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Karyomorphology of two subspecies of *Anthemis maritima* (Asteraceae) from Algeria

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Abstract. The chromosome number and karyomorphology of two subspecies of the *Anthemis maritima* complex collected from two different coastal localities in the Skikda region (northeastern Algeria) are reported in this study: *Anthemis maritima* subsp. *maritima*, which is common throughout the Mediterranean, and *A. maritima* subsp. *bolosii*, a strict Algerian endemic recently rediscovered after 100 years of disappearance. The Feulgen staining method indicated that *Anthemis maritima* subsp. *maritima* is a tetraploid with $2n=4x=36$ chromosomes ($2n=11m+3sm+4st$), and *Anthemis maritima* subsp. *bolosii* is a diploid with $2n=2x=18$ chromosomes ($2n=4m+3sm+2st$). Both taxa have symmetrical karyotypes, 2A and 1A, respectively, according to Stebbins classification. These findings are novel for both the subspecies *Anthemis maritima* subsp. *bolosii* and the Algerian population of *Anthemis maritima* subsp. *maritima*.

Keywords: *Anthemis maritima* subsp. *bolosii*, *Anthemis maritima* subsp. *maritima*, karyotype, chromosome, symmetrical, Skikda.

INTRODUCTION

The Asteraceae is an extremely diverse family of flowering plants, representing a large part of the world's flora with approximately 1,900 genera and 32,000 species (Mandela *et al.* 2019) distributed across 40 tribes (Funk *et al.* 2009). The tribe Anthemideae, which is a member of this family, stands out with 111 genera and nearly 1,800 species, making it one of the most important tribes of the Asteraceae family (Oberprieler *et al.* 2006; Oberprieler *et al.* 2007). Following a thorough revision based on molecular phylogenetic analyses, six new subtribes of the Asteraceae -Anthemideae family were identified, which are: the Anthemidinae, Artemisiinae, Glebionidinae, Handeliinae, Leucantheminae, and Leucanthemopsidinae. Five other monotypic sub-

tribes were also added. As a consequence, this important tribe currently contains 19 subtribes (Oberprieler *et al.* 2022a). One of the most important genera in the Anthemideae tribe and a member of the Anthemidinae subtribe is *Anthemis*, which has 175 species distributed throughout the Mediterranean and Southwest Asia. However, due to the huge micromorphological differentiation of its species and those of related genera, the taxonomy of this genus is often challenging. Hybridization and polyploidy may have played crucial roles in the evolution of this genus, further complicating its taxonomy (Oberprieler 1998; Oberprieler 2001; Lo Presti *et al.* 2010). Phylogenetic analyses based on ITS sequences have improved our understanding of the evolutionary relationships among *Anthemis* species, revealing that some species previously included in the subgenus *Anthemis* were actually more closely related to species in adjacent genera (*Tripleurospermum* and *Nananthea*) than to those in the subgenus *Cota*. This finding resulted to the reclassification of several species, as well as the exclusion of the subgenus *Cota* as a distinct genus (Greuter *et al.* 2003; Oberprieler 2001; Oberprieler *et al.* 2006; Oberprieler *et al.* 2009).

Anthemis appears to be one of the few genera with an ancestral base number of $x=9$, evolved from $x=10$ fairly early in the evolutionary process (Bremer and Humphries 1993). In spite the fact that cytogenetic events such as ascending dysploidy (*Leptinella*, *Artemisia*) and descending dysploidy (*Cotula*, *Artemisia*, *Athanasia*, and *Ursinia*) are common in the Anthemideae tribe, resulting in base number alterations ranging from $x=5$ to $x=11$, 13, and 17, and driving their evolution.

Indeed, all chromosomal counts in *Anthemis* sections indicated a majority of diploid species ($2n=2x=18$) with an $x=9$ base number. Thus, polyploidization is primarily responsible for the evolution in some of its species (Oberprieler 1998; Inceer and Hayirlioglu-Ayaz 2007; Pellicer *et al.* 2007; Javadi *et al.* 2013; Shariat *et al.* 2021). *Hiorthia* is regarded as one of the six sections of the genus that have undergone true evolution due to the occurrence of polyploid species ($2n=4x=36$) (Oberprieler 2001). One of these is the North African *A. maritima*, specifically the subspecies *A. maritima* subsp. *maritima* (Oberprieler 1998).

Despite the revision of (Oberprieler 1998) several taxa of the genus in North Africa are still poorly understood. This concerns not only the morphological aspects but above all the karyological and molecular ones. The study of ploidy levels within the various subspecies allows us to better clarify the phylogenetic relationships between the investigated taxa. Although Algeria holds 20 taxa (12 species and 8 subspecies)

among North African species (Dobignard and Chatelain 2011), but unfortunately, karyological information on these taxa is scarce.

The *Anthemis maritima* complex is present with three subspecies: *A. maritima* subsp. *maritima* and two strictly endemic subspecies to Algeria, *A. maritima* subsp. *bolosii* Benedí et Molero and *A. maritima* subsp. *Pseudopunctata* Oberpr. (Dobignard and Chatelain 2011; Tison and De Foucaul 2014). *A. maritima* subsp. *bolosii* was first distinguished by Benedi Gonzalez and Molero Briones (1990) from *A. maritima* subsp. *maritima* by its more erect stem. Oberprieler (1998) reported the total absence of hairs on the peduncle and involucre of *A. maritima* subsp. *bolosii*, and Sakhraoui *et al.* (2021) added other salient features, namely color and leaf appearance, mode of reproduction, habitat, and aromaticity.

A. maritima subsp. *maritima* occurs on coastal dunes throughout the Mediterranean region (Dobignard and Chatelain 2010–2011; Tison and De Foucaul 2014). In Algeria, the subspecies is given as quite common on the littoral of the small and great Kabylia (Quézel and Santa 1963; Boulemtafes *et al.* 2018). *A. maritima* subsp. *bolosii* is considered rare, particularly since its distribution is very restricted. According to Sakhraoui *et al.* (2021), the plant has been reported only at two coastal localities (Annaba and Skikda) in northeastern Algeria, and the presence of the subspecies has not been recorded for more than a century. The oldest record dates back to 6/23/1920 (specimen: P 03697947). The same author reported the rediscovery of the subspecies in the Stora locality (Skikda region).

A. maritima subsp. *bolosii* is a perennial plant with a woody stump, a hairless stem and pinnate leaves that are sessile, or petiolate. The flowers with ligules and yellow centers are arranged into capitula that range in diameter from 14 to 35 mm and are carried by glabrous peduncles, the plant grows on cliffs and sea rocks (Sakhraoui *et al.* 2021) unlike *A. maritima* subsp. *maritima*, which prefers coastal dunes and sandy beaches (Boulemtafes *et al.* 2018; Sakhraoui *et al.* 2021). This taxon includes aromatic plants of 20 to 70 cm in size with creeping stems rising during flowering and achenes with smooth ribs. The spangles of the receptacle are oblong-lanceolate, and the pinnate leaves are more or less fleshy, weakly to strongly puberulent (Quézel and Santa 1963; Tison and De Foucaul 2014; Sakhraoui *et al.* 2021).

The aim of this contribution is to investigate and characterize, from a karyological point of view, *A. maritima* subsp. *bolosii* in comparison with the geographically closest populations of *A. maritima* subsp. *maritima*. In fact, no cytogenetic investigation of these subspecies from Algeria has been conducted. This study is therefore

Table 1. The location of the two *A. maritima* subspecies.

Subspecies	Locality	Latitudes	Longitudes	Altitude	Month of collection
<i>A. maritima</i> subsp. <i>maritima</i>	Larbi ben M'Hidi	36°53'33"N	7°00'25"E	10 m	November 2022
<i>A. maritima</i> subsp. <i>bolosii</i>	Stora	36°53'11"N	6°53'55"E	4 m	August 2021

the first to report on the chromosome number and karyological features of *A. maritima* subsp. *bolosii* as well.

MATERIALS AND METHODS

The seeds used in this study were collected from wild plants growing in two separate locations in Skikda's region (Table 1). Chromosome observation and detection were achieved by the standard Feulgen staining. Roots of 0.5-1.5 cm in length were obtained from the germinated seeds, then pretreated with 0.03% 8-hydroxyquinoline for 4 h at 4°C before being fixed in a 3 ethanol/1 acetic acid solution for 24 h. Hydrolysis was performed in 1M hydrochloric acid for 5 min at 60°C. Root tips were stained with Schiff's reagent for 2 h before being crushed in a drop of 45% acetic acid between the slide and coverslip. Five metaphase plates for each subspecies were analyzed to obtain the different measurements indicated in this work. The IdeoKar software was used for calculating karyotype parameters (Mirzaghaderia and Marzangib 2015).

RESULTS AND DISCUSSION

The karyomorphological results for *A. maritima* subsp. *maritima* and *A. maritima* subsp. *bolosii* are presented in Tables 2 and 3, respectively. The studied population of *A. maritima* subsp. *maritima* was identified as tetraploid, with a chromosomal number of $2n=36$. The subspecies has a total haploid length of 67.54 μm . The total length of chromosomes ranges from 2.50 μm to 5.14 μm , and the ratio between the long arm and short arm varies from 1.12 to 3.31. The karyotype consists of 18 pairs of chromosomes, of which eleven (1, 2, 3, 4, 5, 6, 8, 10, 12, 15, 16) are metacentric, three (9, 14, 17) are submetacentric, and four (7, 11, 13, 18) are subtelocentric. The chromosomal formula of *A. maritima* subsp. *maritima* is $2n=4x=36=11m+3sm+4st$ (Figure 1).

A. maritima subsp. *bolosii* has nine pairs of chromosomes, four pairs (1, 3, 6, 9) are metacentric, three pairs (2, 4, 7) are submetacentric, and two pairs (5, 8) are subtelocentric. Based on these data, it was determined that this subspecies is diploid, with $x=9$ basic

Table 2. Karyomorphological analysis of *A. maritima* subsp. *maritima*.

N	L (μm)	S (μm)	LT (μm)	AR	RL%	CI	CT
1	3.02±0.11	2.12±0.71	5.14±0.80	1.42	7.59	40.30	m
2	2.90±0.28	1.88±0.16	4.78±0.44	1.54	7.03	39.44	m
3	2.60±0.07	1.98±0.08	4.58±0.14	1.31	6.74	43.32	m
4	2.60±0.10	1.88±0.06	4.48±0.16	1.38	6.59	42.33	m
5	2.18±0.04	1.94±0.08	4.12±0.11	1.12	6.09	47.05	m
6	2.32±0.21	1.74±0.50	4.06±0.70	1.33	6.00	40.33	m
7	2.96±0.16	0.98±0.08	3.94±0.20	3.02	5.83	23.18	st
8	2.42±0.06	1.48±0.08	3.90±0.20	1.63	5.77	37.80	m
9	2.50±0.10	1.34±0.08	3.84±0.12	1.86	5.68	35.22	sm
10	2.28±0.06	1.56±0.10	3.84±0.16	1.46	5.48	40.50	m
11	2.84±0.08	0.88±0.08	3.72±0.15	3.22	5.47	23.86	st
12	2.06±0.13	1.50±0.08	3.56±0.15	1.37	5.24	40.20	m
13	2.50±0.24	0.82±0.08	3.32±0.34	3.04	4.91	24.22	st
14	2.00±0.17	1.18±0.40	3.18±0.57	1.69	4.71	37.30	sm
15	1.80±0.17	1.18±0.36	2.98±0.50	1.52	4.41	39.87	m
16	1.74±0.21	1.08±0.32	2.82±0.52	1.61	4.18	38.57	m
17	1.88±0.11	0.90±0.40	2.78±0.50	2.08	4.12	32.55	sm
18	1.92±0.11	0.58±0.20	2.50±0.30	3.31	3.69	23.36	st

Length of chromosome (L: long arm, S: short arm, LT: total length), AR: Arm ratio, RL: Relative length, CI: Centromeric index, CT: Chromosome type.

Table 3. Karyomorphological analysis of *A. maritima* subsp. *bolosii*.

N	L (μm)	S (μm)	LT (μm)	AR	RL%	CI	CT
1	2.79±0.06	2.39±0.11	5.07±0.17	1.16	14.1	46.07	m
2	2.96±0.16	1.56±0.08	4.52±0.24	1.89	13.0	34.61	sm
3	2.40±0.18	1.64±0.20	4.04±0.39	1.46	11.6	40.67	m
4	2.55±0.18	1.37±0.12	3.92±0.31	1.86	11.2	35.03	sm
5	2.98±0.25	0.82±0.07	3.80±0.33	3.63	10.9	21.65	st
6	2.14±0.21	1.58±0.11	3.72±0.32	1.35	10.7	42.49	m
7	2.28±0.27	1.36±0.08	3.64±0.35	1.67	10.4	37.38	sm
8	2.52±0.10	0.64±0.06	3.16±0.16	3.93	9.11	20.61	st
9	1.80±0.23	1.22±0.13	3.02±0.37	1.47	8.65	40.55	m

Length of chromosome (L: long arm, S: short arm, LT: total length), AR: Arm ratio, RL: Relative length, CI: Centromeric index, CT: Chromosome type.

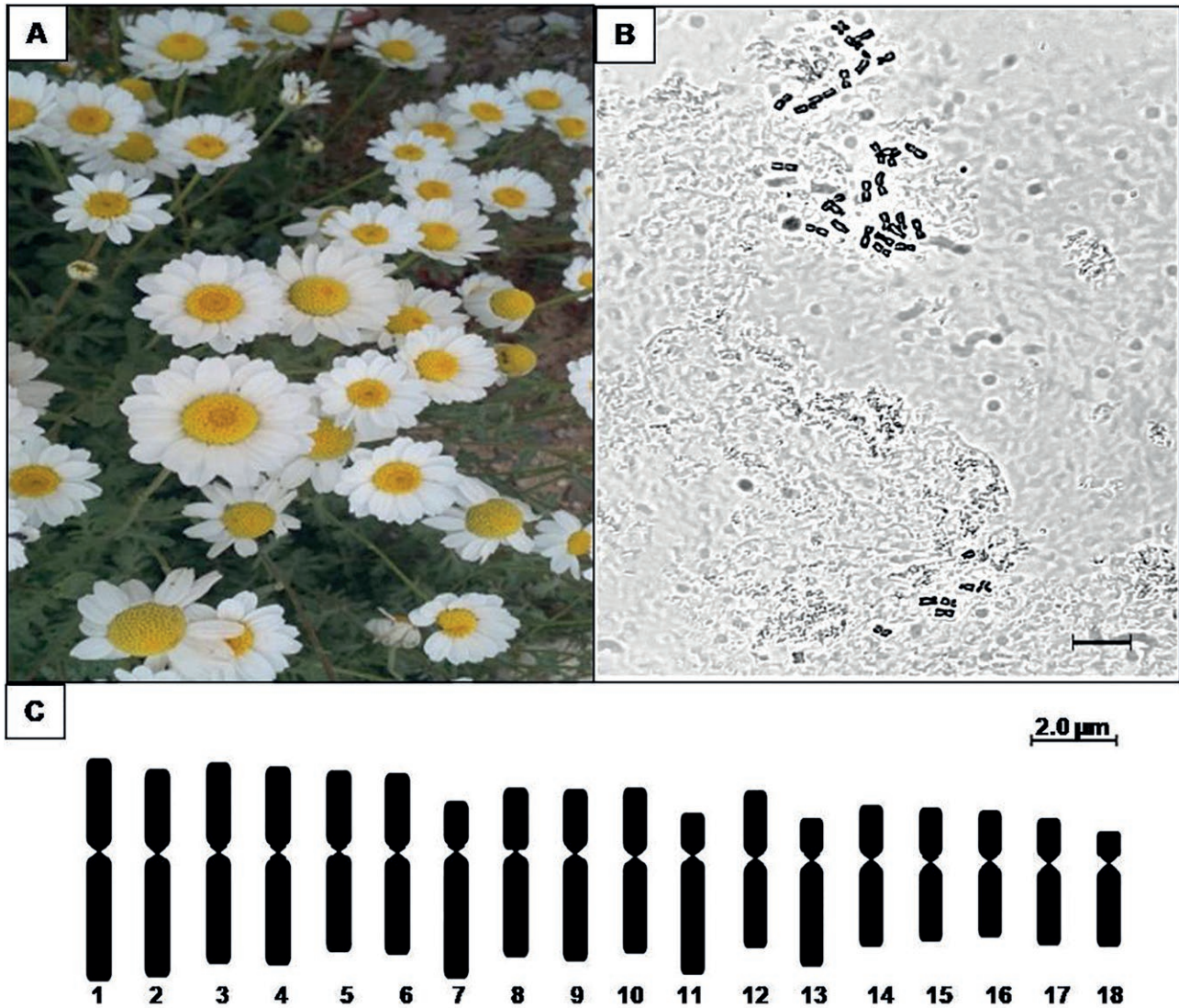


Figure 1. Karyotype of *Anthemis maritima* subsp. *maritima*. A: The plant, B: Metaphase plate, scale bars= 10 μm , C: Idiogram.

chromosomes number and a karyotype formula of $2n=2x=18=4m+3sm+2st$ (Figure 2). The chromosomal lengths range from 3.02 μm to 5.07 μm , the haploid genome is 34.87 μm long, and the long arm to short arm ratio ranges from 1.16 to 3.93. These findings agree with prior research on the basic number $x=9$ and the degree of ploidy.

Among the 28 species of *Anthemis* studied in North Africa (from Morocco and Tunisia), 23 were diploid, and the remaining species were tetraploid ($2n=4x=36$), including *A. maritima* subsp. *maritima* (Tunisian population) (Oberprieler 1998).

All karyotypes were symmetrical, with metacentric, submetacentric, and subtelocentric chromosomes and at least two satellites. In our case, for both subspecies, all

chromosomes were metacentric, submetacentric, and subtelocentric without satellites or secondary constrictions. Generally, species in the genus *Anthemis* contain at least one chromosomal pair bearing a satellite (Oberprieler 1998; Goula *et al.* 2022). The absence of satellites for the two studied taxa could be due to strong chromosomal condensation that prevented their appearance.

The asymmetry index (AsI) for *A. maritima* subsp. *maritima* and *A. maritima* subsp. *bolosii* is 63.40% and 64.02%, respectively. According to Stebbins' classification (1971), both karyotypes are symmetrical 2A and 1A, types respectively. Other research on the karyology of the genus *Anthemis* has already confirmed the dominance of the base $x=9$ (Inceer and Hayirlioglu-Ayaz 2007; Chehregani and Mehanfar 2008; Javadi *et al.* 2013; Qari

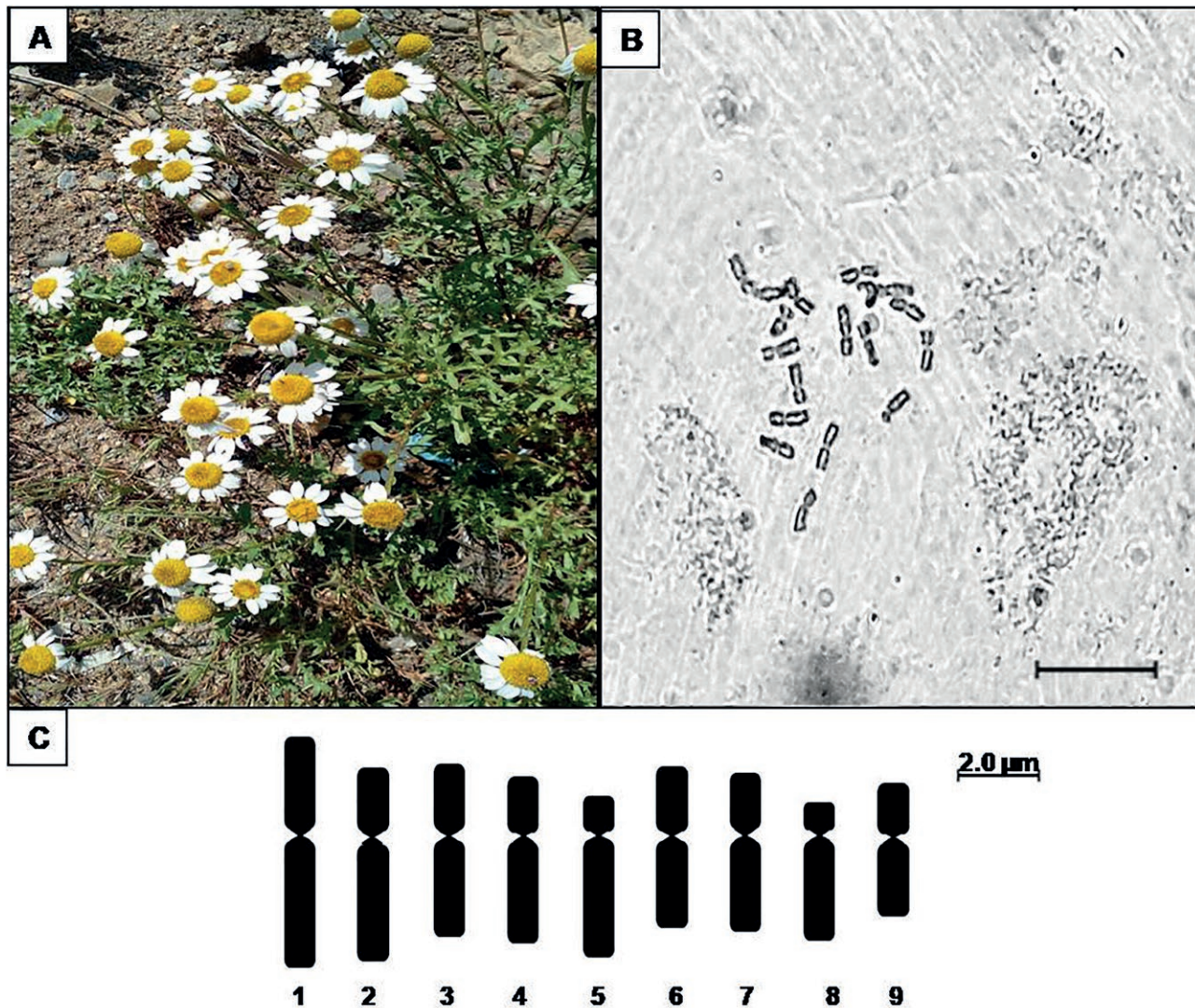


Figure 2. Karyotype of *Anthemis maritima* subsp. *bolosii*. A: The plant, B: Metaphase plate, scale bars= 10 µm, C: Idiogram.

et al. 2016; Shariat *et al.* 2021; Goula *et al.* 2022). The base $x=9$ appears to be constant in the genus *Anthemis*, implying that disploidy, which is often the origin of chromosomal number changes, remains very low.

Several asymmetry indices were calculated to assess the evolutionary linkages between the two subspecies, with values presented in Table 4 for intrachromosomal asymmetry indices (A_1 , TF%, CV_{CI} , and M_{CA}) and interchromosomal indices (A_2 and CV_{CL}). These criteria allowed for a more accurate description of their distinct karyotypes. The intrachromosomal asymmetry indices A_1 , CV_{CI} , and M_{CA} are higher in the diploid subspecies (*A. maritima* subsp. *bolosii*) than in the tetraploid subspecies (*A. maritima* subsp. *maritima*). In contrast, the tetraploid subspecies (*A. maritima* subsp. *maritima*) pos-

sess higher interchromosomal indices A_2 and CV_{CL} than the diploid subspecies (Table 4).

The M_{CA} and CV_{CL} indices are considered reliable quantitative parameters capable of estimating even minor intrachromosomal and interchromosomal variations in the chromosomal complement (Peruzzi *et al.* 2009; Peruzzi and Eroglu 2013; Baeza *et al.* 2015; Genc and Firat 2016; Phukan and Saha 2019; Eroglu and Budak 2020). CV_{CL} and M_{CA} values for *A. maritima* subsp. *maritima* and *A. maritima* subsp. *bolosii* are 19.65, 17.36, 24.3, and 28, respectively. When compared to its tetraploid relative, the diploid subspecies had higher CV_{CI} and M_{CA} values, indicating more variability in centromere location. The tetraploid subspecies, on the other hand, exhibits higher CV_{CL} and A_2 values,

Table 4. Asymmetry indices of *A. maritima* subsp. *maritima* and *A. maritima* subsp. *bolosii*.

Subspecies	HCL	TF%	AsI%	CV _{CL}	CV _{CI}	AI	A ₁	A ₂	M _{CA}	S Cl
<i>A. maritima</i> subsp. <i>maritima</i>	67,54	36,5	63.4	19,65	25,18	4,94	0.41	0.19	24,3	2A
<i>A. maritima</i> subsp. <i>bolosii</i>	34.87	35.97	64.02	17.36	26.56	4,61	0.42	0.17	28	1A

HCL: Haploid total length, TF: Total form percentage (Huziwaru 1962), AsI: Karyotype asymmetry index (Arano and Saito 1980), CV_{CL}: Coefficient of variation of chromosome length, CV_{CI}: Relative variation in centromeric index, AI: Asymmetry index (Paszko 2006), A₁: Intrachromosomal asymmetry, A₂: Interchromosomal asymmetry (Romero Zarco 1986), M_{CA}: Mean centromeric asymmetry (Peruzzi and Eroglu 2013), S Cl: Stebbins classification (Stebbins 1971).

indicating more variation in chromosomal size. Furthermore, the IA index (Paszko 2006), another statistic parameter used to assess karyotype coherence, is higher in the tetraploid subspecies (4.94) than in the diploid subspecies (4.61). A higher IA index value reflects increased chromosomal variability. As a result, it appears that the karyotype of *A. maritima* subsp. *maritima* has evolved more than that of *A. maritima* subsp. *bolosii*. Additionally, Stebbins' (1971) classification classified the karyotypes of the subspecies *A. maritima* subsp. *bolosii* and *A. maritima* subsp. *maritima* in the symmetrical classes 1A and 2A, respectively, indicating some similarity between the two taxa but also a more primitive karyotype of the diploid subspecies compared to the tetraploid one. According to Oberprieler (1998), the karyotypes of *Hiorthia* section species are consistently of type A2, with less variation in intrachromosomal symmetry but higher variation in interchromosomal symmetry. As a consequence, our findings are consistent with previous research, particularly for the tetraploid subspecies. The differences in interchromosomal indices between the two subspecies are quite considerable, as are the differences in intrachromosomal indices, especially M_{CA}, which can be induced by chromosomal mutations (translocations and inversions) resulting in changes between the two taxa. Despite the fact that 82.14% of North African species are diploid (Oberprieler, 1998), practically all species of the section *Hiorthia*, to which the *A. maritima* complex belongs, have been found to be tetraploid (Oberprieler, 1998; Presti *et al.* 2010). Mitsuoka and Ehrendorfer (1972) raised doubts about limiting the *A. maritima* complex to its tetraploid cytotype, suggesting that this plant may also possess a diploid cytotype, like its Moroccan counterpart, the *Anthemis pedunculata* complex.

Thus, our data show unequivocally that the subspecies *A. maritima* subsp. *bolosii* represents the diploid cytotype for the *A. maritima* complex. Furthermore, *A. pedunculata* and *A. maritima*, are the only species that exhibit both diploid and tetraploid cytotypes. Phylogenetic investigations have also shown that the two com-

plexes are closely related (Oberprieler 2001; Presti *et al.* 2010; Oberprieler *et al.* 2022b). The similarity in terms of chromosome size and type between the karyotypes of both subspecies leads us to propose the following hypothesis: the tetraploid cytotype of *A. maritima* may have resulted from either duplication (autopolyploidy) of the diploid cytotype of *A. maritima* subsp. *bolosii* or potentially from an allopolyploidy event involving *A. maritima* subsp. *bolosii* and another related species. Knowing that hybridization between related *Anthemis* species is not new as evidenced by various studies (Uitz 1970; Mitsuoka and Ehrendorfer 1972; Nagl and Ehrendorfer 1974; Oberprieler 1998; Oberprieler 2001; Tison and De Foucaul 2014; Oberprieler *et al.* 2022b). This is also the case for the tetraploid *A. cupaniana*, which is native to Sicily, and has been shown to be the result of allopolyploidization (during the pleistocene). Specifically, this involved a maternal parent from the *A. cretica* group and a paternal parent from the North African complex *A. pedunculata* (*Hiorthia* section), namely the diploid Sicilian *A. ismelia* (Oberprieler *et al.* 2022b).

The comparative palynological study conducted by Oberprieler (1998) suggested that the subspecies *A. maritima* subsp. *bolosii* is the only diploid subspecies (based on pollen size) among the three subspecies in the *A. maritima* complex, and *A. maritima* subsp. *maritima* and *A. maritima* subsp. *pseudopunctata* were identified as tetraploid.

Furthermore, Algeria is the only territory where all three representatives of the *A. maritima* complex have been found. As mentioned above, *A. maritima* subsp. *bolosii* is interesting due to its rarity resulting from its limited distribution along coastal rocks and cliffs. All these information support the hypothesis that *A. maritima* subsp. *bolosii* is the ancestor of the *A. maritima* complex and that Algeria is the center of its diversification, especially since the Algerian population ($2n=4x=36=11m+3sm+4st$) and the Tunisian population ($2n=4x=36=11m+3sm+4st^{sat}$) (Oberprieler 1998) of *A. maritima* subsp. *maritima* appear to be identical from a cytological point of view. According to Greuter (1979),

populations of the same taxon that are geographically separated but have karyotype stability are remnants of an ancient Mediterranean flora present during the Messinian period. Therefore, *A. maritima* complex might represent one of the groups that are ancient polyploids. In contrast to the diploid subspecies, the tetraploid *A. maritima* subsp. *maritima* is found in a number of coastal locations in Algeria (Quézel and Santa 1963; Boulemtafes *et al.* 2018) and across the Mediterranean area (Oberprieler 1998, 2001; Oberprieler *et al.* 2009). According to Winter *et al.* (1999), polyploid species spread more easily than their diploid ancestors, which grow rare and eventually survive only in isolated places shielded from hybridization. Stebbins (1971) asserted that polyploid plants are more adapted to changing environmental circumstances than their diploid ancestors. This adaptation has evolved over millions of years as a result of genetic changes such as mutations and genetic recombination (Adams and Wendel 2005; Alix *et al.* 2017).

Indeed, such a hypothesis requires strong molecular evidence to be confirmed, especially given that the data presented in this study is preliminary and insufficient to prove it.

CONCLUSION

The karyomorphological data of the Algerian population of *A. maritima* subsp. *maritima* and the subspecies *A. maritima* subsp. *bolosii* are reported for the first time and are the only data for the Algerian *Anthemis* taxa. Morphological differences between the two species previously documented by numerous authors are closely related to karyological differences, namely the ploidy level, proportions of each chromosomal type, and chromosomal formula. However, a few similarities between the two subspecies have been identified, including the degree of asymmetry and chromosomal size. As a result, *A. maritima* subsp. *bolosii* might be the origin of the *A. maritima* complex.

Molecular phylogenetic studies need to be considered to confirm this hypothesis and establish the evolutionary links between the subspecies of the *Anthemis maritima* complex in Algeria, and effectively contribute to the understanding of the cytotaxonomy of the *Anthemis* genus in the Mediterranean region. We underline the importance of enacting actual conservation actions to protect the taxa in issue in order to avoid extinction, given their extremely low germinative power, as found during this study.

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