

EVOLUTION OF PERICARP SURFACE STRUCTURE IN *NEPETA* S. S. (LAMIACEAE) AS INFERRED FROM ANALYSIS OF ITS DATA

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Nutlet pericarp structure is important in the taxonomy of Lamiaceae (Labiatae) at different taxonomic levels. Within the family it has also been found that variation in pericarp structure is strongly correlated with the phylogenetic results obtained from molecular DNA analyses. The genus *Nepeta* L., with more than 200 species mainly centred in SW Asia, is one of the taxonomically most complex genera within the family. Traditional taxonomic treatments of *Nepeta* are mainly based on gross morphology. As in other groups of Lamiaceae, pericarp structure provides some of the diagnostic characters in this genus. In order to investigate patterns of pericarp evolution within *Nepeta*, we used scanning electron microscopy to examine nutlet surfaces and pericarp cross sections and explored variation of these characters against a molecular phylogeny based on ITS sequences. Based on this phylogenetic analysis, *Nepeta* in its present circumscription is not monophyletic. Evolutionary trends in structure of nutlet pericarps are apparent although they require confirmation with more robust phylogenies. In particular, nutlets with tuberculate/thorny-like pericarp may have evolved once within this genus, in the common ancestor of five of the six subclades identified within *Nepeta*, and have been lost independently several times. We also show that evolution of tubercles in *Nepeta* is not related to plant life span. Our results also indicate that more genetic markers (both plastid and nuclear) are necessary to reconstruct a reliable and robust organismal phylogeny.

Key words: character evolution, Iran, molecular phylogeny, scanning electron microscopy

INTRODUCTION

The genus *Nepeta* L. (Lamiaceae; subfamily Nepetoideae (Dumort.) Luerss.; tribe Mentheae Dumort.; subtribe Nepetinae (Dumort.) Coss. and Germ.), with more than 200 species distributed in Eurasia and introduced in East and North Africa (Harley *et al.* 2004, Serpooshan *et al.* 2018). While the new concept of *Nepeta* (Serpooshan *et al.* 2018) includes genera such as *Hymenocrater*, we stick to the classic concept of *Nepeta* (*Nepeta* s. s.) in this work. The greatest diversity and species richness within the genus are found in two regions: southwestern Asia, especially Iran where species representative of most sections occur (12 sections; Rechinger 1982), and the western Himalayas, includ-

ing the adjacent Hindu Kush (Harley *et al.* 2004, Jamzad *et al.* 2003, Kaya and Dirmenci 2008, Poyarkova 1954)

Based on the most recent taxonomic treatments, Lamiaceae is distributed worldwide and contains more than 7,000 species arranged into 12 subfamilies (Zhao *et al.* 2021). Subfam. Nepetoideae is subdivided into three tribes: Elsholtzieae (Burnett) Sanders and Cantino, Mentheae and Ocimeae Dumort. (Harley *et al.* 2004, Stevens 2001). Mentheae is the largest tribe within the family, and includes more than 60 genera grouped into five subtribes: Menthinae, Prunellinae, Lycopinae, Salviinae, and Nepetinae (Drew and Sytsma 2012). Earlier taxonomic treatments of the tribe Mentheae, mainly based on morphological characters, have been controversial. However, pericarp structure has been reported to be one of the most reliable sources of taxonomic characters (Ryding 2010). Nutlet pericarps have been the subject of many studies in Mentheae (Budantsev and Lobova 1997, Duletic-Lausevic and Marin 1999, Hedge 1970, Makarova 1967, Moon and Hong 2006, Ryding 1995, 2009, Wagner 1914, Wojciechowska 1958, 1961a, b, 1966). Though Mentheae can hardly be distinguished from the rest of the subfamily Nepetoideae by differences in pericarp structure alone, within the tribe variation in pericarp structure is strongly correlated to phylogeny and gross morphological characters (Ryding 2010). Within subtribe Salviinae, pericarp structure was shown to be a diagnostic character that distinguished groups with different number of stamens (Ryding 2010).

Recent molecular studies (Bräuchler *et al.* 2005, 2010, Drew and Sytsma 2011, 2012, Edwards *et al.* 2006, Paton *et al.* 2004, Trusty *et al.* 2004, Walker and Sytsma 2007, Walker *et al.* 2004) showed that the boundaries of three “redefined” subtribes (Menthinae, Nepetinae, and Salviinae) within the Mentheae differ from other treatments (e.g. Harley *et al.* 2004). According to Drew and Sytsma (2012), tribe Mentheae and its five subtribes originated in the Mediterranean/SW Asia region. Subtribe Nepetinae, with *ca* 14 genera, has been shown to be monophyletic in multiple studies (Drew and Sytsma 2012, Wagstaff *et al.* 1995, Walker and Systma 2007). It is distinguishable from the other tribes especially by having two posterior stamens longer than two anterior ones, and also exhibiting a different pericarp structure (Ryding 2010).

The patterns of morphological and karyological diversity in *Nepeta* have resulted in an unstable history of infrageneric classification, from Bentham’s (1848) eight sections and five subsections, Briquet’s (1896) two sections and 15 subsections, Budantsev’s (1993) 19 sections and 13 subsections, to Jamzad’s (2012) arrangement of Iranian species into six “natural groups”. *Nepeta* species are annual or perennial herbs or subshrubs for which one of the following chromosome numbers have been reported: $2n = 14, 16, 18, 30, 32, 34, 36$ and 54 (Harley *et al.* 2004). Some characters such as indumentum, calyx morphology, and leaf shape and size are variable within the genus *Nepeta*, and even

variable within species (Jamzad 2009). Nutlet morphology, including nutlet surface, has been reported to be one of the most reliable characters in species recognition as well as infrageneric taxonomy (Budantsev and Lobova 1997, Hedge 1992, 1970, Hedge and Lamond 1968, Jamzad *et al.* 2000, Kaya and Dirmenci 2008, Mosquero *et al.* 2002, Rechinger 1982, Ubera and Valdes 1983).

Nutlets in *Nepeta* are elliptic, ovate or obovate, trigonous or rounded-trigonous in transverse section, rounded or truncate at the apex, and exhibit a whitish lateral straight or bilobed areole (Budantsev and Lobova 1997). Nutlet surface in *Nepeta* varies from smooth (undulate, reticulate or with a bead-like pattern) to sculptured (verrucose, tuberculate, tuberculate-cellular, or with thorn-like appendices) (Budantsev and Lobova 1997, Hedge 1970). The pericarp includes three layers, with the innermost layer (endocarp) consisting of sclerenchyma cells. Appearance of the nutlet depends on the structure and form of the exocarp cells (Budantsev and Lobova 1997).

Phylogenetic relationships among species of *Nepeta* and some of their close genera have been studied using the nuclear ribosomal internal transcribed spacer (ITS) marker by Jamzad *et al.* (2003). They found that phylogenetic relationships among monophyletic groups identified within the genus were congruent with the distribution of some floral characters, including corolla shape, bract texture, and colour and pollen exine ornamentation. Serpooshan *et al.* (2018) analysed the phylogenetic relationships of *Hymenocrater* and closely related genera using nrITS and plastid *trnL* intron and *trnL-F* intergenic spacer DNA sequences. Their results confirm the monophyly of Nepetinae and the division of this subtribe into two main clades, one of which includes *Drepanocaryum*, *Hymenocrater*, *Nepeta*, *Lophanthus* and *Marmoritis* and the other *Agastache*, *Glechoma*, *Meehania*, *Schizonepeta*, *Lallemantia*, *Cedronella* and *Dracocephalum*. They proposed to include *Hymenocrater*, *Lophanthus* and *Marmoritis* in *Nepeta*. In order to explore evolutionary trends of pericarp in *Nepeta* s. s., we conducted a molecular analysis of ITS data based largely on the *Nepeta* ITS dataset from Jamzad *et al.* (2003), but with ten additional newly sequenced species, and examined nutlet surface and pericarp cross sections using Scanning Electron Microscopy (SEM). In this work we also examine the possible relationship between the presence of nutlet tubercles and plant life span.

MATERIAL AND METHODS

Study of pericarp

Nutlet pericarps of 14 species were examined for the first time in this study and their characteristics described (Table 1). For the remaining species included in this study, characters whose evolutionary trends are explored were taken from previous works (Budantsev and Lobova 1997, Hedge and

Lamond 1968, 1982, Jamzad 2012, Jamzad *et al.* 2000, Kaya and Dirmenci 2008, Rechinger 1982). Three to five ripe nutlets from each species were taken from herbarium material, washed and dried. In order to examine the periderm cells, cross sections were made at the middle of the nutlets. Complete nutlets plus cross sections were directly mounted on stubs and coated with a tiny layer of gold using a SCDOOS sputter coater (BAL-TEC, Switzerland). Observations were performed using a XL30 Scanning Electron Microscope (Philips, the Netherlands). Terminology used for nutlet surface ornamentation mainly follows Stearn (1992) and Budantsev and Lobova (1997).

Molecular phylogeny

Our dataset included 49 accessions of *Nepeta* s. s. species from 11 of the 19 sections recognised in Budantsev and Lobova (1997) for the ingroup and 15 close allies of *Nepeta* s. s. considered as outgroups (Table 1). Phylogenetic relationships among species of *Nepeta* and some of these close generic allies previously were examined in separate studies (Barber *et al.* 2002, Jamzad *et al.* 2003, Mattner 2002, Prather *et al.* 2002, Serpooshan *et al.* 2018, Steane *et al.* 1999, Upson 1997, Upson and Jury 2002). Specifically, *Origanum vulgare* L. (subtribe Mentheae), *Satureja montana* L. (subtribe Mentheae), *Cedronella canariensis* (L.) Webb et Berthel. (subtribe Nepetinae), *Schizonepeta multifida* Briq. (subtribe Nepetinae), *Hyssopus officinalis* L. (subtribe Mentheae), *Lallemantia canescens* Fisch. et C. A. Mey. (subtribe Nepetinae), *Dracocephalum parviflorum* Nutt. (subtribe Nepetinae), *Agastache pallida* (Lindl.) Cory (subtribe Nepetinae), *Glechoma hederacea* L. (subtribe Nepetinae), *Meehania urticifolia* (Miq.) Makino (subtribe Nepetinae), *Drepanocaryum sewerzowii* (Regel) Pojark. (subtribe Nepetinae), *Hymenocrater bituminosus* Fisch. et C. A. Mey. (subtribe Nepetinae), *Marmoritis complanatum* Ik.-Gal. et Nevski (subtribe Nepetinae), *Marmoritis decolorans* (Hemsl.) H. W. Li (subtribe Nepetinae) and *Lophanthus lipskyanus* Ik.-Gal. et Nevski (subtribe Nepetinae) were in the analysis following Drew and Sytsma (2012).

Total genomic DNA was extracted from either silica-gel dried leaves or herbarium specimens following a modified CTAB protocol of Doyle and Doyle (1987). DNA extraction was performed using the DNeasy Plant Mini Kit (Qiagen, Valencia, California, and USA) following the manufacturer's protocol. The entire ribosomal ITS region (ITS1 + 5.8s + ITS2) was amplified using either primer pairs AB 101 (forward, 5'-ACG AAT TCA TGG TCC GGT GAA GTG TTC G-3') and AB 102 (reverse, 5'-TAG AAT TCC CCG GTT CGC TCG CCG TTA C-3') (Douzery *et al.* 1999), or primer pairs 18 S (forward, 5'-CCT TMT CAT YTA GAG GAA GGA G-3') and 28 S (reverse, 5'-CCG CTT ATT KAT ATG CTT AAA-3') (Muir and Schlötterer 1999). PCR protocol for primer

Table 1

Locality, life span, and nutlet surface of species included in the ITS phylogeny and SEM microscopy. Exact locality information is given for the newly sequenced material and that used for SEM microscopy only. Sections according to Reehinger (1982) are indicated in parentheses. Herbarium acronyms follow Thiers (2015). Abbreviations: Ls = life span, an = annual, pe = perennial

Species	Locality	Ls	Nutlet surface	Sclerid cavity form	ITS analysis (Genbank code)
<i>Agastache pallida</i> (Lindl.) Cory	Mexico	pe	tuberculate	–	JQ669075
<i>Cedronella canariensis</i> (L.) Webb et Berthel.	USA: cultivated at UCBC	pe	smooth	–	JQ669079
<i>Dracocephalum parviflorum</i> Nutt.	USA	pe	smooth	–	JQ669097
<i>Drepanocaryum setozavii</i> (Regel) Pojark.	Tajikistan	an	tuberculate	–	DQ667328
<i>Glechoma hederacea</i> L.	USA	pe	tuberculate	–	JQ669099
<i>Hymenocrater bituminosus</i> Fisch. et C. A. Mey.	SW Asia: Armenia	pe	tuberculate	–	JQ669105
<i>Hyssopus officinalis</i> L.	Eurasia: cultivated at DBG	pe	smooth	–	JQ669106
<i>Lallemantia canescens</i> Fisch. et C. A. Mey.	Eurasia: cultivated at DBG	pe	tuberculate	–	JQ669108
<i>Lophanthus lipskyanus</i> Ik.-Gal. et Nevski	C Asia: Uzbekistan	pe	smooth	–	JQ669109
<i>Marmoritis complanatum</i> Ik.-Gal. et Nevski	C Asia: China	pe	smooth	–	JQ669111
<i>Marmoritis decolorans</i> (Hemsl.) H. W. Li	C Asia: Nepal	pe	smooth	–	GQ456151
<i>Meehania urticifolia</i> (Miq.) Makino	C Asia: China	pe	tuberculate	–	JQ669113
<i>N. (Spicate) assurgens</i> Hausskn. et Bornm.	Iran: SE, Kerman	pe	smooth-reticulate	–	AJ515316
<i>N. (Spartonepeta) balouchestanica</i> Jamzad et Ingr.	Iran: SE, Balouchestan	pe	tuberculate	–	AJ515606
<i>N. (Spicate) binaludensis</i> Jamzad	Iran: NE, Khorassan	pe	smooth	–	AJ515311
<i>N. (Micranthae) bornmuelleri</i> Hausskn. ex Bornm.	Iran: SE, Kerman	an	smooth	–	AJ515310
<i>N. (Micronepeta) bracteata</i> Benth.	Iran: NE, Khorassan, Birjand, Darmin, 2,265 m, 10 May 2010, Sabaghti 1129 (IAUH).	an	smooth-undulate	large vertical elliptic	OL985936
<i>N. (Cataria) cataria</i> L.	Iran: N, Mazandaran	pe	smooth	–	AJ515313

Table 1 (continued)

Species	Locality	Ls	Nutlet surface	Sclerid cavity form	ITS analysis (Genbank code)
<i>N. (Cataria) cataria</i> L.	Eurasia: cultivated at WIS (USA)	pe	smooth	–	JQ669126
<i>N. (Capituliferae) cephalotes</i> Boiss.	Iran: C, Kashan	pe	smooth-reticulate	–	AJ421037
<i>N. (Oxynepeta) congesta</i> Fisch. et C. A. Mey. var. <i>cryptantha</i> (Boiss.) Hedge et Lamond	Iran: SW, Bakhtiari	pe	tuberculate	–	AJ515161
<i>N. (Stenostegia) crassifolia</i> Boiss. et Buhse	Iran: N, Tehran	pe	tuberculate	–	AJ515307
<i>N. (Cataria) crispa</i> Willd.	Iran: N, Tehran, Pulo; 2,200 m, 28 July 2009, Haji-Mirzaie 5411 (IAUH)	pe	tuberculate	small compressed rounded	–
<i>N. (Cataria) crispa</i> Willd.	Iran: W, Hamedan	pe	smooth	–	AJ515159
<i>N. (Micronepeta) daenensis</i> Boiss.	Iran: SE, Kerman, Rayen, Aabshar, 3,600 m, 10 July 2009, Rajaei 667 (IAUH)	an	smooth-reticulate	large vertical and horizontal elliptic	OL985933
<i>N. (Spartonepeta) afgersiana</i> Schewinf. ex Hedge	Arabia: Saudi Arabia	pe	thorny-like	–	KF765442
<i>N. (Psilonepeta) denudata</i> Benth.	Iran: N, Tehran	pe	smooth-bead like	–	AJ420996
<i>N. (Micronepeta) eremophila</i> Hausskn. et Bornm.	Iran: SE, Kerman	an	tuberculate	–	AJ5153151
<i>N. (Schizocalyx) fissa</i> C. A. Mey.	1. Iran: N, Tehran	pe	smooth or tuberculate	–	AJ421035
	2. Iran: W, Azarbaijan, Takab, Takht-e Soleyman Road, Shirmard river around, roadside, 1,836–1,900 m, 12 Aug. 2002, Sonboli 27 (MPH)	pe	smooth	elliptic	–
<i>N. (Capituliferae) glocephala</i> Rech. f.	Iran: C, Kashan	pe	smooth-reticulate	–	AJ515308
<i>N. (Spartonepeta) glomerulosa</i> Boiss.	Iran: SE, Kerman	pe	tuberculate	–	AJ515317

Table 1 (continued)

Species	Locality	Ls	Nutlet surface	Sclerid cavity form	ITS analysis (Genbank code)
<i>N. (Spartonepeta) glomerulosa</i> Boiss.	Iran: SE, Kerman, Rayen, Deh morteza, 3,400 m, 25 June 2009, Rajaei 669 (IAUH)	pe	tuberculate	small compressed rounded	OL985937
<i>N. (Glechomanthe) glutinosa</i> Benth.	C Asia: Pamir	pe	smooth-reticulate	–	GQ456153
<i>N. (Oxynepeta) heliotropifolia</i> Lam.	Iran: W, Zanjan	pe	tuberculate	–	AJ515312
<i>N. (Capituliferae) hormozganica</i> Jamzad.	Iran: S, Hormozgan	an	tuberculate	–	AJ515160
<i>N. (Spartonepeta) isaurica</i> Boiss. et Helder.	Turkey: cult. at RBG, Kew	pe	tuberculate	–	AJ515306
<i>N. (Micronepeta) ispathanica</i> Boiss.	Iran: C, Tehran	an	tuberculate	–	AJ515318
	Iran: Kerman, Rayen, Rask, 3,000 m, 5 May 2009, Rajaei, 670 (IAUH)	an	tuberculate	small compressed rounded	OL985929
<i>N. (Stenostegia) kurdica</i> Hasskn. et Bormm.	Iran: W, Kordestan	an	tuberculate	–	AJ515320
<i>N. (Psilonepeta) laxiflora</i> Benth.	Iran: W, Bakhtiari	pe	smooth	–	AJ420995
<i>N. (Glechomanthe) longibracteata</i> Benth.	C Asia: Pamir	pe	smooth-reticulate	–	GQ456154
<i>N. (Capituliferae) mahamensis</i> Jamzad et Simmonds.	1. Iran: SE, Kerman, Rayen, Rask, 3,000 m, 5 May 2009, Rajaei 671 (IAUH) 2. Iran: SE, Kerman	an	smooth	small compressed rounded	OL985928
<i>N. (Cataria) menthoides</i> Boiss. et Buhse	1. Iran: NW, Azerbaijan 2. Iran: Ardebil, Sabalan Mt, Ghotur-Soee, 18 Jun 2007, Gholipour 1183 (MPH) 1. Iran: NW, Azerbaijan	an pe pe an	smooth smooth-undulate smooth-undulate tuberculate	– – yes –	AJ421003 AJ421002 2. no AJ421042

Table 1 (continued)

Species	Locality	Ls	Nutlet surface	Sclerid cavity form	ITS analysis (Genbank code)
<i>N. (Micranthae) meyeri</i> Benth.	2. Iran: N, Tehran, Shahid Beheshti university campus, 1,800 m, Sonboli 1664 (MPH)	an	tuberculate	small rounded	no
<i>N. (Micranthae) mirzayanii</i> Rech. f.	Iran: SE, Balouchestan	an	tuberculate	–	AJ515309
<i>N. (Stenostegia) mussini</i> Spreng. ex Henck.	SW Asia: cult. At Kew	pe	tuberculate	–	AJ515305
<i>N. (Capituliferae) natanzensis</i> Jamzad	Iran: SE, Kerman, Rayen, Ordikan, 3,800 m, 10 July 2009, Rajaei 673 (IAUH)	an	smooth	–	OL985930
<i>N. (Psilonopeta) oxydonta</i> Boiss.	1. Iran: C, Fars	pe	smooth-reticulate	–	AJ420996
	2. Iran: C, Esfahan, Semirom, 1,900 m, 4 June 2005, Sonboli <i>et al.</i> 901 (MPH)	pe	smooth-reticulate	large vertical elliptic	no
<i>N. (Stenostegia) persica</i> Boiss.	Iran: C, Fars, Abadeh, Shahrman Mt., 2,500 m, 25 May 2006, Sonboli & Kanani 983 (MPH)	pe	thorny like	large, vertical, and elongated elliptic	OL985931
<i>N. (Spicate) pogonosperma</i> Jamzad et Assadi	Iran: N, Alburz Mts range	pe	smooth	–	AJ421041
<i>N. (Micronepeta) pungens</i> Bunge	Iran: SE, Bakhtiari	an	smooth-reticulate	–	AJ42103
<i>N. (Stenostegia) racemosa</i> Lam.	Iran: N, Tehran, Shahrestanak, Khor, 2,100 m, 3 Jun 2002, Yousefzadi 497 (MPH)	pe	tuberculate	small compressed rounded	OL985935
<i>N. (Micranthae) saccharata</i> Bunge	1. Iran: SE, Baluchistan	an	tuberculate	–	AJ515314
	2. Iran: SE, Kerman, Rayen, Mirshadi, 3,200 m, 25 May 2009, Rajaei 675 (IAUH)	an	tuberculate	narrowly elongated elliptic	OL985932

Table 1 (continued)

Species	Locality	Ls	Nutlet surface	Sclerid cavity form	ITS analysis (Genbank code)
<i>N. (Micron.) satuireioides</i> Boiss.	Iran: NE, Khorassan, Birjand, Darmin, 2,265 m, 10 May 2010, Sabaghi 1131 (IAUH)	an	tuberculate (looking smooth in immature achenes)	large compressed rounded	OL985938
<i>N. (Psilonepeta) sessilifolia</i> Bunge	Iran: C, Esfahan, 25 km from Ghamsar on the road to Gohrud, 2,700 m, 14 Jun. 2007, Somboli & Gholipour 1153 (MPH)	pe	smooth-undulate	large vertical elliptic	OL985934
<i>N. (Micronepeta) schiraziana</i> Boiss.	1. Iran: SW, Fars 2. Iran: C, Fars, Abadeh, Dahaneh, 2,100 m, 8 Jun 2004, Kanani 762 (MPH)	an an	thorny like thorny like	– –	AJ421034 –
<i>N. (Psilonepeta) scrophularioides</i> Rech. f.	Iran: NW, Azarbayejan	pe	smooth	–	AJ515319
<i>N. (Schizocalyx) sibirica</i> Bunge	C Asia: Cult. at RBG	pe	smooth	–	AJ421039
<i>N. (Micranthae) straussii</i> Hausskn. et Borrm.	Iran: W, Lorestan	an	tuberculate	–	AJ421039
	Iran: W, Lorestan	an	tuberculate	–	JF301404
<i>N. (Micranthae) wettsheimii</i> H. Braun	Iran: NW, Azarbayejan, Ghahremani 80476 (TAR)	an	tuberculate	–	AJ421038
<i>Origanum vulgare</i> L.	[Eurasia]: cultivated at Las Vueltas, La Palma	pe	smooth	–	AY506647
<i>Satureja montana</i> L.	Cultivated at UCBC	pe	tuberculate	–	JQ669135
<i>Schizonepeta multifida</i> Briq.	Eurasia: Siberia	pe	smooth	–	DQ667313

pair AB101–AB102 followed a pretreatment of 5 min at 95 °C, 35 cycles of 30 s at 95 °C, 30 s at 50 °C, and 1 min 30 s at 72 °C, and a final extension of 7 min at 72 °C. PCR protocol for primer pair 28 S-18 S followed a pretreatment of 3 min at 94 °C, 35 cycles of 18 s at 94 °C, 30 s at 55 °C, and 60 s at 72 °C, a post-treatment of 78 s at 55 °C, and a final extension of 7 min at 72 °C.

Forward and reverse sequences were visually compared and edited, and then initially aligned using Sequencher 4 software (Gene Codes Corporation, Ann Arbor, MI USA). In addition to our sequences, 15 ITS sequences from other taxa were retrieved from GenBank (Table 1). Sequences were assembled and aligned using MacClade 4 (Maddison and Maddison 2000). Maximum parsimony (MP) analysis of the ITS dataset was performed with PAUP* (Swofford 2002) using 100 heuristic search replicates, random stepwise addition of taxa, and tree-bisection reconnection (TBR) branch swapping. Bootstrap support (BS) for clades was calculated using PAUP* (Swofford 2002) with 100 replicates of heuristic searches, and random stepwise addition of taxa. Bayesian analysis (BA) of the ITS dataset was performed using MrBayes v3.1.2 (Huelsenbeck and Ronquist 2001). In order to find the appropriate model of DNA substitution for our ITS dataset the Akaike information criterion (AIC; Akaike 1974) was applied as implemented in the program Model-Test v3.7 (Posada and Crandall 1998). The GTR (general time-reversible) + I + G model was chosen with gamma distribution set to 0.6653 and proportion of invariable sites set to 0.3571. Substitution rates were set to A-C = 0.6831, A-G = 1.1245, A-T = 1.3404, C-G = 0.2598, C-T = 3.5853, and G-T = 1.000.

RESULTS

Structure of pericarp in Nepeta s. s.

Nutlet shape in *Nepeta* species studied here ranges from oblong (in *N. oxyodonta*), to elliptic (in *N. bracteata*), to narrowly ovate (in *N. ispahunica*; Figs 1–2). They are trigonous or rounded-trigonous in transverse section, and apically rounded or truncate, and usually possess a whitish lateral bilobed areole. Nutlets of all 14 species newly examined here are glabrous but exhibit a variable pericarp surface. The results are summarised in Table 1. Pericarp surfaces of our material are smooth, partly smooth, or sculptured. Nutlet pericarp consists of three layers: exocarp, mesocarp and endocarp, and cells of the outer layer (exocarp) define the type of pericarp surface. *Nepeta saccarata* and *N. meyeri*, both from the section *Micranthae* have a sculptured surface with tubercles (Fig. 1A–C) or protuberclcs (Fig. 1E–G), covered with tiny vesicles. Tubercles are formed by exocarp cells (Budantsev and Lobova 1997). The sclerenchymatous endocarp mainly consists of sclerids with rounded to elongated cavities and poral canals running towards the apical and partly the basal parts

of cells. Sclerid cavities in our species of section *Micranthae* are elliptic to elongate (Table 1). Of five species from the section *Micronepeta*, *N. ispanhanica* and *N. saturojoides* possess a pericarp with a tuberculate exocarp and different form of cavities (Fig. 1Q–T, V–BB). *Nepeta bracteata* and *N. daenensis* have pericarps

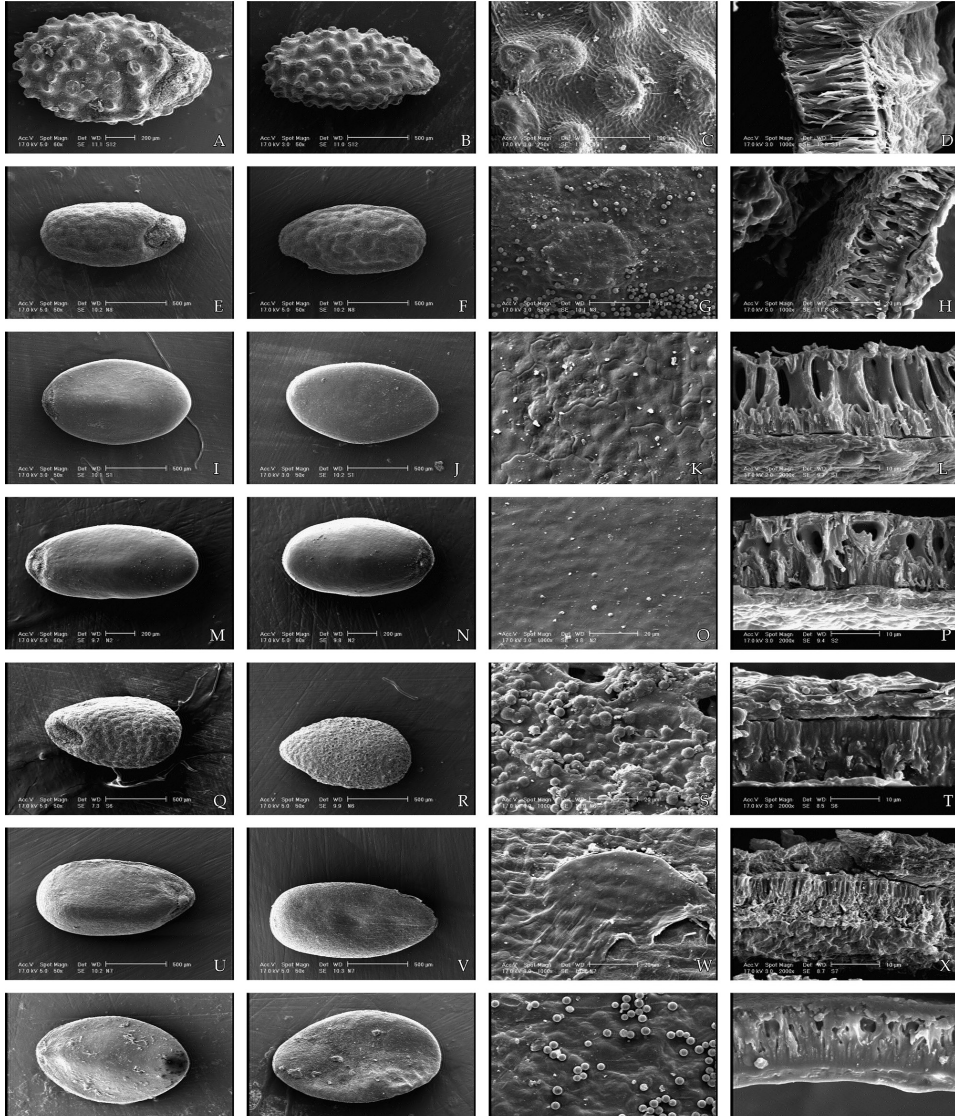


Fig. 1. SEM micrographs of nutlets of *Nepeta* s. s. First column: ventral view; second column: dorsal view; third column: surface of dorsal view in higher magnification; fourth column: pericarp in cross section. A–D: *N. saccharata*; E–H: *N. meyeri*; I–L: *N. bracteata*; M–P: *N. daenensis*; Q–T: *N. ispanhanica*; U–X: *N. mahanensis*; Y–BB: *N. saturojoides* (immature)

with smooth surface and rounded to elliptic cavities (Fig. 1I–L, M–P). *Nepeta mahanensis* has pericarp with smooth surface and tightly compressed sclerids with few elongated cavities (Fig. 1U–X). Two species belonging to the section *Psilonepeta* (*N. oxyodonta* and *N. sessilifolia*) have smooth pericarps (Fig. 2A–D,

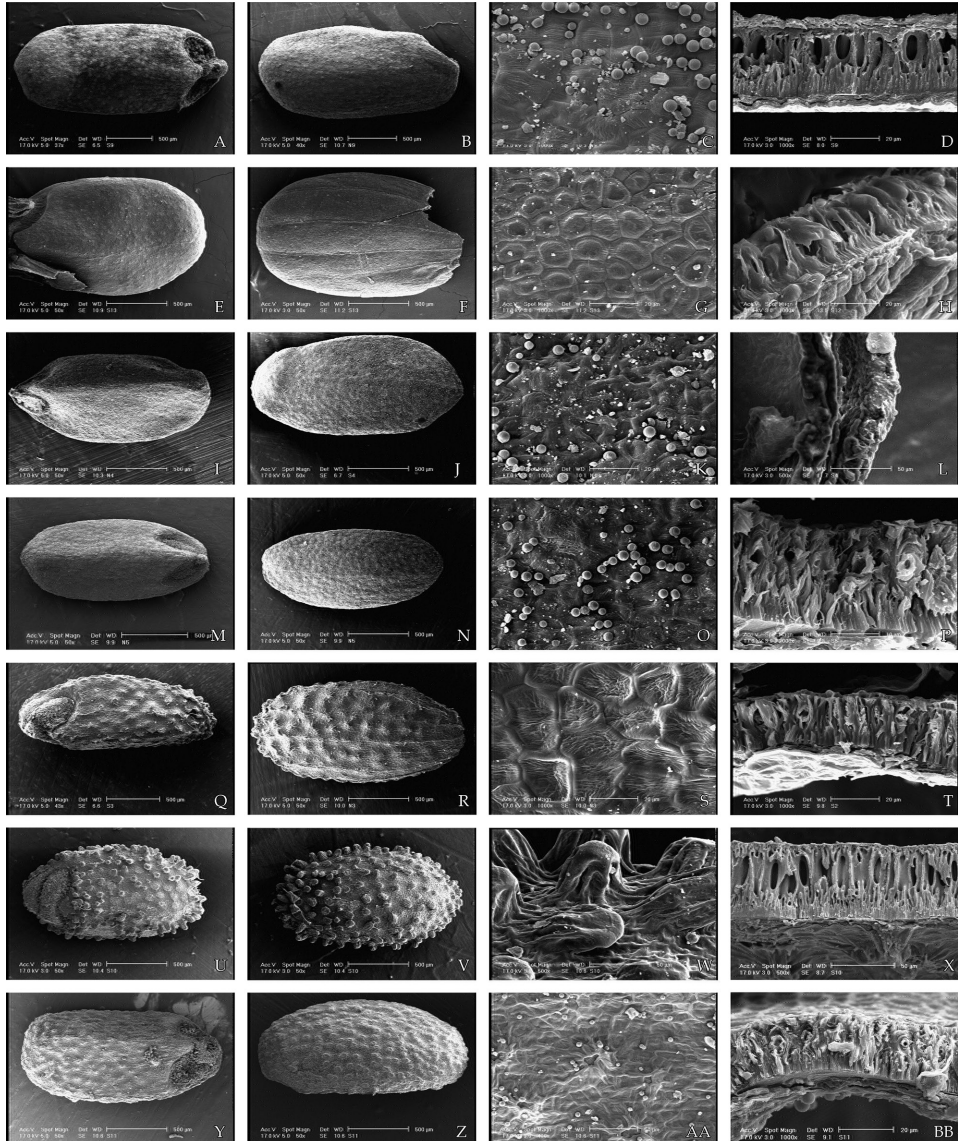


Fig. 2. SEM micrographs of nutlets of *Nepeta* s. s. First column: ventral view; second column: dorsal view; third column: surface of dorsal view in higher magnification; fourth column: pericarp in cross section. A–D: *N. oxyodonta*; E–H: *N. sessilifolia*; I–L: *N. fissa*; M–P: *N. glomerulosa*; Q–T: *N. crassifolia*; U–X: *N. persica*; Y–BB: *N. racemosa*

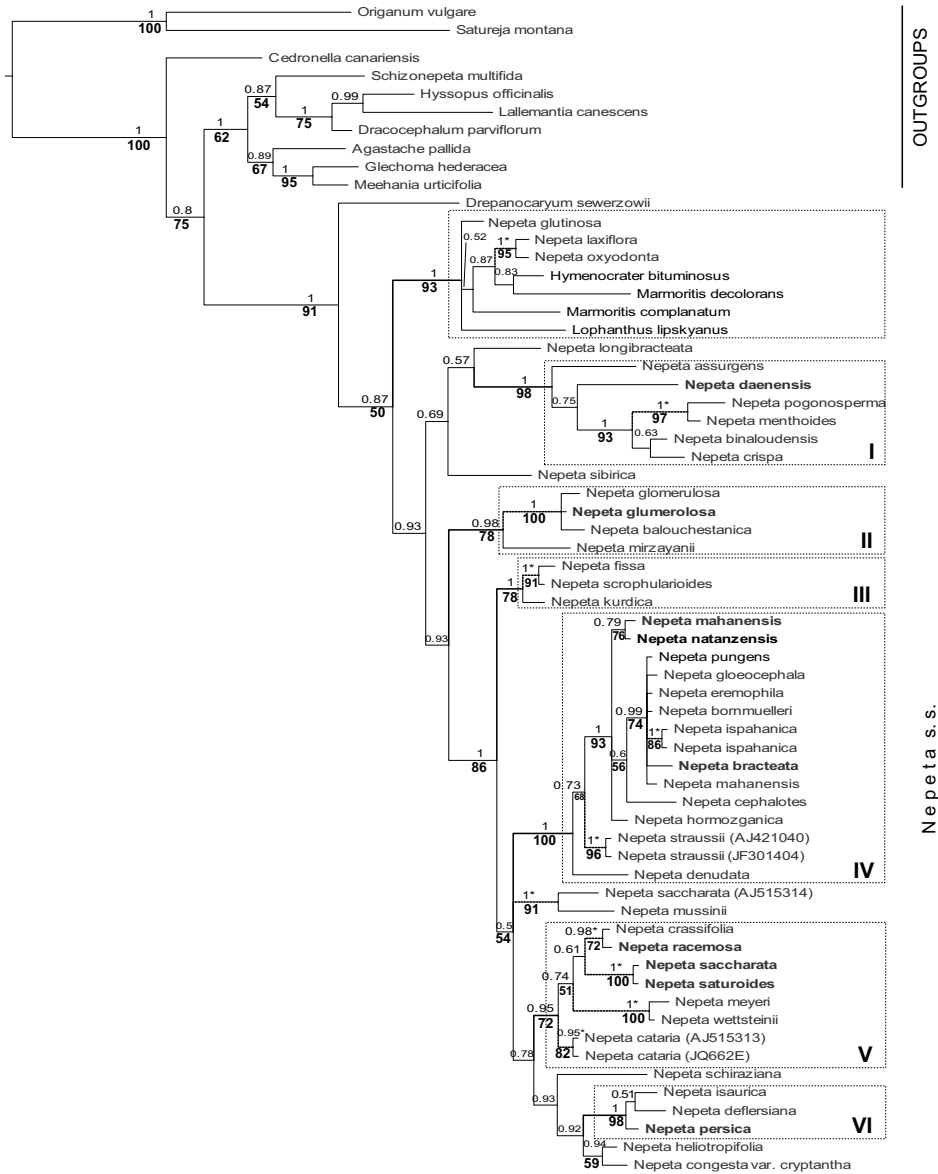


Fig. 3. 50% majority-rule consensus tree obtained from the Bayesian analysis of an ITS dataset focused on *Nepeta* s. s. Numbers above branches represent posterior probabilities (PP). Numbers below branches indicate bootstrap support (BS) of those clades retrieved in the maximum parsimony strict consensus tree. The Roman numerals I–VI indicate major subclades with PP = 1 except for V (0.95). Asterisks indicate small nested two-taxon subclades with PP \leq 1. For each *Nepeta* species, the section in which it is placed in Rechinger (1982) infrageneric taxonomy is given on the right panel together with the informal natural group (designed by Arabic numerals in brackets) in Jamzad *et al.* (2003)

E–G). Our samples from three sections *Schizocalyx* (*N. fissa*), *Spartonepeta* (*N. glomerulosa*) and *Stenostegia* (*N. crassifolia*, *N. persica* and *N. racemosa*) have sculptured pericarps with different types of sclerid cavities (Fig. 2I–L, M–P, Q–T, U–X, Y–BB). Species from different sections have pericarps with tiny glands.

Phylogeny of Nepeta s. s. based on ITS sequences

Maximum Parsimony (MP) analysis – Our aligned ITS (ITS1 + ITS2) dataset (including ingroup and outgroup taxa) included 664 nucleotides of which 119 (17.9%) were parsimony-uninformative and 213 variable characters (32%) were potentially parsimony informative. Parsimony analysis resulted in 2,778 MP trees with length = 778 steps, consistency index (CI) = 0.536 and retention index (RI) = 0.749. All minimal trees were combined into a strict consensus tree with length = 825 steps, CI = 0.505 and RI = 0.716. Topology of the strict consensus tree (not shown) obtained from MP analysis is highly similar to that of the 50% majority rule tree obtained from the Bayesian Analysis (Figs 3–5, discussed below).

Bayesian Analysis (BA) – The 50% majority rule tree obtained from the Bayesian analysis of our dataset is shown in Figure 3. This tree was chosen as the basis for analysis of character evolution (Figs 4 & 5). All posterior probabilities are shown above the branches. Numbers below branches show the bootstrap supports obtained from MP analysis of same dataset. Posterior probability (PP) supports equal or higher than 0.95 were considered as firm support. As shown in Figure 3, all *Nepeta* species are grouped into a few clades. Most interestingly, three species of *Nepeta* i.e., *N. glutinosa*, *N. oxyodonta* and *N. laxiflora* alongside with *Hymenocrater bituminosus*, *Lophanthus lipskyanus*, *Marmoritis decolorans* and *M. complanatum* formed a well-supported clade with PP = 1 and BS = 93%. The remaining species of *Nepeta* s. s. fell in a moderately supported clade (PP = 0.93) that is sister to the aforementioned one. Within this *Nepeta* s. s. clade, there are six subclades with PP = 0.95–1.00, each formed by more than two taxa which, however, do not match the sectional classification (Fig. 3, I–VI). Subclade I is formed by six species from tree different sections, II by three species from two different sections, and III by three species from three different sections. Subclade IV is the largest subclade and contains 12 mostly annual species from four different sections. Subclade V includes seven species from four different sections, and VI includes three species from two different sections.

There are also 10 smaller well-supported (PP \geq 0.95) nested subclades formed by two taxa (Fig. 3, indicated by asterisks). Three of them are formed by different accessions of same species and three others are formed by different species from same section. Two other subclades are formed by species

from one or more sections. Annual species of *Nepeta* s. s. (mainly sections *Micranthae* and *Micronepeta*) are scattered across the tree.

Evolution of characters in Nepeta s. s. – In order to trace character evolution in *Nepeta* s. s., the 50% majority rule tree obtained from the Bayesian analysis (BA) of our dataset was used (Figs 4 & 5). Two characters, life span and nutlet surface sculpture, were traced on the BA tree using MacClade 4. As shown in Figure 4, annual life span and tuberculate/thorny-like surface are apomorphies that evolved mainly in the *Nepeta* s. s. clade. Of 10 outgroups studied in this work, all of them are perennial and six of them have nutlets with non-tuberculate surfaces. Five out of the six main subclades (Fig. 3, I–V) include both annual and perennial species (Fig. 4). Subclade VI is the only well-supported subclade completely formed by perennial species, but no clade is exclusively formed by annual species, which are instead scattered across the *Nepeta* s. s. clade. In contrast, three out of the six main subclades consist of species having nutlets only with either smooth (subclade I) or tuberculate/thorny-like (subclades II, VI) surfaces (Fig. 5).

DISCUSSION

Phylogeny of Nepeta s. s.

Our ITS sequence results do not support the monophyly of *Nepeta* s. s. in its present circumscription. However, the trees obtained do not have high support, particularly in the inner nodes. This is consistent with Jamzad *et al.* (2003), who did not obtain a robust species phylogeny based on the ITS data alone. These authors found that their ITS phylogeny was partially correlated with some morphological characters, which they considered could be used as the foundation for a new phylogenetic infrageneric classification for the genus. Based on those results, Jamzad (2012) ignored the classic sectional divisions, the last of which is that by Rechinger (1982), and grouped all Iranian *Nepeta* in six natural groups (Fig. 3). However, our results show that these groups (Jamzad *et al.* 2003) are not completely recovered in our phylogeny. It seems that more markers (both plastid and nuclear) are necessary to reconstruct a reliable and robust phylogeny.

Annual life history in Nepeta s. s.

All outgroups studied here are perennials except for *Drepanocaryum sewerzowii* and *Dracocephalum parviflorum*. But *Nepeta* includes both annual and perennial species (Fig. 4). Annual species are scattered from C Asia to SW Asia. They grow mainly in harsh and mountainous environments of the

Irano-Turanian Region. Therefore, multiple acquisitions of an annual life history seem to be due to a parallel response to environmental factors. In these regions, growth cycle of annual species is usually limited to spring and very early summer (Hedge and Lamond 1982, Jamzad 2012) due to the very cold temperature of winters and harsh, hot and dry summers.

Evolution of pericarp surface in Nepeta s. s.

Tubercles and thorns are formed by exocarp cells (Budantsev and Lobova 1997), which are the ones responsible for the pericarp surface. In the smooth type, the length of exocarp cells is uniform whereas in the tuberculate/thorny-like type some cells, which form tubercles and thorns, are longer than others. Early diverging clades of our phylogeny have mostly nutlets with smooth/non-tuberculate surfaces. Within the Nepetoideae, few other genera have the tuberculate/thorny-like pericarp surface, mainly *Agastache* Gronov., and some species of *Hymenocrater* Fisch. et C. A. Mey. (Budantsev and Lobova 1997, Ryding 2010). In contrast, the genus *Nepeta* s. s. includes species with either smooth/non-tuberculate or tuberculate/thorny-like pericarp surface. The earliest diverging subclade within *Nepeta* s. s. (subclade I) shows this ancestral state (Fig. 4). Since nodal supports for some clades are low (Fig. 3), we cannot reconstruct character evolution with certainty. However, should the topology in Figure 3 be confirmed, the tuberculate/thorny-like pericarp surface could have evolved once within *Nepeta* s. s. in the common ancestor of subclades II, III, IV, V and VI, and then lost independently several times within subclades III, IV and V.

Nutlet pericarps have been widely studied in Lamiaceae and shown to provide useful taxonomic characters within the family at different taxonomic levels (Budantsev and Lobova 1997, Duletic-Lausevic and Marin 1999, Moon and Hong 2006, Moon *et al.* 2009, Ryding 1995, 2009, 2010). In one of the most comprehensive and recent studies in this field, Ryding (2010) found that variation in pericarp structure in tribe Mentheae was strongly correlated with phylogeny based on DNA analyses and gross morphological characters. Moon *et al.* (2009) recognised five different pericarp surface types in Mentheae based on differences in cell outline, anticlinal walls, cell boundary relief, and curvature of the outer periclinal wall. They suggested that representative nutlet features were phylogenetically informative within Mentheae mainly at the generic level. As discussed above, our study shows that pericarp characters of nutlets in *Nepeta* s. s. maybe phylogenetically informative below the generic level. However, a robust phylogeny of the group based on a thorough sampling for both nuclear and plastid molecular markers would be needed to better understand the evolution of the nutlet surface as well as other morphological and anatomical features of the group.

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