplo de las disyunciones intraespecíficas Este-Oeste en la región mediterránea. *Anales Jard. Bot. Madrid* 65(2): 415-426 (en inglés).

Microcnemum es un género monotípico de Salicornioideae que consiste en hierbas higrohalófilas, anuales, raras, que crecen en cuencas endorréicas hipersalinas del interior y salares. *Microcnemum coralloides* muestran una disyunción Este-Oeste en la región mediterránea: *M. coralloides* subsp. *coralloides* aparece en el centro y el levante español, mientras que *M. coralloides* subsp. *anatolicum* crece en Turquía, Siria, Armenia e Irán. Estudiamos la filogenia, la biogeografía y la diferenciación morfológica de *M. coralloides*. Los análisis moleculares, empleando cinco adquisiciones occidentales y ocho orientales de la especie, se basaron en tres marcadores distintos (ITS nuclear, espaciadores plástidos *atpB-rbCL* y región *trnT/F*) analizados con Máxima Parsimonia y Máxima Verosimilitud. Las estimaciones de tiempos divergentes se calcularon empleando una Prueba de Verosimilitud (LRT) y el método de Verosimilitud Penalizada. Las dos subspecies se distinguen claramente por la diferencia en la superficie de su envoltura. No se encontraron otras características de diferenciación. Los datos moleculares (ITS y análisis ML de la región *trnT/F*) indican que *M. coralloides* subsp. *coralloides* originó de dentro de *M. coralloides* subsp. *anatolicum*, lo cual implica un origen en el este del Mediterráneo y su posterior dispersión hacia el oeste. Las estimaciones de edad para la separación de las dos subspecies data desde hace 2,8 a 0,5 millones de años. En vista de la diferenciación genética, relativamente baja y la reducida edad del grupo terminal (0,7-0,1 millones de años) de *M. coralloides* subsp. *coralloides* en comparación con *M. coralloides* subsp. *anatolicum*, favorecemos la hipótesis de que la parte ibérica de la gama de la especie se estableció durante periodos fríos del Bajo Pleistoceno y que la gama de la especie se fragmentó durante un periodo más cálido no mucho después de su llegada a la Península.

Palabras clave: Biogeografía, disyunción, *Microcnemum*, reloj molecular, Mediterráneo oriental, España.

Introduction

Plant species that show an extreme East-West disjunction in the Mediterranean region have been noticed for a long time (Engler, 1879; Willkomm, 1896; Braun-Blanquet & Bolòs, 1957; Davis & Hedge, 1971). More recently, also some examples of disjunctly distributed lichens were reported in the literature (Barreno, 1991; Egea & Alonso, 1996). Additional striking examples come from studies of cave-dwelling Crustaceae (Brehm, 1947), freshwater zooplankton (Miracle, 1982), Coleoptera (Sanmartin, 2003) and from a detailed survey of the insect fauna of the central Monegros region of Spain which listed 62 species showing a disjunct distribution between NE Spain and the steppes in the eastern Mediterranean area or Central Asia (Ribera & Blasco-Zumeta, 1998). Although molecular and analytical tools are now available to study the origin and age of disjunct distribution patterns, the list of molecular studies which focus on extreme disjunctions of plant species in the Mediterranean is relatively short (but see: Castro & al., 2002; Carine & al. 2004; Rosselló & al., 2007; Thompson, 1999).

Three main explanations have been put forward for extreme East-West disjunctions in the Mediterranean region: 1. The extant populations have been interpreted as relict populations of a formerly (pre-Pleistocene) continuous or at least wider distribution area, and probably survived in isoclimatic areas of the Mediterranean (Taberlet & al., 1998) from where some of them again expanded. 2. The extant populations in one part of the disjunct distribution area are the result of colonization through long distance dispersal. The direction of dispersal is usually inferred from the asymmetrical distribution of the specific taxon with the newly colonized area having a smaller expansion (Davis & Hedge, 1971; Ribera & Blasco-Zumeta, 1998; Thompson, 1999). 3. Some taxa are interpreted to be early and undocumented introductions by man (Willkomm, 1896).

Microcnemum is a monotypic genus of Chenopodiaceae subf. Salicornioideae that shows an extreme East-West disjunction in the Mediterranean region. Like most members of Salicornioideae it grows in hygrohalophytic habitats and has succulent, apparently leafless, articulated stems and spike-like inflorescences. Among salicornioidean genera, *Microcnemum* is characterised by comprising small annual herbs. The bracts subtending three flowers that are free from each other are connate and form a cup-shaped structure. The perianth is reduced to a tiny membranous lobe and the seeds are ovoid, flattened and have a granulose or papillose surface (Aellen, 1967; Molero, 1986, 2000). Of these characters only the minute peri-

anth rudiment which disappears in the fruiting stage represents an autapomorphy and is unique in Salicornioideae (Kadereit & al., 2006).

Microcnemum occurs along the shores of flat, stepic lagoons in extremely saline sites. It often grows on thick salt crusts together with a few other species that are salt-tolerant and able to cope with extreme hypersaline conditions during the dry season. Associated with *Microcnemum coralloides* in Turkey are, for example, *Kalidium foliatum* Moq., *K. wagenitzii* (Aellen) Freitag & G. Kadereit, *Halocnemum strobilaceum* M. Bieb., *Halimione verrucifera* (M. Bieb.) Aellen and *Salicornia* L. spp. (all Chenopodiaceae), while *M. coralloides* in Spain often is associated with *Halopeplis amplexicaulis* Ung.-Sternb., *Halocnemum strobilaceum* M. Bieb., *Arthrocnemum macrostachyum* (Moric.) K. Koch and several species of *Sarcocornia* A.J. Scott and *Salicornia* (also all Chenopodiaceae). The presence of *Microcnemum* seems to fluctuate from year to year probably in response to seasonal fluctuations in rainfall (E. Yaprak, pers. obs.; B.M. Crespo, pers. comm.).

Microcnemum coralloides comprises two subspecies. *Microcnemum coralloides* subsp. *coralloides* is restricted to inland salt lakes or salt pans in central and eastern Spain (Molero, 1986; Blanché & Molero, 1990). For some time after its discovery in 1959, *M. coralloides* subsp. *anatolicum* was only known from the type locality or nearby (Aellen, 1967). Later it was also recorded from central and NW Iran, the Syrian Desert, Armenia and several new localities in Turkey (Mouterde, 1966; Botschantzev & Barsegjan, 1972; Gabrielian, 1981; Akhiani, 1988; Akhiani & Ghorbani, 1993; Hamzaoglu & al., 2005; Yaprak, 2008). Apart from their disjunct distribution, the two subspecies differ only in the structure of the seed testa, which is densely and distinctly papillose in *M. coralloides* subsp. *anatolicum* (Wagenitz, 1959) but only granulose or slightly papillose in *M. coralloides* subsp. *coralloides* (compare Figs. 1-4 in Molero, 1986). Other differences between the two subspecies have never been identified. The type collection of *M. coralloides* subsp. *anatolicum*, however, was incomplete because the plant was collected in the fruiting stage only (Wagenitz, 1959).

A recent molecular systematic study of subf. Salicornioideae demonstrated that, based on nuclear DNA (ITS), *Microcnemum* is most closely related to *Arthrocnemum* Moq. (Kadereit & al., 2006). *Arthrocnemum* contains low shrubs that differ from *Microcnemum* in several characters (e.g., growth form, number of stamens) but also shows some similarities (e.g., shape of the connate bracts, free flowers, membranous perianth; Kadereit & al., 2006). It is a ditypic genus that is distributed in inland and coastal halo-

phytic habitats throughout the Mediterranean area, SW Asia and North America. Statistical support for the sister group relationship of these two genera was low, and the topology of chloroplast DNA based trees (*atpB-rbcL* spacer; Kadereit & al., 2006) suggests that the closest living relatives of *Microcnemum* are members either of the *Salicornia/Sarcocornia* lineage or of the Australian *Salicornieae*. However, the branches of these four lineages (*Arthrocnemum*, *Microcnemum*, *Salicornia/Sarcocornia* lineage, Australian *Salicornieae*) are long and diverged more or less simultaneously in the Middle Miocene (19.6-14.6 mya; Kadereit & al., 2006).

The finding that the two subspecies of *Microcnemum* represent a lineage that originated during the Middle Miocene, show an extremely disjunct distribution but, at the same time, are morphologically very similar, leads to the following questions: 1. Are the two subspecies supported by molecular evidence? 2. Does flower morphology provide additional characters to distinguish the two subspecies? 3. When did the two subspecies separate? 4. Is the distribution found today the result of recent dispersal or is it better explained as a relict of a formerly wider distribution area?

To address these questions we sequenced three different markers from the chloroplast and nuclear genome for five accessions of *M. coralloides* subsp. *coralloides* and eight accessions of *M. coralloides* subsp. *anatolicum*. Where a likelihood ratio test (LRT) rejected a model of strict clock-like evolution, we applied a Penalized Likelihood (PL) approach to obtain divergence dates of interest. Additionally, we studied the morphology of the poorly known and rare *M. coralloides* subsp. *anatolicum*.

Material and methods

Plant material for molecular and morphological studies.—The plant material included in the molecular study is listed in Table 1 and the accessions of *Microcnemum* included in the molecular analyses are mapped in Fig. 1. Altogether 13 accessions of *Microcnemum* were sampled for ITS and the *atpB-rbcL* spacer, five from Spain, six from Turkey, one from Iran and one from Armenia. For the *trnT/F* region, one Spanish *Microcnemum* sample and the Armenian sample failed to amplify and for two samples only a partial sequence was obtained (compare Table 1). Additionally, two accessions of *Arthrocnemum macrostachyum*, *Halosarcia indica* (Willd.) Paul G. Wilson, *Sarcocornia utahensis* (Tidest.) A.J. Scott and *Halopeplis amplexicaulis* were included in all analyses. According to the results of Kadereit & al. (2006), *Halopeplis* served as outgroup in this study. The other lineages included in the analysis are most closely related to *Microcnemum*.

The material of *M. coralloides* subsp. *anatolicum* listed in Table 1 was also used for morphological examination.

DNA isolation, amplification, and sequencing.—Total genomic DNA was either isolated from silica gel dried stems or young herbarium material using 20-50 mg. For DNA extraction the NucleoSpin plant DNA extraction kit (Macherey-Nagel) was used following the manufacturer's specifications.

Standard PCR amplification was performed using standard reaction mixes (e.g., Kadereit & al., 2003) including 0.25-1% DMSO and c.1 ng/μl DNA with a Grant Autogene II-Thermocycler programmed as described in Kadereit & al. (2005). Amplification products were checked on 0.8% agarose gels. PCR pro-

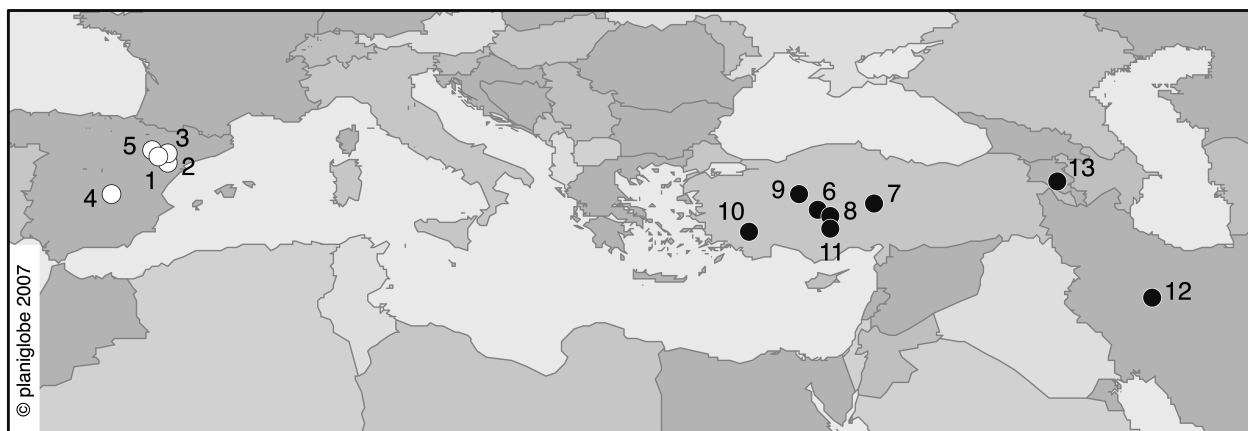


Fig. 1. Localities of *Microcnemum coralloides* included in the molecular analyses (numbers refer to population numbers listed in Table 1). *Microcnemum coralloides* subsp. *coralloides* shows a scattered distribution in central and eastern Spain (compare also <http://www.programanthos.org>) and *M. coralloides* subsp. *anatolicum* has been recorded from a few localities in Turkey, Syria, Armenia and central, eastern and northwestern Iran.

Table 1. Taxa sampled, vouchers, markers and their GenBank accession numbers (newly generated sequences in **bold** face, others taken from *Schütze & al., 2003; ** Kadereit & al., 2005, and ***Kadereit & al., 2006).

Taxon	Lab.No. (sample)	voucher	ITS	atpB-rbcl spacer	trn T/L/F region
<i>Halopeplis amplexicaulis</i> Ung.-Stemb. ex Ces., Passer & Gibelli	2002/14	Spain, Aragón, Laguna de Guallar, G. Kadereit et al. 2002/14 (MIG)	AY489237**	DQ340093***	EU544010
<i>Halosarcia indica</i> (Willd.) Paul G. Wilson	chen 262	subsp. <i>julacea</i> Paul G. Wilson: Australia, New South Wales, S. Jacobs 8691 (NSW)	AY489243** (subsp. <i>julacea</i>)	DQ340103*** (subsp. <i>bidens</i>)	EU544011 (subsp. <i>bidens</i>)
<i>Sarcocornia utahensis</i> (Tidestr.) A. J. Scott	chen 558	subsp. <i>bidens</i> (Nees) Paul G. Wilson: Western Australia, Mortlock River, S. Jacobs 9135 (NSW)	DQ340173***	DQ340129***	EU544012
<i>Arthrocnemum macrostachyum</i> (Morici.) K. Koch	chen 353	USA, Utah, Utah County, E. H. Zacharias 855 (UC)	AY489240**	DQ340101***	EU544014
<i>Microcnemum coralloides</i> (Loscos & Pardo) Buen subsp. <i>coralloides</i>	2002/7	Turkey, Seyhan Prov., SE Adana, H. Freitag & M. Adigüzel 28846 (KAS, GAZI); coord.: 36°58'N, 35°15' E	AY489239**	EU484419	EU544013
	2002/15 (1)	Spain, Tarragona Prov., Delta del Ebro, G. Kadereit 2002/7 (MIG) coord.: 40°42' N, 0°43' E	AY489245**	DQ340106***	no results obtained
	chen 642 (2)	Spain, Aragón, Acaniz, Laguna de Guallar, G. Kadereit 2002/15 (MIG); coord.: 41°02'56" N, 0°07'50" W			
	chen 1344 (3)	Spain: Zaragoza, Huesca, Penalba, Amariga Baja, 356 m, G. Sanz & J. Cervantes s.n. (01.09.2002; Yaprak 120) (ANK); coord.: 41°30' N, 0°02' W	EU544000	EU484420	EU544015
	chen 1345 (4)	Spain, Azaila, Laguna de la Salada, M.B. Crespo & E. Camuñas 35749 (ABH); coord.: 41°17' N, 0°29' W	EU544001	EU484421	EU544016 (412 bp missing)
	chen 1460 (5)	Spain, Mota del Cuervo, Laguna de Manjavacas, M.B. Crespo 16115 (ABH); coord.: 39°30' N, 2°52' W	EU544002	EU484422	EU544017 (294 bp missing)
	chen 1354 (6)	Spain, Zaragoza, Velado de Peñafior, P. Catalán s.n., 22.6.2007 (MIG); coord.: 41°39' N, 0° 52' W	EU544003	EU484423	EU544018
<i>Microcnemum coralloides</i> (Loscos & Pardo) Buen subsp. <i>anatolicum</i> Wagenitz	chen 607 (7)	Turkey, Konya Prov., Cihanbeyli, Vural 7558 (GAZI, KAS); coord.: 38°39' N, 32°55' E	AY181876*	AY181811*	EU544019
	chen 608 (8)	Turkey, Kayseri, Büyüktuzhisar, Tuzla Lake, South shore of the lake, 1124 m, A.E. Yaprak 2004- 251 (=Yaprak 31) (ANK); coord.: 38°59' N, 35°48' E	EU544004	EU484424	EU544020
	chen 609 (9)	Turkey, Aksaray, Eskil to Yenikent, 904 m, A.E. Yaprak 2004- 268 (Yaprak 43) (ANK); coord.: 38°25' N, 33°29' E	EU544005	EU484425	EU544021
	chen 682 (10)	Turkey, Konya prov., 8 km NE Karapınar; dry saltmarshes "Sultaniye Sazıslı", c. 900 m; H. Freitag 28.736 (KAS); coord.: 37°49' N; 29°44' E	EU544006	EU484426	EU544022
	chen 734 (11)	Turkey, Konya prov., 8 km NE Karapınar; dry saltmarshes "Sultaniye Sazıslı", c. 1000 m; H. Freitag 28.777 (KAS); coord.: 37°43' N, 33°33' E	EU544007	EU484427	EU544023
	chen 1709 (12)	Turkey, Eskişehir, Günyüzü, Kavuncu Village, 707 m, A.E. Yaprak 2006-7 (Yaprak 51) (ANK); coord.: 39°25' N; 31°57' E	EU544008	EU484428	EU544024
	chen 1788 (13)*	Iran, Arak, N of Kavir-e Migham, near Deh-Namak; near the margin of Arak salt lake; H. Akhiani 1112 (TARI); coord.: 34.23° N, 49.869° E	EU544009	EU484429	EU544025
		Armenia, S of Yerevan; J. Akopian s.n. 18.09.1976 (ERE); coord.: c. 40° N, 44° E (see "Red data book of Armenian SSR: rare and endangered species of plants", 1988, p. 96-97)	only 300 bp obtained, seq. identical with (12)	seq. complete, seq. identical with (12)	no results obtained

*The Armenian sample arrived only after the completion of the analyses. The obtained sequence fragments (together c. 1000bp), however, are identical with the sequences of the Iranian sample.

ducts were directly purified with the PCR product purification kit (Macherey-Nagel). PCR primer sequences used for ITS and the *atpB-rbcL* spacer can be found in Kadereit & al. (2006). The *trnT/F* region was amplified in three fragments (primer sequences after Taberlet & al. (1991) if not indicated otherwise): 1. *trnT-f* (= *ucp-a*, forward) and *trnL-b* (modified for this study, reverse): 5' AAT TCC AGG GTT TCT CTG AAT TTG -3'; 2. *ucp-c* (forward) and *ucp-d* (reverse); 3. *trnL-460* (after Worberg & al., 2007) and *trn-F* (= *ucp-f*, reverse).

The purified PCR products were sequenced using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer) and five-fold diluted PCR primers as sequencing primers. For cycle sequencing, thermocyclers were programmed as described in Kadereit & al. (2005). Extension products were sequenced on ABI 373 or 377 automated sequencers.

Alignment.—Forward and reverse sequences were edited and assembled to consensus sequences which then were aligned using Sequencher™ 4.1. The alignments of all markers were straightforward and required little manual correction. Indels were unambiguous and coded in a 0/1 matrix for all markers (compare Table 2). Mononucleotide microsatellites in the cp markers were excluded from the analyses.

Maximum Parsimony analyses.—The matrices were analysed under maximum parsimony (MP) criteria separately and combined. A partition homogeneity

test (Farris & al., 1994; implemented in PAUP* 4.0b10) was conducted with 1000 homogeneity replicates and the same heuristic search conditions as described below. All analyses (markers separately and combined) included heuristic searches with 1000 replicates of random taxon addition and tree-bisection-reconnection (TBR) branch swapping. MP Bootstrap was performed under the same conditions as the heuristic search with 1000 replicates of 100 random taxon additions.

Maximum Likelihood analyses.—Maximum likelihood (ML) analyses were conducted for all three data sets and the combined data set using the best-fit substitution model determined by Modeltest 3.06 (Posada & Crandall, 1998). Maximum likelihood searches were performed with the settings outlined in Table 3. The heuristic search was performed with TBR branch swapping and 100 random sequence additions. Bootstrap support values were calculated from 100 replicates of heuristic searches again using TBR swapping and 100 random additions of sequences.

Molecular clock analysis.—Age estimates for Salicornioideae published in Kadereit & al. (2006) were used to calibrate the basal node as indicated in Fig. 2. The age estimates calculated in Kadereit & al. (2006) used *Salicornites massalongoi* from Oligocene deposits (35.4-23.3 Mya, Chiavù, Italy; Principi, 1926) as a fossil calibration point and was based on a broad sample of Salicornioideae. Because the study of Kadereit & al. (2006) was based on ITS and the *atpB-rbcL* spacer, the age estimates obtained from these two markers were used for the corresponding data sets here. For the calibration of the *trnT/F* tree and the tree resulting from the combined analysis the age estimates for ITS and the *atpB-rbcL* spacer in Kadereit & al. (2006) were used in combination (22.2-17.8 mya; compare Fig. 2C,D,G,H).

For the estimation of divergence times we used two different approaches: 1. Likelihood ratio test (LRT; Felsenstein, 1988) and 2. Penalized Likelihood (PL) as implemented in “r8s” (Sanderson, 2002). The latter was conducted twice: 1. based on the tree resulting from the MP analyses and 2. based on the ML trees.

LRT: A likelihood ratio test (Felsenstein, 1988) was conducted for all three data sets and the combined data set. Rate-constancy was tested by calculating log-likelihood scores for trees with and without a molecular clock enforced using PAUP. Non-significance at the 0.01 level between tree topologies, indicating that a molecular clock can not be rejected, was assessed with Modeltest 3.06 (Posada & Crandall, 1998).

PL: The MP and ML tree topologies shown in Fig. 2 (except the ML tree of the *atpB-rbcL* spacer

Table 2. Sequence characteristics of the molecular markers used and tree statistics of the Maximum Parsimony analyses.

Marker	ITS	<i>atpB-rbcL</i> spacer	<i>trnT/F</i> region	combined data
Taxa included <i>Microcnemum</i> /other Salicornioideae	12/5	12/5	11*/5	11*/5
Aligned bp	663	744	1876	3283
Within <i>Microcnemum</i>				
Σ variable sites	10	8	31	49
Indels/ point mutations	1/9	3/5	13/18	17/32
Parsimony informative	4	6	19	29
All taxa analysed				
Σ variable sites	157	52	178	387
Parsimony informative	79	21	78	177
Tree length	207	54	204	473
Number of shortest trees	1	1	21	18
Tree indices	CI 0.9 RI 0.89	CI 0.96 RI 0.97	CI 0.90 RI 0.91	CI 0.89 RI 0.89

*1 No amplification results for sample 2002/15 of *Microcnemum coralloides* subsp. *anatolicum* for the *trnT/F* region, but sample in the ITS data set identical with sample 642.

Table 3. Settings and results of the Maximum Likelihood analyses and results of a Likelihood ratio test (LRT) conducted for all data set and the combined data set (df = degrees of freedom).

Marker	ITS region	<i>atpB-rbcL</i> spacer	<i>trnT/F</i> region	combined data sets
Substitution model	GTR+I	GTR	GTR+G	GTR+I
Nucleotide frequencies	A 0.19 C 0.296 G 0.315 T 0.2	A 0.394 C 0.139 G 0.115 T 0.322	A 0.383 C 0.140 G 0.151 T 0.327	A 0.346 C 0.171 G 0.184 T 0.299
Rate matrix	AC 1.052 AG 6.703 AT 1.748 CG 0.422 CT 2.597 GT 1.0	AC 0.467 AG 0.917 AT 0.082 CG 0.994 CT 0.917 GT 1.0	AC 1.158 AG 0.903 AT 0.203 CG 0.645 CT 0.903 GT 1.0	AC 0.932 AG 01.943 AT 0.251 CG 0.703 CT 1.261 GT 1.0
Fraction invariable sites or gamma distribution	0.6368	0	0.239	0.76
No of trees/ML score	1/1738.89	1/1189.69	1/3363.37	1/6576.38
ML score with clock enforced	1754.714	1200.05	3393.0	6606.53
Results of LRT	df = 15 ratio 31.65 p = 0.0072 not clock-like	df = 15 ratio 20.73 p = 0.146 clock-like	df = 14 ratio 59.34 p < 0.0000001 not clock-like	df = 14 ratio 66.3 p < 0.0000001 not clock-like

which was clock-like) were implemented in the r8s command block together with the following settings: method Penalized Likelihood (PL), algorithm Truncated-Newton (TN) method. For each analysis we performed two runs: 1. a preliminary run estimating the optimal smoothing parameters with cross validation start set to 0, cross validation increase set to 0.2 and number of steps $k = 50$; 2. the second run implemented the optimal smoothing parameters (MP trees: 1 000 000 each for ITS, *atpB-rbcL* spacer and the *trnT/F* region and 4 for the combined analysis; ML tree: 4 for ITS and combined data set and 10 for the *trnT/F* region) and estimated the ages of the nodes using the constraints (minimum age, maximum age) as indicated in Fig. 2 (shaded box).

Confidence intervals were calculated for the age estimates of the combined ML tree using r8s (Sander-son, 2002) and the r8s bootstrap kit (Eriksson, 2002). Relative branch length from 100 bootstrapped trees were transformed into 100 absolute ages. This age distribution was then used to calculate 95% confidence intervals for the crucial nodes in *Microcnemum*.

Results

Morphological data.—Comparison of the morphology of the two subspecies revealed a great overall similarity. After the survey of more material from Turkey (listed in Table 1), the seed testa remains the only unambiguous character to distinguish the two sub-

species (Fig. 3, see also Molero, 1986: Figs. 1-4). The Iranian and Armenian specimens included here also show a distinctly papillose seed testa as typical for *M. coralloides* subsp. *anatolicum*. Whether the populations growing in Syria have papillose seeds, too, has not been investigated. A more delicate and less branched habit of the eastern subspecies does not consistently distinguish the two taxa because dwarf forms of *M. coralloides* subsp. *coralloides* can also be observed in Spain (G. Kadereit, per. obs.; Molero, 2000: 535, Fig. 160a). We found that fertile segments of “well-grown” individuals are usually larger (2.5–3.5 mm long) in *M. coralloides* subsp. *coralloides* than in *M. coralloides* subsp. *anatolicum* (1.5–2.2 mm long). Although we also saw flowering material from Turkey, which was unavailable to Wagenitz (1959), we did not find any additional character to delimitate the two subspecies. The number of stamens is one in both. Regarding coloration, we found that although *M. coralloides* subsp. *anatolicum* never turns crimson, coloration is variable and can not be used as a diagnostic character.

Molecular data.—The partition homogeneity test showed a p-value of 0.03 which means that only 3% of the artificial partitions resulted in shorter or equally long trees than the original partitions. Therefore, the three data sets do not show a significantly different phylogenetic signal when a level of 0.01 is used.

Sequence characteristics of the molecular markers used and tree statistics of the Maximum Parsimony

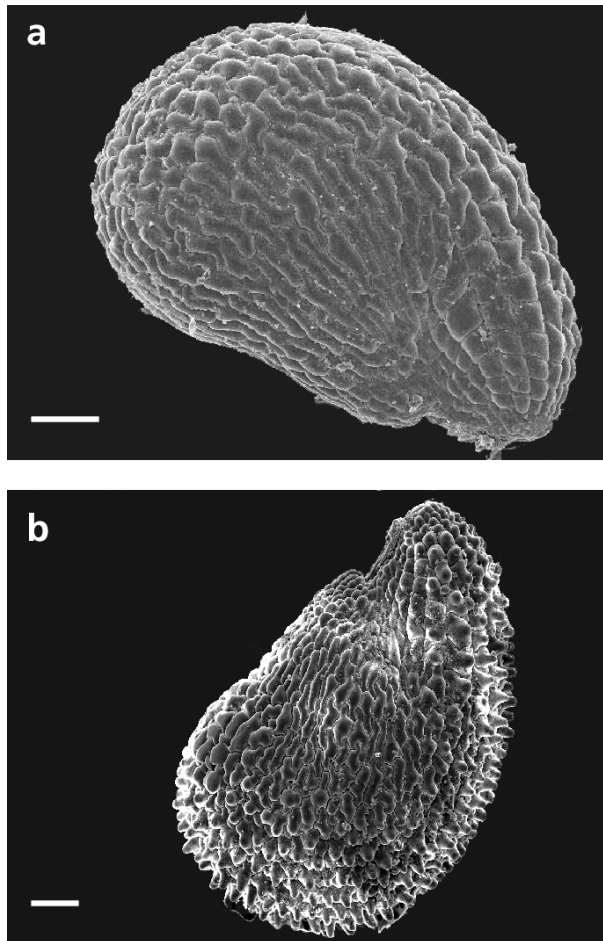


Fig. 3. SEM pictures of seeds of *Microcnemum coralloides* subsp. *coralloides* (**a**, seed taken from sample 2 in Table 1) and *Microcnemum coralloides* subsp. *anatolicum* (**b**, seed taken from sample 8 in Table 1). Scale bars in a and b: 100 μ m.

analyses are summarized in Table 2. The trees are shown in Fig. 2A-D. The ITS and *atpB-rbcL* spacer data sets each resulted in only one shortest tree (Fig. 2A,B), and the analysis of the *trnT/F* region resulted in 21 shortest trees that differed in those clades within the subspecies of *Microcnemum* which did not receive bootstrap support (Fig. 2C). The combined analysis resulted in 18 equally parsimonious tree topologies, and again differences in topology was found in those branches within the subspecies of *Microcnemum* that did not receive bootstrap support (Fig. 2D). The results of the Maximum Likelihood analyses are summarized in Table 3. The trees are shown in Fig. 2E-H.

The main results of the molecular clock analyses are indicated in Fig. 2 and summarized in Tables 3 (LRT) and 4 (age estimates for *Microcnemum* and confidence intervals for age estimates in the combined ML). Only the *atpB-rbcL* spacer data set was clock-

like. Therefore, the PL method was not conducted for the ML tree for this marker. Generally ML tree based age estimates and MP tree based age estimates did not differ strongly except for the *atpB-rbcL* spacer (Fig. 2B,F).

Discussion

Morphological differentiation between the two subspecies of Microcnemum

In 1957 Wagenitz (1959) discovered a Turkish population of *Microcnemum* along the shores of Tuz Gölü (Nigde prov., Turkey). He compared his collections with some Spanish specimens of *Microcnemum coralloides* and found a great overall similarity. The only differences Wagenitz noted were: 1) a more delicate and less branched habit in the Turkish specimens, 2) papillose seeds in the Turkish versus granulate seeds in the Spanish specimens (Fig. 3), and 3) a yellow coloration of the specimens versus crimson or blue-greenish in the Spanish ones. Although Wagenitz collected his material mainly in the fruiting stage, he was able to observe mature anthers which also did not show any differences to the Spanish material. Despite the great similarity of the Turkish and Spanish specimens Wagenitz regarded the differences found sufficient to describe the Turkish material as a new subspecies, *M. coralloides* subsp. *anatolicum*.

The results of our own morphological study of *M. coralloides* subsp. *anatolicum* which included several new populations from Turkey and material from Iran and Armenia (Fig. 1, Table 1) fully support the findings of Wagenitz (1959). We also found great overall morphological similarity of *M. coralloides* subsp. *anatolicum* with *M. coralloides* subsp. *coralloides*, including flower morphology. The seed testa is the only consistent character to distinguish them (Fig. 3; Molero, 1986: Figs. 1-4). Slight differences in habit (height, succulence and branching) and coloration do not distinguish the two subspecies reliably and are probably plastic and strongly influenced by seasonal fluctuations of rainfall and salinity. Both subspecies of *M. coralloides* show variation, and grow to only very small size under unfavourable conditions.

Molecular phylogeny of Microcnemum

The molecular analyses presented here recover the same conflicting phylogenetic relationships of *Microcnemum* as found by Kadereit & al. (2006). The ITS data set shows a sister group relationship to *Arthrocnemum*, albeit with very low support (MP: BS 57%, ML: BS 52%), while the *atpB-rbcL* spacer data set moderately supports a sister group relationships of *Microcnemum* to a clade comprising *Sarcocornia uta-*

hensis (representing the *Sarcocornia/Salicornia* lineage) and *Halosarcia indica* (representing the Australian *Salicornieae*). The analysis of the *trnT/F* region supports a sister group relationship of *Microcnemum* to *Sarcocornia* (MP: BS 61%; ML: BS 83%) and the two clades are sister to *Halosarcia indica* (MP: BS 78%; ML: BS 96%). In the combined analysis the phylogenetic signal of the *atpB-rbcL* spacer data set prevails (Fig. 2D,H). This conflict in the relationships of *Microcnemum* is smaller than it seems. Bootstrap support is low in all three data sets and *Microcnemum* is best regarded as part of a polytomy including the *Sarcocornia/Salicornia* lineage, *Arthrocnemum* and Australian *Salicornieae*. All four clades originated during the Middle Miocene (Fig. 2) probably in the Tethys area, where three of the four clades are still found today. The molecular data presented here show that *Microcnemum* originated 20.9-13.8 mya (Table 4).

The relationship between the two subspecies of *M. coralloides* is resolved in three different ways (Table 4): 1. They are sister to each other [combined analysis (ML; MP), *trnT/F* region (MP) and *atpB-rbcL* spacer (MP)], 2. *M. coralloides* subsp. *anatolicum* is paraphyletic in relation to *M. coralloides* subsp. *coralloides* [ITS (ML, MP), *trnT/F* region (ML)], 3. relationships are not resolved [*atpB-rbcL* spacer (ML)]. Considerable genetic diversity among the accessions of *M. coralloides* subsp. *coralloides* can only be found in the *trnT/F* region while the accessions of *M. coralloides* subsp. *anatolicum* show genetic diversity in all three markers. Therefore, the crown group age of *M. coralloides* subsp. *anatolicum* is distinctly older (4.1-0.7 mya) than that of *M. coralloides* subsp. *coralloides* (0.7-0.08 mya, Fig. 2, Table 4). Considering the geographical isolation of the two taxa, their molecular differentiation and the presence of at least one distinct diagnostic character, their recognition as subspecies is fully justified.

Biogeography of *Microcnemum*

A major aim of this study was to infer the time of separation between *M. coralloides* subsp. *coralloides* and *M. coralloides* subsp. *anatolicum*. This node received age estimates ranging from 2.8-0.5 mya based on the ML and MP trees and different dating methods (Fig. 2, Table 4). The age of 7.9 mya calculated for the *atpB-rbcL* spacer based on the MP tree (Fig. 2B) is a clear outlier compared to the other estimates obtained and may well have resulted from the low number of characters of this data set (Tables 2, 4). The ITS age estimates are the youngest, and the ML tree dated with the PL method of the *trnT/F* region yielded the oldest estimates. Recent intersubspecific introgress-

sion of ITS as a possible explanation of the young age estimates obtained from this marker seems unlikely in view of the great geographical distance between the two subspecies.

The young age estimate for the ITS data set may also result from an unusual mutation rate. However, comparison with the ITS mutation rates (average: 4.13×10^{-9} subs/site/year; range: $1.72-8.34 \times 10^{-9}$ subs/site/year) found for herbaceous plants (Kay & al., 2006) shows that the estimated rates for all *Microcnemum* samples ($3.0-4.3 \times 10^{-9}$ subs/site/year) lie well within this range and close to the average rate.

Probably the most objective and possible accurate estimate with the present data is that of the combined analysis, which incorporates all characters available and balances marker specific rate variation among clades. In the two combined trees the split is dated to 2.5 (MP) and 1.7 (ML) mya, respectively. In the combined ML analysis the split of the two subspecies is dated to 1.7 mya with the 95% confidence interval ranging from 2.17-1.28 mya (Table 4).

A split between the two subspecies around 2.5-1.5 mya as indicated by the combined analyses would imply a separation shortly before, or at the onset of, the Pleistocene. Like Wagenitz (1959), we therefore exclude recent long distance dispersal or anthropogenic introduction to Spain as explanations for the disjunction of *Microcnemum*. Both explanations can be rejected because of the genetic distinctness of *M. coralloides* subsp. *coralloides* (12 mutations in the combined analysis, Fig. 2D) and its intrasubspecific diversification at least in the *trnT/F* region (Fig. 2C,G).

Microcnemum coralloides shows an extremely scattered distribution in both parts of its range. As outlined in the introduction, the species is restricted to inland lagoons with extreme salinity regimes where it is associated with a few other plant species (mostly *Chenopodiaceae*) able to tolerate hypersaline conditions. The habitat of *Microcnemum* is likely to have been available only in isolated localities throughout the past, although it may have been more widespread during the Messinian crisis of the Late Miocene (Krisman & al., 1999; Duggen & al., 2003; Thompson, 2005) and during cooler and drier periods of the Pleistocene (Shackleton & al., 1984; Lang, 1994; Van Andel & Tzedakis, 1996).

Two explanations can be offered for the disjunct distribution of *Microcnemum*:

1) *Microcnemum* was widely and more or less continuously distributed in the Mediterranean area since the Late Miocene. Such distribution range was fragmented by the onset of the climatic oscillations at the beginning of the Pleistocene. Together with other non frost-tolerant plants *Microcnemum* was restricted to areas with suitable climatic conditions. Such areas ex-

Table 4. Age of *Microcnemum* and dating the split of the two subspecies (in my): **1.** Results of molecular clock analyses [ML clock = age estimates are derived from the clock-enforced ML tree (LRT = Likelihood Ratio Test), PL method = Penalized Likelihood method as implemented in r8s, anat. = *M. coralloides* subsp. *anatolicum*, cora. = *M. coralloides* subsp. *coralloides*]; **2.** Confidence intervals for the combined ML tree (Fig. 2H).

1. Marker	ITS region	<i>atpB-rbcL</i> spacer	<i>trnT/F</i> region	combined data sets
Relationships of the two subspecies of <i>M. coralloides</i> (see also Fig. 2)	<i>M. coralloides</i> subsp. <i>coralloides</i> nested within <i>M. coralloides</i> subsp. <i>anatolicum</i>	Sister group relationship in MP analysis not resolved in ML analysis	Sister group relationship in MP; <i>M. coralloides</i> subsp. <i>coralloides</i> nested within <i>M. coralloides</i> subsp. <i>anatolicum</i> in ML	Sister group relationship
ML clock				
LRT (Table 3) stem age genus stem age subsp. split betw. subsp.	not clock-like	clock-like 18.1-16.8 both 2.2-2.0 2.2-2.0	not clock-like	not clock-like
PL method with ML tree				
stem age genus stem age subsp. split betw. subsp.	18.3 anat. 1.5 cora. 0.8 0.8	*1	16.2 anat. 4.1 cora. 2.3 2.2	20.9 both 1.7 1.7
PL method with MP tree				
stem age genus stem age subsp. split betw. subsp.	16.6 anat. 0.7 cora. 0.5 0.5	18.4 both 7.9 7.9	13.8 both 2.8 2.8	17.2 both 2.5 2.5
2. Confidence Intervals (CI) for the combined ML tree (Fig. 2H)				
<i>Microcnemum</i> stem CI: 19.0-22.5 (mean = 20.8) subsp. <i>coralloides</i> crown CI: 0.33-0.72 (mean = 0.53) subsp. <i>anatolicum</i> crown CI: 0.71-1.26 (mean = 0.98) split of the two subspecies CI: 1.28-2.17 (mean = 1.73)				

*1 PL estimate not needed because LRT was clock-like

isted in southern Spain, southern Sicily, southern Anatolia, SW Asia and N Africa (Van Andel & Tzedakis, 1996). This would imply that extant sites of the species in central and eastern Spain were re-colonized from localities in southern Spain.

2) Alternatively, it seems possible that the Iberian part of the distribution range of *Microcnemum* was established only at the onset of the Pleistocene. In cold periods of the Pleistocene the sea level fell and large coastal plains emerged (Shackleton & al., 1984; Lang, 1994; Van Andel & Tzedakis, 1996). At such times saline lagoons probably were frequent along the lower and shallow coast lines and could have allowed migration of hygrohalophytic taxa such as *Microcnemum*.

Considering the parphyly of *M. coralloides* subsp. *anatolicum* in relation to *M. coralloides* subsp. *coralloides* in the ITS and *trnT/F* trees (Fig. 2A,E,G), the stronger genetic differentiation of subsp. *anatolicum* in comparison to subsp. *coralloides* in all three markers, and the higher crown group age of the former

(Fig. 2), we prefer the second of the above two hypotheses and postulate that the Iberian part of the range of *M. coralloides* was established at the onset of the Pleistocene. Such explanation requires that although conditions during cold parts of the early Pleistocene allowed migration over long distances, gene flow between different parts of the distribution range was interrupted soon after the arrival of the species in Iberia. The alternative explanation, Pleistocene fragmentation of a wider distribution area, fails to explain the asymmetrical distribution of genetic diversity seen today, unless additional factors such as population bottlenecks are advocated.

Although *Microcnemum* is a rather extreme example of an intraspecific East-West disjunction in the Mediterranean region, it is not a singular case. More than 70 examples in c. 25 different plant families can be found in the literature (Engler, 1879: 53-57; Willkomm 1896: 103-104; Tab. 1 "oriental plants"; Davis & Hedge, 1971; Greuter & al., 1984, 1986, 1989).

However, only few examples have been analysed

with molecular tools. Sanmartin (2003) concluded that the Pachydeminae (Coleoptera, Scarabaeoidea), a group of beetles disjunctly distributed in the East and West Mediterranean area, originated in the south-east Mediterranean region and initially diversified in the Middle East and Iran-Afghanistan region. The presence of the group in the Iberian Peninsula was interpreted as a result of migration through North Africa and Gibraltar during the Late Miocene. A molecular study of *Buxus balearica* Lam. (Rosselló & al., 2007) using ITS sequence data found a clear geographical split between western and eastern accessions of the species. The molecular data and the ecology of *B. balearica* as a non cold-tolerant shrub restricted to mesic environments support the hypothesis that the few extant populations in Anatolia (formerly separated as *Buxus longifolia* Boiss.) are relicts of a formerly wider distribution area. Unfortunately, this study does not estimate the date of the split between western and eastern populations.

Acknowledgements

We are grateful to J. Akopian (Yerevan), M. Assadi (Teheran), P. Catalán (Zaragoza), M.B. Crespo (Alicante), H. Freitag (Kassel) D. Gómez (Jaca) and J. Mehregan (Mainz) for supplying the rare and hard to find material of *M. coralloides*. We thank N. Munevver Pinar (Ankara), B. Niethard (Mainz), D. Franke (Mainz) and E. Westberg for various technical help. We thank J.J. Aldasoro (Madrid), F. Blattner (Gatersleben), P. Catalán (Zaragoza), H. Freitag (Kassel), J. W. Kadereit (Mainz), G. Schneeweiss (Vienna) and P. Vargas (Madrid) for critically reading and substantially improving the manuscript. A research visit of A.E. Yaprak to Mainz in 2005 was supported by the DAAD (German Academic Exchange Service).

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Associate Editor: P. Catalán

Received: 4-III-2008

Accepted: 21-VII-2008