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Phylogenetic placement, floral anatomy, and morphological characterization of the North African pastoral halophyte *Atriplex mollis* Desf. (Amaranthaceae)

Abderrazak TLILI[1], Imed SBISSI[1,*], Fayçal BOUGHALLEB[1], Hassen GOUJA[1], Teresa GARNATJE[2], Joan VALLÈS[3], Mohamed NEFFATI[1]

[1]Arid Lands Institute, Laboratory of Pastoral Ecosystems and Valorization of Spontaneous Plants and Associated Microorganisms, University of Gabes, Medenine, Tunisia

[2]Botanical Institute of Barcelona (IBB, CSIC-ICUB), Barcelona, Spain

[3]Laboratory of Botany, Faculty of Pharmacy and Food Sciences, University of Barcelona, Barcelona, Spain

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Abstract: *Atriplex mollis* Desf. (Amaranthaceae), a North African endemic halophytic species, is further described in this study. Phylogenetic analysis based on a combined dataset of ITS and ETS rDNA and *atp*B-*rbc*L and *trn*K cpDNA showed that *A. mollis* is closely related to the Malta- and Gozo-endemic *Cremnophyton lanfrancoi* Brullo & Pavone. Given this close phylogenetic relationship, *A. mollis* is also considered among the oldest species of *Atriplex*, together with *C. lanfrancoi*. Molecular data also suggest that *A. mollis* in North Africa, *C. lanfrancoi* on Malta Island, and *Atriplex cana* Ledeb. in Eurasian semideserts constitute a separate clade within the tribe Atripliceae. As an 18-month-old shrub, *A. mollis* can reach a mean height of 44.06 ± 8.09 cm with a leaf area around 1.24 ± 0.15 cm^2, and can produce seeds in order of 113.08 ± 28.52 g $plant^{-1}$. The anatomy of *A. mollis* shows the presence of male and female developed flowers. Hermaphroditic flowers that may lead to the appearance of male flowers with underdeveloped female organs were rarely found. Three main shapes of ovule (campylotropous, amphitropous, and orthotropous) were found in *A. mollis*.

Key words: *Atriplex*, *Cremnophyton*, floral anatomy, ITS, ETS, atpB-rbcL, *trn*K, phylogeny, morphology

1. Introduction

The genus *Atriplex* is among 12 genera belonging to the tribe *Atripliceae*. The 11 genera in addition to *Atriplex* are: *Archiatriplex* G. L. Chu, *Axyris* L., *Ceratocarpus* L., *Endolepis* Torrey, *Exomis* Fenzl ex Moq., *Grayia* Hooker & Am., *Krascheninnikovia* Gueldenst., *Microgynoecium* Hooker, *Spinacia* L., *Zuckia* Standley, and *Proatriplex* (Weber) H. C. Stutz & G. L. Chu (Flores and Davis, 2001). A large distribution has been recorded for the genus *Atriplex* around the world. Typically, in subtropical and temperate regions *Atriplex* species can be annual or perennial subshrubs or shrubs growing on steppes and in deserts and coastal habitats (Kadereit et al., 2010). Their basic chromosome number is x = 9 (Nobs, 1975) with a variable ploïdy level, usually diploid (2n = 18), and polyploid only in a few cases.

Cremnophyton lanfrancoi, described by Brullo and Pavone (1987) as a new genus of the Amaranthaceae, was subsequently included in the genus *Atriplex* (synonym: *Atriplex lanfrancoi*), according to Kadereit et al. (2010). This species is the exception of the *Atriplex* genus due to its different chromosome number (2n = 20) (Brullo and Pavone, 1987). *Atriplex confertifolia* (Stutz and Sanderson, 1983), *Atriplex cana* (Sukhorukov, 2006), and *Cremnophyton lanfrancoi* (Brullo and Pavone, 1987) are mentioned as ancient lineages of *Atriplex* and represent the Pleistocene and Oligocene/Miocene, respectively.

Regarding the morphological features, small seeds with bracteoles and flowers usually unisexual are the principal characters of *Atriplex* species (Pottier-Alapetite, 1979; Flores and Davis, 2001). This genus contains about 400 species. Among these, 48 species are Mediterranean and habitually used as a fodder reserve (Ortíz–Dorda et al., 2005). According to Le Houerou (1992), the arid lands include 6 autochthone saltbushes, and the introduced, exotic species are mainly from Australia and America.

Among the native species on the southern border of the Mediterranean basin (North Africa), *Atriplex mollis* Desf. has been reported in Tunisia, Algeria, and Libya as endemic (Greuter et al., 1984) and was later observed in Greece (Crete, Chania, and the Island of Gavdopoula) as a supralittoral shrub (Greuter and Raus, 1999). It has been

* Correspondence: imed.sbissi@ira.agrinet.tn

introduced in Malta (Haslam et al., 1977; Greuter et al., 1984), mistakenly reported in Sardinia (Greuter et al., 1984; Conti et al., 2005), and later corrected in Euro+Med (2006). It is a perennial reaching 50 cm with many branches from the base and not very fleshy leaves exceeding 2 cm (Pottier-Alapetite, 1979). It is a potential fodder plant in periods of scarcity. It can tolerate salinity, in particular by excreting salts through swollen vesicular hairs (Walter and Breckle, 1986). In Tunisia, the range of the species is limited to the south. It grows on the littoral and inland salty soils (Pottier-Alapetite, 1979). It is usually used as a forage species for livestock and wildlife and to rehabilitate degraded and saline areas. Considering the importance of *Atriplex mollis* in enhancing the productivity of salt-affected soils and its resemblance to other *Atriplex* species, this work aims to provide information about morphological and anatomical characters, geographical distribution, and phylogenetic placement of this species using molecular markers.

2. Materials and methods

2.1. Plant material

Fresh leaves and seeds of *A. mollis* were collected from 9 natural populations located in Southern Tunisia (Table 1). Additionally, fresh leaves of *C. lanfranco* from Migra l-Ferha (southwestern coast of Malta) were collected from members of Argotti Botanic Garden and Resource Center in Malta (Gnien tal-Argotti). Sampled leaves were put in wet paper to ensure leaf rehydration and stored in a refrigerator before DNA extraction. Seeds of *A. mollis* were sown in a plot inside the pastoretum of the Arid Regions Institute of Medenine (33°30′01.1″N, 10°38′30.7″E). Flowers and inflorescence branches were collected from this plot for anatomical and architectural pattern study.

2.2. Morphological analysis

Morphological characteristics were studied in 18-month-old plants (n = 36) belonging to the 9 studied populations. Height and minimum and maximum aboveground diameters were measured directly. Leaf area and seed diameter were determined using Image J software (Schneider et al., 2012). Leaf and stem dry matter were measured after drying the fresh material at 105 °C for 24 h. All ripe fruits of each individual were collected and weighted.

Geographical distribution was determined by a random prospection of sites located in the central and the southern areas of Tunisia (Table 1). The phenological stages [vegetative growth (VG), flower bud formation (FBF), flowering (F), fruit growth (FG), and seed dispersal (SD)] were observed weekly during 2017. All measurements were made at the Arid Lands Institute of Medenine experimental plot, which was installed according to randomly complete block design (RCBD). Photos were taken by digital camera, either directly or coupled with a binocular loupe (Figure 1).

2.3. Anatomical study

Floral samples were collected in November 2017 during the period of flower anthesis from the collection plot. The material was immediately fixed in FAA (formaldehyde, glacial acetic acid, 70% ethanol; 5:5:90 by volume) overnight at room temperature and then transferred into 70% ethanol. One hundred flowers were collected randomly from 10 individuals then analyzed anatomically. The material was passed through a tertiary butyl alcohol series (15%–100%) for dehydration and embedded in warm paraffin (56–58 °C). Transverse and longitudinal sections 10 µm thick were obtained using a rotary microtome (Sakura, Japan) and stained with toluidine blue (O'Brien et al., 1964) and/or with safranin and fast green (Zarlavsky,

Table 1. Locations of *Atriplex mollis* populations from Tunisia and *Atriplex lanfrancoi* from Malta.

Accessions	Populations	Locations	Coordinates
Djerba (DJ)	DJ	Djerba Island	33°43′34.1″N, 10°59′02.4″E
ElOuara (OAA)	KET	El Ketf	33°10′44.8″N, 10°29′30.3″E
	OS	Oued Sabeug	33°03′12.5″N, 11°01′19.8″E
	AOR	Aïn Oum Rkhis	32°38′24.3″N, 11°24′33.3″E
Kébili (KEB)	ZIG	Zigzaou	33°43′55.7″N, 9°12′49.6″E
	ECH	Echareb	33°57′05.5″N, 9°06′45.5″E
Tozeur (TOZ)	HAZ	Hazoua	33°40′56.1″N, 9°12′49.1″E
	CH	Chbika	34°10′08.2″N, 7°50′11.7″E
El Mahres-Sfax (MAH)	MAH	El Mahres	34°30′22.7″N, 7°10′34.7″E
Malta (MAL)	MAL	Malta	Argotti Botanic Garden and Resource Center, Malta

2014). All slides were mounted in Canada balsam, and the images were captured with a Leica digital camera coupled to a light microscope (Leica, Wetzlar, Germany).

2.4. DNA extraction, PCR amplification, and sequencing

Total genomic DNA of 2 studied species was extracted from 50 mg of freeze-dried leaves using a CTAB-based protocol (Doyle and Doyle, 1987) and stored in 50 µL of TE buffer at –20 °C. Standard polymerase chain reaction (PCR) protocols and primer sets (Table 2) were used to amplify and sequence 4 DNA regions: the internal and 3' region of the external transcribed spacer ribosomal RNA gene (ITS and ETS, respectively), the 3' end of the *mat*K gene and adjacent 3' *trn*K intron, and *atp*B-*rbc*L intergenic spacer of cpDNA. The amplification was performed in a total volume of 25 µL containing 1 µL of DNA extract, 2.5 µL of 10X *Taq* polymerase reaction buffer (Invitrogen), 1.5 mmol/L of $MgCl_2$, 200 µmol/L of each dNTP (Invitrogen), 1.0 µmol/L of each primer pair, and 2.0 units of *Taq* polymerase DNA (Invitrogen).

The PCR fragments were treated with exonuclease I and shrimp alkaline phosphatase at 37 °C, to eliminate excess primers and nucleotides in the PCR reaction mixture, and then used for sequencing analysis as template DNA. The purified PCR products were sequenced using the ABI Prism Dye-Terminator Cycle-Sequencing Ready Reaction kit (PerkinElmer) and PCR primers as sequencing primers. Extension products were subject to electrophoresis on an AB 3500 DNA sequencer (Applied Biosystems).

2.5. Phylogenetic analysis

The ITS and ETS rDNA and atpB-*rbc*L and *trn*K (including 3' *mat*K) cpDNA spacer nucleotide sequences were initially aligned individually for each locus (Table 3) with sequences retrieved from GenBank in MUSCLE (Edgar, 2004). Phylogenetic trees were recovered according to the maximum likelihood (ML), maximum parsimony (MP) (Cavalli–Sforza and Edwards, 1967; Fitch, 1971), and Bayesian approaches (Huelsenbeck and Ronquist, 2001). MP and ML analysis was performed by PAUP* v4.0b10 (Swofford, 2002) using heuristic searches with 1000 replicates of random-addition sequence, tree-bisection-reconnection (TBR) branch-swapping algorithm. We conducted phylogenetic analyses on individual and combined datasets. A combined data matrix of 1383 characters of the ITS, ETS, and *trn*K partitions including 610, 338, and 435 characters, respectively, and another matrix of 392 ITS and 669 *atp*B-*rbc*L characters were constructed. The number of included taxa was 18 for the first matrix (ITS-ETS-*trn*K) and 30 for the second. The best-fit nucleotide substitution model was selected using ModelTest v3.06 (Posada and Crandall, 1998), considering the selection of Akaike Information Criterion (AIC). All characters were unordered and equally weighted, and the gaps were treated as missing data. Bayesian analysis was conducted with MRBAYES v3.1.2 (Huelsenbeck et al., 2001; Ronquist and Huelsenbeck, 2003) with 2 independent runs conducted for 70,000,000 Markov chain Monte Carlo (MCMC) generations using the default setting of MRBAYES. Average standard deviations of split frequency (ASDSF) values were far lower than 0.01 at the end of the generations. Clades were considered strongly supported if they had bootstrap values equal to or greater than 70% and Bayesian Posterior Probabilities (PP) equal to or greater than 95%. Trees obtained are the results of combined data analyses. Sequences for ITS, ETS rDNA, *trn*K, and atpB-rbcL cpDNA were submitted to GenBank under the accession numbers MH823893, MH823895, MH823897, and MH823899 for *A. mollis* and MH823892, MH823894, MH823896, and MH823898 for *C. lanfrancoi*, respectively.

3. Results

3.1. Morphological description

Based on the results of a long field survey, the geographical distribution of *A. mollis* in Tunisia is considered limited to the southern part of the country. During this prospection, it was inventoried from the border with Libya to El

Table 2. List of primers used for PCR amplification.

DNA region primer name		Primer sequence	Annealing	Reference
ITS	ITS1	5'-TCCGTAGGTGAACCTGCGG-3'	53 °C	White et al., 1990
	ITS4	5'-TCCTCCGCTTATTGATATGC- 3'		
ETS	18S-E	5'-GCAGGATCAACCAGGTAGCA-3'	60 °C	Baldwin and Markos, 1998
	ETS-Atr	5'-CACGTGTGAGTGGTGATTGGTT-3'		
*trn*K	*trn*K-2R	5'-AACTAGTCGGATGGAGTAG-3'	62 °C	Steele and Vilgalys, 1994
	*mat*K8	5'-CTTCGACTTTCTTGTGCT-3'		
*atp*B-*rbc*L	*atp*B-*rbc*L F	5'-GAAGTAGTAGGATTGATTCTC-3'	58 °C	Xu et al., 2000
	*atp*B-*rbc*L R	5'-CAACACTTGCTTTAGTCTCTG-3'		

Table 3. Taxa used in the phylogenetic analysis and their corresponding culture collections and accession numbers.

Taxon name	Collection ID	GenBank accession numbers			
		ITS	ETS	3'*trn*K	*atp*B-*rbc*L
Atriplex altaica	EM320	HM587479			HM587615
A. crassifolia	EM321	HM587496			HM587625
A. dimorphostegia	chen377	HM587499			HM587626
A. imbricata	chen1997	HM587515			HM587632
A. isatidea	chen538	HM587519			HM587633
A. latifolia	EM412	HM587523			HM587636
A. leucophylla	EHZ-JeGr3	HM587526			HM587639
A. moneta	EM322	HM587530			HM587641
A. oblongifolia	EM333	HM587535			HM587645
A. parryi	EHZ-585	HM587539			HM587648
A. patagonica	chen1995	HM587541			HM587649
	Schroder 9.2.2003	HM005866	HM005826	HM005786	
A. prostrata	EM335	HM587545			HM587653
	Zacharias 204	HM005857	HM005817	HM005777	
	Zacharias 309	HM005856	HM005816	HM005776	
A. sibirica	EM312	HM587554			HM587659
A. sphaeromorpha	EM338A	HM587555			HM587660
A. suberecta	chen836	HM587561			HM587663
	Greenhouse s.n.	HM005863	HM005823	HM005783	
Cremnophyton lanfrancoi	chen1895	HM587568			HM587680
A. australasica	chen842	HM587482			HM587617
A. billardierei	chen564	HM587485			HM587618
A. calotheca	EM316	HM587486			HM587619
A. cana	EM310	HM587487			HM587620
A. centralasiatica	chen920	HM587490			HM587621
A. micrantha	EM319	HM587529			HM587640
A. patula	EM324	HM587542			HM587650
	Oswald And Ahart 9581	HM005859	HM005819	HM005779	HM005859
A. phyllostegia	Zacharias992	HM005870	HM005830	HM005790	HM587651
	Tiehm 13806	HM005869	HM005829	HM005789	
A. powellii	EHZ-529	HM587544			HM587652
A. recurva	EM391	HM587548			HM587654
A. spongiosa	chen158	HM587558			HM587661
A. halimus	chen 1876	HM587508			HM587630
Halimione verrucifera	chen470	HM587575			HM587695
H. pedunculata	chen471	HM587573			HM587694
A. hortensis	Zacharias 1027	HM005855	HM005815	HM005775	
A. lentiformis	Zacharias 37A	HM005873	HM005833	HM005793	
A. amnicola	Stutz 95446	HM005861	HM005821	HM005781	
A. cinerea	Kuschel 325	HM005864	HM005824	HM005784	
A. watsonii	Greenhouse s.n.	HM005871	HM005831	HM005791	

Table 3. (Continued).

A. serenana	Zacharias 495	HM005868	HM005828	HM005788	
A. leucoclada	Ertter 18793	HM005860	HM005820	HM005780	
A. rosea	Zacharias 563	HM005858	HM005818	HM005778	
A. rusbyi	Beck 11.335	HM005865	HM005825	HM005785	
A. californica	Zacharias 1025	HM005850	HM005810	HM005770	
Extriplex joaquinana	Ertter 17303	HM005853	HM005813	HM005773	
	Zacharias 306	HM005852	HM005812	HM005772	
A. peruviana	Bentley 338	HM005867	HM005827	HM005787	
A. rosea	Zacharias 563	HM005858	HM005818	HM005778	
Chenopodium murale	Ertter 18872	HM005835	HM005795	HM005755	

Mahres (Sfax), Gafsa, and Tozeur (Table 1). *A. mollis* was located in the south with relic populations on the borders of salt marshes (sebkhas and chotts) and coastal sandy soils. As a result of long observations in natural sites and collection from experimental plots, measurements of plant shape were found to vary slightly within and between populations. The height of all measured adults in natural sites ranges from 50 to 100 cm. After 18 months of growth in the experimental plot, the average aboveground minimum diameter of plants is 43.68 ± 10.16 cm, the mean maximum diameter is 56.38 ± 12.84 cm, and the mean height is 44.06 ± 8.09 cm. The mean leaf area is 1.24 ± 0.15 cm^2, and the mean seed diameter is 1.9 ± 0.33 mm. The mean dry matter of leaves (LDM) and stem (SDM) are 14.24 ± 2.22 and 12.28 ± 3.46%, respectively. The mean weight of fruits is approximately 113.08 ± 28.52 g $plant^{-1}$ (Table 4). The plant is much branched from the base, and the leaves covering the branches are fleshy and elongated (Figure 1a). At the same branch extremities and during the flowering phase, male and female flowers appear with male flowers dominating at the extremities and female at the base (Figures 1b–1f) and rarely hermaphrodite flowers (Figure 1f). Two equal and orbicular bracteoles surround seeds, with diameters ranging from 4 to 8 mm, welded at the base and opened at the upper (Figures 1g and 1h). Phenological monitoring showed that this perennial shrub has a life cycle divided into a broad period of vegetative growth from late January to mid-August. Flower buds begin to appear in mid-August, and the flowering starts from September and continues until the end of November. Fruit growth and seed maturity are from late November to early December. Seed dispersal occurs from late December to mid-February (Figure 2).

3.2. Floral anatomy

The anatomical study of *A. mollis* showed the presence of male and female developed flowers. In Figure 3a, it seems that some male flowers are initially hermaphroditic with underdeveloped female organs. The male flower has 5 tepals arranged in 1 whorl (Figure 3b). In addition, the flowers possessing 5 free stamens situated at the base of

Table 4. Morphological characterization (±SD, n = 36) of 9 accessions of *A. mollis* Desf. **d**: Canopy minimal diameter, **D**: canopy maximal diameter, H: canopy height, La: leaf area, LDM: leaf dry matter, SDM: stem dry matter, SD: seed diameter, and FW: fruit weight.

Accessions	d (cm)	D (cm)	H (cm)	La (cm^2)	LDM (%)	SDM (%)	SD (mm)	FW (g)
DJ	40.83 ± 7.72	52.52 ± 9.06	43.91 ± 7.00	1.16 ± 0.11	14.24 ± 2.47	13.80 ± 3.38	1.9 ± 0.32	144 ± 7.16
KET	39.08 ± 10.87	48.72 ± 14.05	39.88 ± 9.89	1.12 ± 0.20	14.42 ± 2.30	12.21 ± 3.26	1.7 ± 0.41	273.75 ± 74.36
OS	41.16 ± 9.52	54.75 ± 14.41	43.29 ± 7.42	1.19 ± 0.10	15.41 ± 1.85	12.75 ± 4.15	1.4 ± 0.29	118.12 ± 41.34
AOR	40.31 ± 9.22	53.89 ± 10.82	41.13 ± 7.72	1.23 ± 0.11	13.99 ± 1.69	12.52 ± 4.48	2.0 ± 0.38	63.33 ± 17.03
ZIG	48.02 ± 13.04	59.33 ± 14.35	49.58 ± 8.76	1.33 ± 0.19	14.68 ± 2.67	12.39 ± 3.85	1.9 ± 0.26	132.5 ± 48.06
ECH	44.25 ± 8.77	56.19 ± 10.99	47.77 ± 6.99	1.30 ± 0.29	13.95 ± 3.14	13.17 ± 3.64	2.3 ± 0.45	33.75 ± 10.27
HAZ	43.52 ± 11.12	58.08 ± 13.81	44.16 ± 7.69	1.32 ± 0.07	13.24 ± 1.47	11.59 ± 4.20	2.1 ± 0.16	161.25 ± 33.88
CH	50.69 ± 10.58	64.02 ± 12.81	45.27 ± 6.41	1.41 ± 0.18	12.84 ± 1.17	13.20 ± 1.85	1.4 ± 0.30	17.85 ± 7.75
MAH	45.3 ± 10.64	59.99 ± 15.25	41.58 ± 10.95	1.18 ± 0.14	15.43 ± 3.24	8.95 ± 2.37	2.0 ± 0.42	73.19 ± 16.8
Mean	43.68 ± 10.16	56.38 ± 12.84	44.06 ± 8.09	1.24 ± 0.15	14.24 ± 2.22	12.28 ± 3.46	1.9 ± 0.33	113.08 ± 28.52

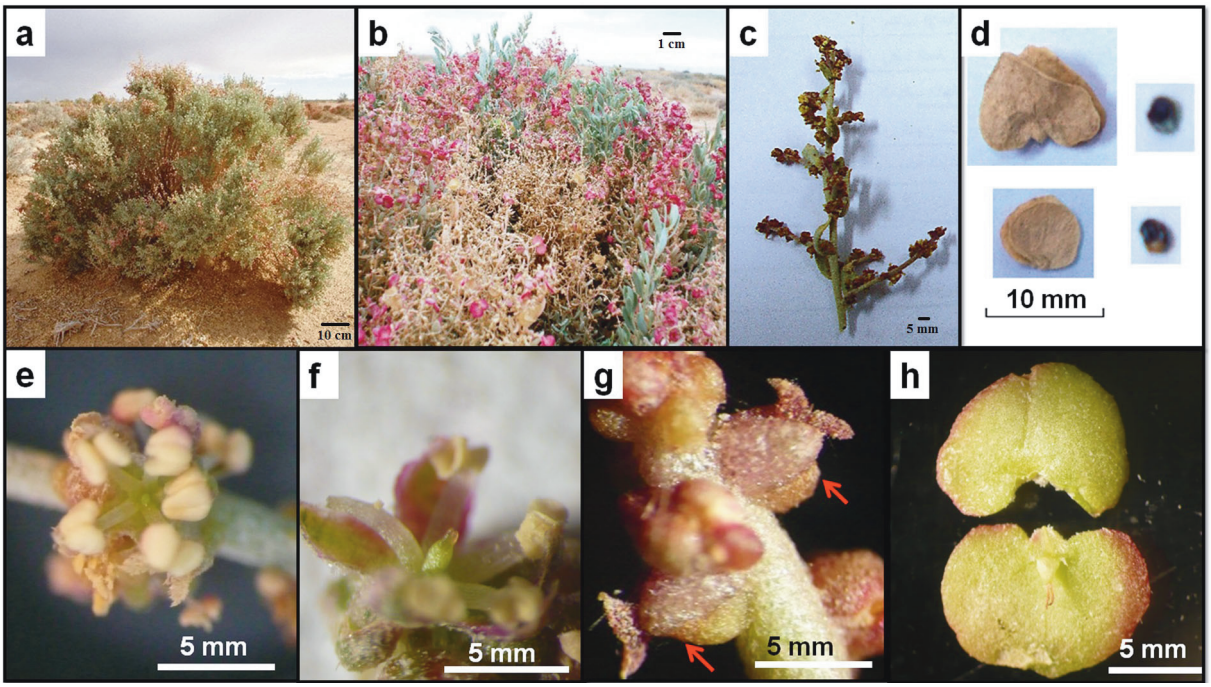

Figure 1. *Atriplex mollis* Desf. plant morphologies. (a) Plant in natural site, (b) inflorescence, (c) floral branch, (d) mature seeds with and without bracteoles, (e) functional male flower with 5 stamens, (f) hermaphroditic flower, (g) functional female flower under arrow, and (h) bracteoles with premature seed.

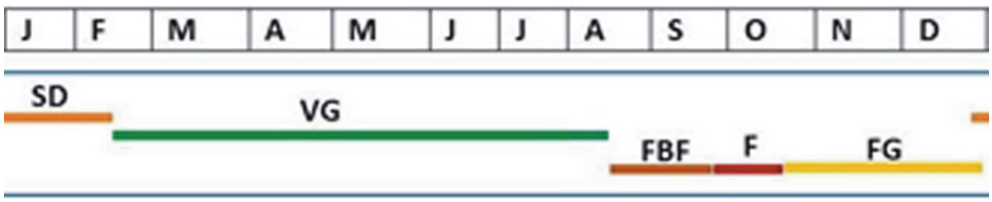

Figure 2. Phenological diagram of the studied populations of *A. mollis* from January to December 2017. VG: vegetative growth, FBF: flower bud formation, F: flowering, FG: fruit growth, and SD: Seed dispersal.

the tepals are arranged in a single whorl. Anatomically, the mature anther wall consists of epidermis and endothecium (Figure 3c). The epidermis has thickened rounded cells. The endothecium is located around each pollen sac, and the cells are thin-walled elongated–quadrangulate in cross section. Each anther has 2 thecae with 1 pollen sac. The female flower is characterized by the presence of 2 opposite bracteoles with a uniovulate ovary. The longitudinal sections of female flowers show 3 ovule types: campylotropous, amphitropous, and orthotropous (Figures 3d–3f). Embryogenesis steps show the beginning of cotyledon formation from the apical meristem at the heart stage (Figures 3g and 3h), which is followed by a cotyledonary stage (Figures 3i and 3j).

3.3. Phylogenetic analysis

Phylogenetic trees from each dataset were generated initially. These were not significantly different (data not shown), and therefore combined datasets were performed. The combined dataset of ITS and *atp*B-*rbc*L consisted of 1061 nucleotide characters, of which 392 characters corresponded to ITS and 669 characters to *atp*B-*rbc*L. Among them, 890 were constant, 86 variable, and 83 informative for parsimony. *Halimione verrucifera* (chen470) and *Halimione pedunculata* (chen471) from the newly-recognized sister group of *Atriplex* (Kadereit et al., 2010) are used as outgroup taxa. The best tree from ML analysis had a log likelihood of –3220.9306 (Figure 4). All phylogenetic analyses yielded trees with similar overall topology to those in the work of Kadereit et al. (2010).

In this study, the examined specimens shared 99.81% sequence identity with *C. lanfrancoi* (IRA-Atr002) and form a coherent cluster well-supported by significant bootstrap and PP (Figure 4) among Amaranthaceae family and are most closely related to the Eurasian semidesert *A.*

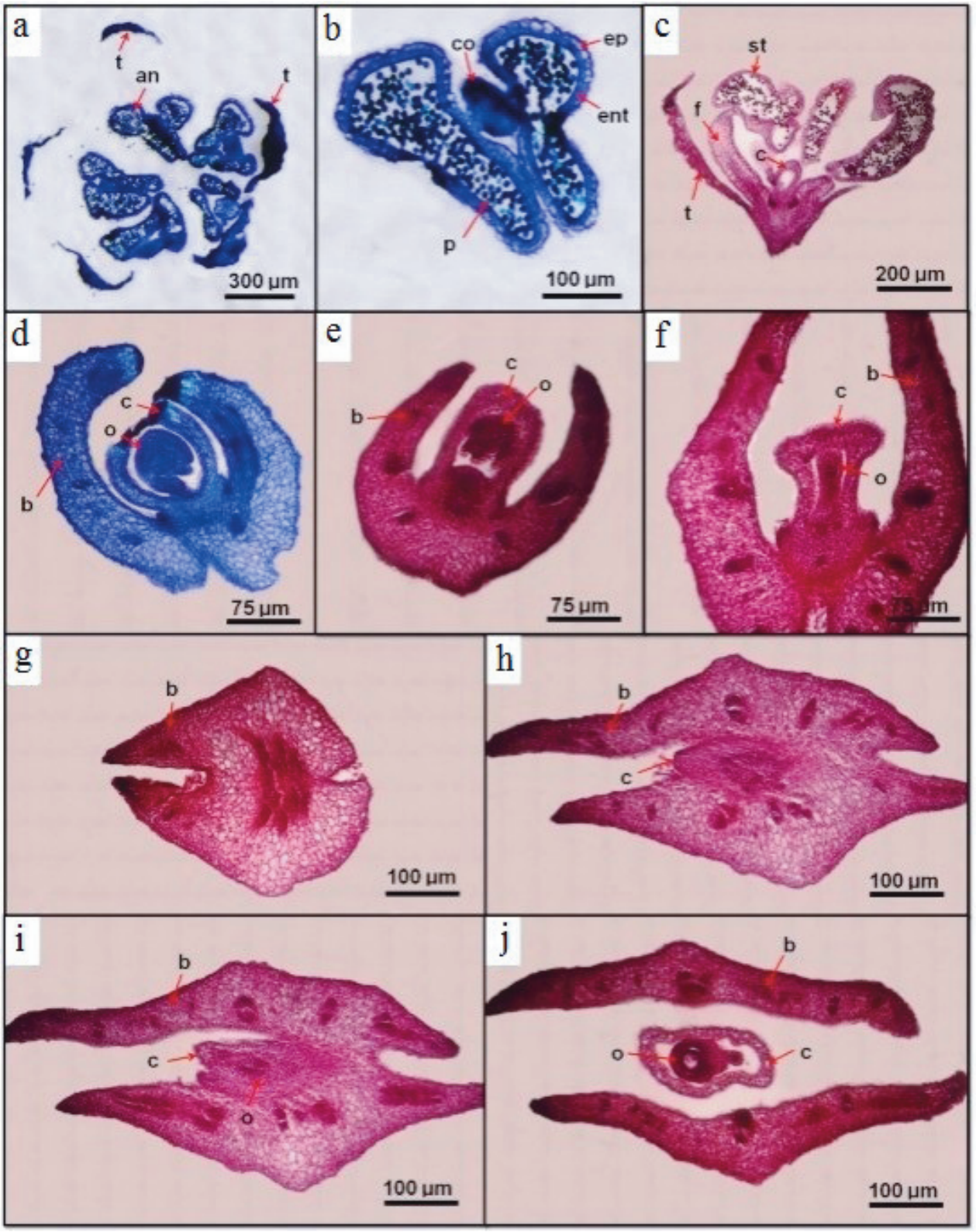

Figure 3. Flower anatomy of *Atriplex mollis* Desf. (a) Longitudinal section of hermaphroditic flower, (b) cross section of male flower, (c) mature anther anatomy, (d–j) longitudinal section of female flower, (d) amphitropous ovule, (e) campylotropous ovule, (f) orthotropous ovule, (g–j) embryogenesis steps, (g) heart shaped embryo, (h and i) carpel emergence, and (j) mature ovary. With b: bracteole, c: carpel, o: ovule, st: style, t: tepal, an: anther, ep: epidermis, co: connective, ent: endothecium, f: filament, and p: pollen.

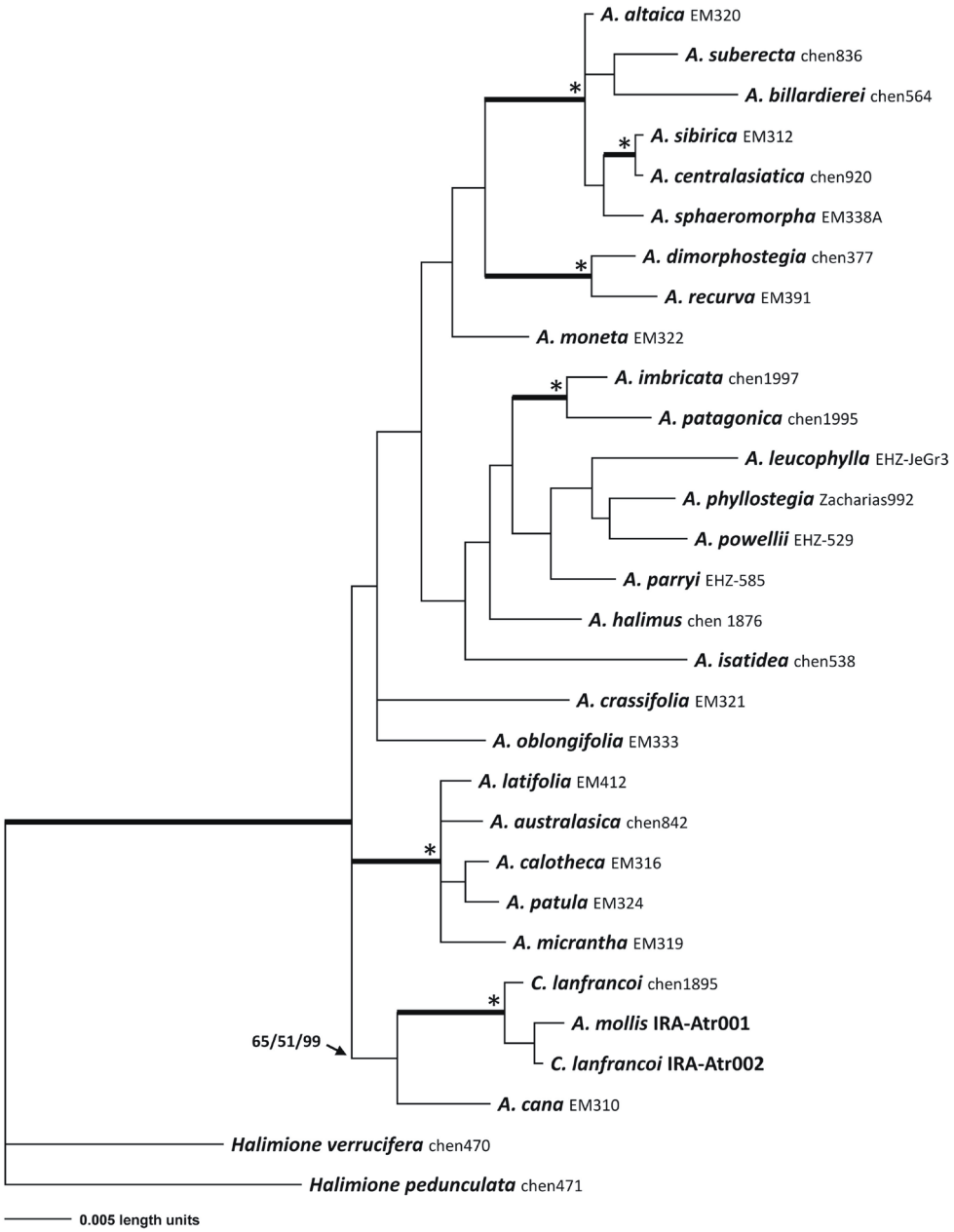

Figure 4. Phylogeny of *Atriplex mollis* derived from combined ITS and *atp*B-*rbc*L gene datasets rooted with *Halimione verrucifera* and *Halimione pedunculata*. Nodes with asterisks (*) are supported with 100% Bayesian posterior probabilities and >70% MP and ML bootstrap values.

cana, with low statistical support (68% ML, 51% MP, and 99% PP).

To further verify the taxonomic positions of our species, an additional analysis was carried out on a combined dataset of ITS, ETS, and 3'*trn*K consisting of 1383 nucleotide characters including 1124 conserved characters, 106 parsimony-informative characters, and 151 variable characters, using *Extriplex joaquinana* (Ertter 17303) from the sister group of *Atriplex* (Kadereit et al., 2010) as outgroup (Figure 5). ML analysis yielded an optimal tree with the best likelihood –4099.0028, which was topologically congruent with both the MP and Bayesian trees. The obtained sequence was highly similar to those of *C. lanfrancoi* (IRA-Atr002) (99.71% sequence identity). They were grouped together into a well-supported, separate clade.

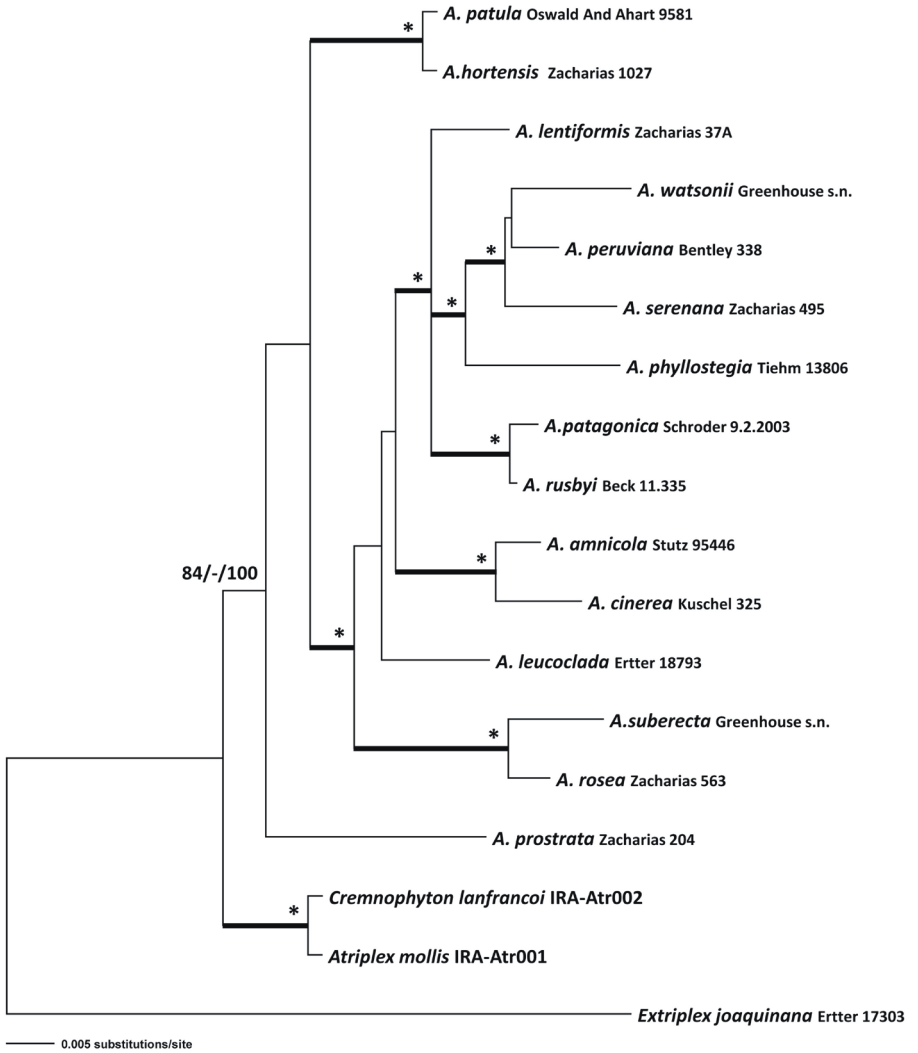

Figure 5. Phylogeny of *Atriplex mollis* derived from combined ITS, ETS, and *trn*K gene datasets rooted with *Extriplex joaquinana*. Nodes with asterisks (*) are supported with 100% Bayesian posterior probabilities and >70% MP and ML bootstrap values.

4. Discussion

The geographical distribution of *A. mollis* in Tunisia found in this work corroborates the distribution previously described by Pottier-Alapetite (1979). Peculiar morphological characters defined in *Atriplex mollis* were also reported by Pottier-Alapetite (1979). The *Atriplex* species reported were monoecious, dioecious, and rarely trimonoecious, such as *A. halimus* (Talamali et al., 2001) and *A. canescens* (McArthur et al., 1992), which contain 3 types of flowers in the same individual. Generally, *Atriplex* flowers were divided into 2 architectural models including male flowers with functional reproductive male organs supported by tepals and an aborted gynoecium (Bisalputra, 1960; Talamali et al., 2003) and functional female flowers containing a single carpel surrounded by 2 bracts. Our investigations confirm the general distribution of flowers in *A. mollis* while indicating the presence of hermaphroditic flowers that could likely be functional male flowers with an aborted gynoecium. The female flowers with bracteoles and the hermaphroditism encountered are signs of the primitiveness of the species, confirming the opinions reported by Hall and Clements (1923) and Talamali et al. (2006). The majority of floral branches showed the dominance of male flowers in the terminal part and female flowers at the base. Talamali et al. (2001) also report this favorable distribution for fecundation in *A. halimus*. The flower (unisexual and bisexual) and the ovule (campylotropous, amphitropous, and orthotropous) types found in *A. mollis* confirm results reported for *A. halimus* (Amel et al., 2007) detailing the diversity of ovule types,

flowers, and seeds in the same individual, as well as the variation in radicle position and its effect on flower type (Stutz et al., 1990).

We used various combinations of datasets and methods of molecular phylogenetic analysis to provide the first combined molecular phylogeny of the studied species. The results suggest that *A. mollis* is the sister of *C. lanfrancoi* and closely related to *A. cana,* which is widely distributed in semideserts from western China to the eastern part of European Russia (Kadereit et al., 2010). These 3 species form a monophyletic group among Amaranthaceae with quite variable support (68% ML, 51% MP, and 99% PP) (Figure 4) and share several morphological characters, including the shape of leaves and bracteoles and floral architecture. However, they differ in stamen number, size of the bracteoles, and chromosome number. Pottier-Alapetite (1979) reported that *A. mollis* is a shrub with upright stems, spathulate and thick leaves (1 to 1.5 cm), and large bracteoles, occupying salty sands in Southern Tunisia, Cyrenaica in Libya, and Fezzan (Hoggar) in Algeria. Its floral morphology, analyzed in this study, showed that *A. mollis* is characterized by staminate flowers with 5 stamens (pentamerous), whereas *C. lanfrancoi*, reported by Brullo and Pavone (1987), displayed flowers predominantly tetramerous and sometimes pentamerous. According to our observations, *A. mollis* is similar to *C. lanfrancoi*; monoecious and having fleshy and spathulate leaves and bracts usually adherent to the pericarp in the lower fruit part. However, they differ mostly by the presence of small bracts, sometimes unequal in *C. lanfrancoi* fruits. This last species was registered by the Maltese botanist Edwin Lanfranco as a new genus in Chenopodiaceae. Based on morphological, ecological, and karyological studies, they assumed that *A. portulacoides* (*Halimione portulacoides*) is the closest species. However, molecular studies conducted by Kadereit et al. (2010) classified *A. portulacoides* among the *Halimione* clade. On the other hand, Brullo and Pavone (1987) reported that *C. lanfrancoi* is a very isolated chasmophyte occupying a specific habitat (coastal limestone cliffs in Malta and Gozo) and characterized by a different chromosome number (2n = 20), compared with *Atriplex*. They proposed that *C. lanfrancoi* was a very primitive taxon [tertiary relict (Oligocene/Miocene)] related to *Atriplex* genus, probably more closely to their ancestors (Brullo and Pavone, 1987), and suggested that the basic chromosome number (n = 9) of *Atriplex* genus (including *A. mollis*) was obtained after an evolution involving the loss of 1 chromosome. *Cremnophyton lanfrancoi*, closely related to *A. cana*, was reassigned by Kadereit et al. (2010) to the genus *Atriplex* based on internal transcribed spacer (ITS) rDNA and *atp*B-*rbc*L data. They proposed *A. lanfrancoi* (Brullo and Pavone) G. Kadereit et Sukhor as a new combination, with *C. lanfrancoi* as the basionym. *Atriplex cana* is also considered an old lineage of *Atriplex* (Sukhorukov, 2006). This species grows in clayey, saline soils in Kazakhstan and is widely distributed in semideserts from South West Asia (Caucasus) and the eastern part of European Russia to Western China (Kochánková and Mandák, 2008; Kadereit et al., 2010).

On August 16, 2017, The International Plant Names Index (IPNI) published new synonyms for *Atriplex mollis*, *Cremnophyton molle* (Desf.) G.L.Chu, and *Atriplex cana*; *Cremnophyton canum* (C.A.Mey.) G.L.Chu. The study of the evolutionary system of Chenopodiaceae around the world published by Zhu and Sanderson (2017) has provided a new systematic organization based on morphological terms and provided the IPNI reference. Our phylogenetic analysis corroborates this systematic organization for these 2 species and the 1 quoted Maltese endemic species.

In addition, according to Kadereit et al. (2010), *C. lanfrancoi* and *A. cana* branch off early within the *Atriplex* genus (11.8–19.25 Ma), and some morphological differences can be illustrated by comparing the 3 related species with other Atripliceae members. For this reason, we suggest that *A. mollis* can be placed among the oldest species of *Atriplex*. Found in scattered populations and occupying littoral and continental salty sand soils, the habitat of *A. mollis* seems different from that of *C. lanfrancoi,* which mainly grows in coastal limestone cliffs, while *A. cana* grows on salty clay soil (Kadereit et al., 2010). While *A. mollis* and *C. lanfrancoi* clearly have a Mediterranean origin, the relationship of these 2 species and *A. cana* remains unresolved.

4.1. Conclusion

Compiling the molecular phylogeny and historical biogeography of North African *Atriplex* is a priority for identifying the relationships between species and their evolutionary story. In the present study, a group of 3 closely-related species under systematic discussion has been detected. Further studies are necessary to determine precisely whether the taxa sharing a clade with *Atriplex mollis* should remain in *Atriplex* or placed in *Cremnophyton*. The production of 4-nucleotide sequence datasets should serve as a framework for more accurate taxonomic placement of halophytic species and aid in understanding the role of dispersal–vicariance events in shaping distribution patterns in the genus *Atriplex*. Further karyological studies in the 3 taxa of the *A. mollis* clade may confirm the existence of different chromosome numbers.

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