Contribution to the phylogeny of Caucasian Nonea (Boraginaceae) inferred from nrDNA ITS and trnL–F data

4 authors, including:

Murat Erdem Güzel
Karadeniz Technical University
19 PUBLICATIONS **124** CITATIONS

Serdar Makbul
Recep Tayyip Erdoğan Üniversitesi
56 PUBLICATIONS **603** CITATIONS

Kamil Coskuncelebi
Karadeniz Technical University
107 PUBLICATIONS **1,099** CITATIONS

Article

Contribution to the phylogeny of Caucasian *Nonea* (Boraginaceae) inferred from nrDNA ITS and *trnL-F* data

MURAT ERDEM GÜZEL[1], VUGAR KARIMOV[2], SERDAR MAKBUL[3] & KAMİL COŞKUNÇELEBİ[1]
[1]*Department of Biology, Faculty of Science, Karadeniz Technical University, 61080 Trabzon, Turkey*
[2]*Institute of Botany of the National Academy of Sciences of Azerbaijan. 40, Padamdar shosse, Baku-AZ1073, Azerbaijan*
[3]*Department of Biology, Faculty of Sciences and Arts, Recep Tayyip Erdoğan University, Rize, Turkey*
Author for correspondence: muraterdemguzel@hotmail.com

Abstract

Nonea (Boraginaceae) with nearly 35 species was divided into four sections based on the shape of mericarpids and the position of the anthers in the corolla tube. Although several comprehensive taxonomic studies have been performed on Turkish and European *Nonea* taxa, Caucasian ones have not been studied well. Therefore Caucasian *Nonea* need close attention with regard to molecular systematics. In this study, 15 Caucasian *Nonea* including *N. cyanocalyx* and *N. bakuensis* were evaluated with nrDNA ITS and cpDNA *trnL-F* sequence data using Maximum Parsimony and Bayesian Inference to reconstruct phylogeny. All examined members of *Nonea* were grouped at three main clades with weak to good support values based on nrITS and at two main clades with moderate to good support values based on *trnL-F*. Both trees did not coincide with the traditional sub-generic delimitation of *Nonea*, but nrITS tree supported monophyly of *Nonea*. On the other hand, *Pulmonaria* is deeply nested in *Nonea* in the *trnL-F* tree. Moreover, present findings support treating *N. cyanocalyx* and *N. bakuensis* as distinct species rather than subspecies and revealed a preliminary phylogenetic structure of little known Caucasian *Nonea*.

Keywords: Azerbaijan, Caucasia, Cryptanthera, phylogenetic, systematics

Introduction

Nonea Medik. with nearly 35 species is a genus of the Boraginaceae tribe Boragineae (Selvi *et al.* 2006). The main diversity center of the genus is the NE Turkey-Caucasian mountain systems and the Irano-Turanian-Anatolian highlands, while fewer species occur in Europe and in the Mediterranean (Selvi *et al.* 2006). The genus was first recognized by Medicus (1789) based on accrescent fruiting calyx and hairy fornices inserted at the throat of the corolla. After him, Candolle (1846) divided the genus into four sections based on the shape of mericarpids and the position of the anthers in the corolla tube (Selvi *et al.* 2002). However, Candolle's (1846) sectional treatment was not supported by a recent molecular study of Selvi *et al.* (2006). In Selvi *et al.* (2006), a new monotypic genus *Melanortocarya* Selvi, Bigazzi, Hilger & Papini was described based on *Nonea obtusifolia* (Willd.) DC. and the genus *Elizaldia* Willk. was transferred to the genus *Nonea*. Recently, these two taxonomic entities were confirmed by Chacón *et al.* (2016).

Nowadays several comprehensive studies (Selvi *et al.* 2002; Selvi *et al.* 2006; Cecchi *et al.* 2009) were performed on Turkish and European representatives of *Nonea*, however the Caucasian ones have been studied mostly floristically (Kusnezow 1915; Kadyrov 1957; Galushko 1980; Mtzchvetadze 1985; Karimov & Ali-zade 2016). Popov (1953) treated *Nonea* with 16 species under the tribe Boragineae Bercht. & J.Presl (=Anchuseae DC.) and followed Candolle's (1846) sectional categories in the Flora of the USSR. He also reported close relationships among *Pulmonaria* L., *Borago* L., *Nonea* and *Brunnera* Steven due to erect nutlet which is a rare morphological character in the tribe Boragineae. Besides these, Popov (1953) paid special attention to *N. caspica* (Willd.) G. Don. distributed from Central Asia to Europe and recognized four varieties (var. *bakuensis* M. Pop., var. *diffusa* M. Pop., var. *picta* M. Pop. and var. *turkestanica* M. Pop.). He also treated *N. pulla* (L.) DC. with five varieties (var. *rossica* (Stev.) M. Pop., var. *taurica* (Ldb.) Kuzn., var. *armeniaca* Kusn., var. *lencoranica* Kusn. and var. *persica* (Boiss.) M. Pop.) and two races (*N. armeniaca-N. lencoranica-N. persica* race and *N. cyanocalyx-N. voronovii* race). Later, Karimov (2016) recognized *N. bakuensis* Popov ex V. Karimov and *N. cyanocalyx* Popov ex V. Karimov as distinct species based on

the morphological examination of several own collections and herbarium specimens held in LE (Komarov Botanical Institute of RAS) and BAK (Azerbaijan National Academy of Sciences). However, *N. bakuensis* and *N. cyanocalyx* endemic to Caucasia are not currently listed as accepted species in online portals such as Euro+Med (2006-) and The Plant List (2010). Therefore, the taxonomical statuses of these two recently re-evaluated taxa need further clarification among the Caucasian *Nonea*. Currently, several molecular studies (Selvi *et al.* 2002; Selvi *et al.* 2006; Cecchi *et al.* 2009) successfully improved our taxonomical knowledge of *Nonea* members except for poorly known Caucasian representatives. In the present study, we aimed to clarify the phylogeny and taxonomy of poorly known Caucasian *Nonea* species, including recently re-evaluated *N. bakuensis* and *N. cyanocalyx*, based on nrDNA ITS and cpDNA 5'*trnL(UAA)-trnF* spacer (referred to as *trnL-F*) data.

Materials and methods

Taxon Sampling
Multiple sampling was used for each taxon whenever possible to observe the intraspecific DNA sequence variation. Eleven Caucasian *Nonea* taxa from 25 individuals were sequenced for the first time with respect to nrITS and *trnL-F*. A total of 60 sequences were generated from 15 *Nonea* taxa. All specimens were collected from Azerbaijan and Nakhchivan between 2008 and 2016 by the second author. Detailed information of vouchers including GenBank accession number is given in Appendix 1. Thiers (2008+) was adopted for the herbarium abbreviations. Specimens of *N. bakuensis* and *N. cyanocalyx* used in the present paper were collected from type localities.

Anchusa officinalis L., *Borago officinalis* L.and *Brunnera orientalis* (Schenk) I. M. Johnst. belonging to tribe Boragineae were used as outgroup taxa for the phylogenetic analysis. Additionally *Melanortocarya obtusifolia* Selvi, Bigazzi, Hilger & Papini (=*Nonea obtusifolia*), *Pulmonaria angustifolia* L., *P. mollis* Hornem., *P. obscura* Dumort., *P. hirta* L. (=*P. picta* Rouy) and *P. cesatiana* (Fenzl & Friedr.) Selvi & al. (=*Paraskevia cesatiana* (Fenzl & Friedr.) W. Sauer & G. Sauer) were included in the analysis due to close relationship between *Nonea* and *Pulmonaria*. In the phylograms, names of taxa whose sequences were imported from GenBank, are followed their currently accepted names to avoid confusion.

DNA extraction, PCR amplification, sequencing, and phylogenetic analyses
Total genomic DNA was extracted from herbarium material following the modified CTAB extraction procedure of Doyle & Doyle (1987). The nrITS region and *trnL-F* were amplified with universal ITSA/ITSB (Blattner 1999) and with the universal primers trnC/trnF (Taberlet *et al.* 1991), respectively, according to the PCR conditions of Gültepe *et al.* (2010). PCR products were sequenced with the aid of Macrogen Inc. (Seoul, Korea) by use of the same primers. For the phylogenetic analysis, 60 newly generated sequences (32 of them belonging to ITS and 28 belonging to *trnL-F*) and 71 sequences (36 of them belonging to ITS and 35 belonging to *trnL-F*) downloaded from GenBank (Appendix 2) were used. Pairwise Distance Analysis (PDA) was performed in MEGA version 7 (Kumar *et al.* 2016) to observe similarity of the studied taxa. The sequences were aligned with Muscle v.3.8.31 (Edgar 2004) and edited in PhyDE v.0.9971 (Müller *et al.* 2010). Indels were coded as informative characters according to the Simple Indel Coding (SIC) method (Simmons & Ochoterena 2000) as implemented in the program SeqState version 1.40 (Müller 2005a) and added at the end of the sequence data set. Phylogenetic relationships were reconstructed using Maximum Parsimony (MP) and Bayesian Inference (BI) analyses. MP analyses were carried out using the Parsimony Ratchet (Nixon 1999) with PRAP (Müller 2004). The generated command files contain standard ratchet settings (200 ratchet iterations with 25% of the positions randomly up-weighted (weight = 2) during each replicate and 10 random addition cycles). The analysis was run in PAUP* version 4.0b10 (Swofford 2003) using heuristic search with the following parameters: all characters have equal weight, gaps are treated as 'missing', simple addition of sequences, TBR branching swapping, maxtrees setting to 100 and auto-increased by 100, one non-binary starting tree arbitrarily dichotomized before branch swapping, only one tree saved. A majority rule consensus tree was calculated from the most parsimonious trees. Jackknife (JK) support values for the nodes found in the MP analysis were calculated in PAUP* applying the optimal jackknife parameters according to Farris *et al.* (1996) and Müller (2005b) with the following parameters: 10,000 jackknife replicates using the TBR branch swapping algorithm with 36.788% of characters deleted and one tree held during each replicate. Prior to BI analyses, the nucleotide substitution model that best fits the datasets were determined for nrITS and *trnL-F* with MrModeltest 2.3 (Nylander 2004), following the Akaike Information Criterion (AIC). BI analyses in MrBayes 3.2 were performed with four simultaneous runs of Metropolis-coupled Markov Chains Monte

Carlo (MCMCMC), each with four parallel Markov chains. Each chain was performed for 20 million generations and, starting with a random tree, one tree was saved every 1000th generation. In initial runs the default temperature (0.1) resulted in low proportion exchanges between cold and heated chains. Therefore heating temperature was reduced to 0.05, which successfully increased the efficiency of the Metropolis coupling. For other parameters the default settings of the program were left unchanged. A conservative burn-in of 0.2 (i.e. discarding the first 20% of the trees) was applied. The remaining trees were used to generate a majority rule consensus tree. The support values calculated from the analyses were gradated as following: full (JK=100, BI=1), strong (JK=90–99, BI=0.9–0.99), good (JK=80–89, BI=0.8–0.89), moderate (JK=60–79, BI=0.8–0.89), weak (JK=50–59, BI=0.5–0.79).

Incongruence Length Difference (ILD) test (Farris *et al.* 1994) was performed to assess the congruence between the nrITS and *trnL-F* datasets in PAUP* version 4.0b10 (Swofford 2003). For this test, the following parameters were used: heuristic search of 1000 replicates, each with 100 random addition searches, maxtrees set to 1 and one tree held each step. As significance threshold for congruence or homogeneity of the partitions a P value of > 0.05 or 0.01 is considered as appropriate by Sullivan (1996) and Cunningham (1997), respectivily.

Results

ILD test resulted in incongruence with high significance (P= 0.001) level between nrITS and *trnL-F* datasets. Therefore, the two datasets were analyzed separately. Results of ILD test is corroborated by the incongruent tree topologies based on nrITS and *trnL-F* datasets as well (Figure 1 and 2). The optimal models revealed for nrITS and *trnL-F* were SYM+G and GTR+G, respectively.

nrDNA ITS dataset

In the present study, 11 Caucasian *Nonea* taxa from 23 individuals were examined for the first time with respect to nrITS region. A total of 32 nrITS sequences (for GenBank accession numbers see Appendix 1) were generated from 15 *Nonea* taxa. The full length of the nrITS (ITS1, 5.8S and ITS2) varied between 637 to 653 bp in the 67 (59 in group +8 outgroup) sequences. Of a total of 655 characters in the aligned data set, 139 bp were parsimony informative. Simple Indel Coding increased the total number of characters to 701 and the number of parsimony informative characters to 185.

Bayesian phylogram (majority rule consensus tree) of *Nonea* based on nrITS with JK support values is presented in Figure 1. According to this phylogram, members of *Nonea* were grouped in three main clades with moderate support values (JK = 52 and PP = 1.0) except for *N. obtusifolia* which has already been transferred to *Melanortocarya*. According to pairwise distance analysis (PDA) based on nrITS, *N. pulla* DC. and *N. atra* Griseb., *N. melanocarpa* Boiss. and *N. pisidica* Selvi, Bigazzi & Hilger, *N. flavescens* (C. A. Mey.) Fisch. & C. A. Mey. and *N. setosa* (Lehm.) Roem. & Schult. are the closest taxa with 100 % similarity. *N. palmyrensis* and *Nonea heterostemon* Murb. (=*Elizaldia heterostemon* (Murb.) I. M. Johnst.) are the farthest taxa with 84.9 % similarity based on nrITS data (Table 1). The most similar taxa to *N. bakuensis* (Figure 3a) and *N. cyanocalyx* (Figure 3c) are *N. caspica* (Figure 3b) and *N. armeniaca* (Kusn.) Grossh. (Figure 3d) with 99.22 % and 99.8 % similarity, respectively (Table 1).

trnL-F dataset

In this study, 10 Caucasian *Nonea* taxa from 25 individuals (for GenBank accession numbers see Appendix 1) were examined for the first time with respect to *trnL-F*. A total of 28 *trnL-F* sequences (for GenBank accession numbers see Appendix 1) were generated from 13 *Nonea* taxa. The length of the *trnL-F* varied between 878 to 859 bp in the 63 (55 in group +8 outgroup) samples. Of a total of 918 characters in the aligned data set, 47 bp were parsimony informative. Simple Indel Coding increased the total number of characters to 941 and the number of parsimony informative characters to 70.

Bayesian phylogram (majority rule consensus tree) of *Nonea* based on *trnL-F* with JK support values is presented in Figure 2. According to the phylogram, members of *Nonea* grouped in two main clades with strong support values (JK = 88 and PP = 1.0) including *N. obtusifolia* which has been transferred to *Melanortocarya* Selvi, Bigazzi, Hilger & Papini. Furthermore all members of *Pulmonaria* used in this study are deeply nested in *Nonea* with strong support values (JK = 95 and PP = 1.0). According to PDA based on *trnL-F* (Table 2), 48 pairs of taxa are share the same *trnL-F* profile with 100 % similarity. *N. stenosolen* Boiss. & Balansa and *N. pulmonarioides* Boiss. & Balansa are the farthest taxa with 95.7 % similarity. The most similar taxa to *N. bakuensis* and *N. cyanocalyx* is *N. pisidica*, *N. polychroma*

Table 1. Pairwise Distance Analysis results based on nrITS among the studied taxa. Important values are shown in boldface.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34
1 N. calycina DQ269669																																		
2 N. heterostemon DQ269671	0.118																																	
3 N. anchusoides DQ269672	0.093	0.127																																
4 N. atra DQ269673	0.085	0.119	0.064																															
5 N. caspica DQ269674	0.100	0.095	0.093	0.086																														
6 N. echioides DQ269675	0.107	0.134	0.100	0.093	0.093																													
7 N. intermedia DQ269676	0.086	0.111	0.064	0.057	0.079	0.086																												
8 N. macrantha DQ269677	0.062	0.079	0.041	0.048	0.063	0.069	0.049																											
9 N. macrosperma DQ269678	0.118	0.145	0.087	0.087	0.110	0.118	0.027	0.079																										
10 N. micrantha DQ269679	0.086	0.120	0.057	0.071	0.086	0.093	0.072	0.035	0.103																									
11 N. monticola DQ269680	0.063	0.087	0.042	0.048	0.056	0.070	0.034	0.013	0.064	0.035																								
12 N. dumanii JQ031034	0.055	0.087	0.034	0.041	0.056	0.062	0.042	0.007	0.071	0.028	0.007																							
13 N. pisidica DQ269684	0.077	0.087	0.070	0.063	0.020	0.084	0.063	0.041	0.094	0.063	0.041	0.034																						
14 N. pulmonarioides DQ269685	0.063	0.094	0.042	0.048	0.063	0.070	0.049	0.013	0.079	0.035	0.013	0.007	0.041																					
15 N. stenosolen DQ269686	0.078	0.111	0.049	0.064	0.079	0.085	0.064	0.027	0.095	0.007	0.028	0.021	0.056	0.028																				
16 N. vesicaria AY383304	0.093	0.104	0.102	0.078	0.109	0.116	0.094	0.070	0.127	0.078	0.071	0.063	0.085	0.071	0.071																			
17 N. pallens FJ590418	0.078	0.111	0.071	0.063	0.071	0.085	0.056	0.041	0.087	0.064	0.041	0.034	0.048	0.041	0.056	0.086																		
18 N. palmyrensis FJ590419	0.123	0.151	0.093	0.108	0.108	0.056	0.109	0.070	0.142	0.085	0.070	0.063	0.100	0.070	0.078	0.132	0.100																	
19 N. pulla AY383317	0.085	0.119	0.064	0.000	0.086	0.093	0.057	0.048	0.087	0.071	0.048	0.041	0.063	0.048	0.064	0.078	0.063	0.108																
20 N. polychroma DQ350133	0.092	0.095	0.085	0.077	0.020	0.084	0.078	0.055	0.109	0.078	0.055	0.048	0.013	0.055	0.070	0.093	0.063	0.100	0.077															
21 N. luthea N11	0.085	0.103	0.078	0.071	0.063	0.092	0.071	0.048	0.102	0.071	0.048	0.041	0.041	0.048	0.064	0.093	0.056	0.108	0.071	0.055														
22 N. bakuensis N20	0.086	0.128	0.087	0.079	0.078	0.086	0.079	0.056	0.111	0.079	0.056	0.049	0.056	0.056	0.072	0.102	0.064	0.117	0.079	0.070	0.070													
23 N. melanocarpa N26	0.077	0.087	0.070	0.063	0.020	0.084	0.063	0.041	0.094	0.063	0.041	0.034	0.000	0.041	0.056	0.085	0.048	0.100	0.063	0.013	0.041	0.056												
24 N. armeniaca N30	0.084	0.110	0.063	0.055	0.070	0.091	0.070	0.034	0.101	0.056	0.034	0.027	0.048	0.034	0.049	0.077	0.063	0.092	0.055	0.062	0.062	0.078	0.048											
25 N. persica N32	0.084	0.110	0.063	0.055	0.070	0.092	0.071	0.034	0.102	0.056	0.034	0.027	0.048	0.034	0.049	0.078	0.063	0.077	0.055	0.062	0.070	0.078	0.048	0.013										
26 N. leucoranika N34	0.100	0.126	0.078	0.070	0.085	0.107	0.086	0.048	0.118	0.071	0.048	0.041	0.063	0.048	0.064	0.093	0.078	0.108	0.070	0.077	0.077	0.093	0.063	0.027	0.027									
27 N. setosa N13	0.085	0.103	0.078	0.071	0.071	0.085	0.071	0.048	0.102	0.071	0.048	0.041	0.048	0.048	0.064	0.093	0.063	0.100	0.071	0.063	0.063	0.079	0.048	0.070	0.070	0.085								
28 N. versicolor N15	0.093	0.103	0.086	0.078	0.071	0.093	0.064	0.055	0.094	0.079	0.041	0.048	0.056	0.056	0.071	0.101	0.071	0.108	0.078	0.070	0.020	0.086	0.056	0.077	0.077	0.093	0.007							
29 N. cyanocalyx N41	0.077	0.110	0.056	0.048	0.078	0.084	0.063	0.027	0.094	0.049	0.027	0.020	0.055	0.027	0.042	0.070	0.056	0.070	0.048	0.069	0.063	0.071	0.055	0.020	0.007	0.034	0.063	0.034						
30 N. diffusa N47	0.084	0.087	0.078	0.070	0.014	0.077	0.071	0.048	0.102	0.071	0.048	0.041	0.007	0.048	0.063	0.093	0.055	0.092	0.070	0.007	0.007	0.063	0.007	0.055	0.055	0.070	0.056	0.063	0.062					
31 N. alpestris N48	0.085	0.103	0.078	0.071	0.071	0.085	0.071	0.048	0.102	0.071	0.048	0.041	0.048	0.048	0.064	0.093	0.063	0.100	0.071	0.063	0.063	0.079	0.048	0.070	0.070	0.085	0.000	0.007	0.063	0.056				
32 N. rosea N49	0.086	0.120	0.094	0.086	0.078	0.108	0.087	0.063	0.119	0.087	0.063	0.056	0.055	0.063	0.079	0.110	0.071	0.124	0.086	0.070	0.070	0.093	0.078	0.085	0.085	0.101	0.042	0.049	0.078	0.063	0.042			
33 N. daghestanica N50	0.063	0.111	0.071	0.064	0.071	0.077	0.064	0.041	0.095	0.064	0.041	0.034	0.048	0.041	0.056	0.086	0.042	0.093	0.064	0.063	0.063	0.064	0.048	0.063	0.063	0.078	0.063	0.071	0.056	0.056	0.063	0.056		
34 N. decurrens N54	0.101	0.127	0.079	0.071	0.094	0.101	0.027	0.056	0.056	0.087	0.049	0.056	0.078	0.056	0.079	0.110	0.071	0.125	0.071	0.093	0.093	0.095	0.078	0.085	0.085	0.101	0.056	0.049	0.056	0.085	0.056	0.071	0.079	
35 N. flavescens N57	0.085	0.103	0.078	0.071	0.071	0.085	0.071	0.048	0.102	0.071	0.048	0.041	0.048	0.048	0.064	0.093	0.063	0.100	0.071	0.063	0.063	0.079	0.048	0.070	0.070	0.085	0.000	0.007	0.063	0.056	0.000	0.042	0.063	0.056

Table 2. Pairwise Distance Analysis results based on trnL-F among the studied taxa. Important values are shown in boldface.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
1	N. calycina DQ269669																															
2	N. heterostemon DQ269663	0.007																														
3	N. pulla AY383275	0.019	0.019																													
4	N. anchusoides AY627869	0.015	0.015	0.011																												
5	N. echioides AY627871	0.015	0.015	0.004	0.015																											
6	N. intermedia AY627872	0.023	0.019	0.011	0.007	0.015																										
7	N. monticola AY627873	0.023	0.023	0.011	0.007	0.015	0.007																									
8	N. pallens AY627875	0.019	0.019	0.000	0.011	0.004	0.011	0.011																								
9	N. pisidica AY627877	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019																							
10	N. polychroma AY627878	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000																						
11	N. pulmonarioides AY627879	0.031	0.031	0.023	0.019	0.023	0.019	0.019	0.023	0.038	0.038																					
12	N. stenosolen AY627880	0.035	0.034	0.023	0.034	0.019	0.034	0.034	0.023	0.019	0.019	0.043																				
13	N. versicolor AY627881	0.019	0.023	0.004	0.015	0.007	0.015	0.015	0.004	0.022	0.022	0.027	0.026																			
14	N. caspica JX976917	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022																		
15	N. atra DQ269664	0.027	0.027	0.007	0.011	0.011	0.011	0.011	0.007	0.026	0.026	0.023	0.030	0.011	0.026																	
16	N. macrantha DQ269665	0.027	0.027	0.015	0.027	0.011	0.027	0.026	0.015	0.019	0.019	0.035	0.023	0.019	0.019	0.023																
17	N. macrosperma DQ269666	0.019	0.019	0.007	0.004	0.011	0.004	0.004	0.007	0.026	0.026	0.015	0.030	0.011	0.026	0.007	0.023															
18	N. micrantha DQ269667	0.015	0.015	0.011	0.023	0.007	0.023	0.023	0.011	0.015	0.015	0.031	0.027	0.015	0.015	0.019	0.019	0.019														
19	N. vesicaria DQ269668	0.015	0.015	0.011	0.023	0.007	0.023	0.023	0.011	0.015	0.015	0.031	0.027	0.015	0.015	0.019	0.019	0.019	0.007													
20	N. persica N31	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015												
21	N. versicolor N15	0.019	0.023	0.004	0.015	0.007	0.015	0.015	0.004	0.022	0.022	0.027	0.026	0.000	0.022	0.011	0.019	0.011	0.015	0.015	0.004											
22	N. daghestanika N37	0.019	0.019	0.000	0.011	0.004	0.011	0.011	0.000	0.019	0.019	0.023	0.023	0.004	0.019	0.007	0.015	0.007	0.011	0.011	0.019	0.022										
23	N. armeniaca N30	0.019	0.026	0.015	0.026	0.011	0.026	0.026	0.015	0.019	0.019	0.035	0.023	0.015	0.019	0.023	0.023	0.023	0.019	0.019	0.019	0.019	0.015									
24	N. cyanocalyx N41	0.027	0.026	0.015	0.026	0.011	0.026	0.026	0.015	0.011	0.011	0.035	0.015	0.019	0.011	0.023	0.015	0.023	0.019	0.019	0.011	0.019	0.015	0.007								
25	N. lencoranica N34	0.026	0.026	0.022	0.034	0.019	0.034	0.034	0.022	0.004	0.004	0.042	0.023	0.026	0.004	0.030	0.023	0.030	0.019	0.019	0.004	0.026	0.022	0.015	0.015							
26	N. bakuensis N20	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015	0.000	0.022	0.019	0.011	0.011	0.004						
27	N. diffusa N47	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015	0.000	0.022	0.019	0.011	0.011	0.004	0.000					
28	N. melanocarpa N26	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015	0.000	0.022	0.019	0.011	0.011	0.004	0.000	0.000				
29	N. lencoranica N34	0.026	0.026	0.015	0.026	0.011	0.026	0.026	0.015	0.004	0.004	0.034	0.015	0.019	0.004	0.022	0.015	0.022	0.019	0.019	0.004	0.019	0.015	0.007	0.007	0.007	0.004	0.004	0.004			
30	N. alpestris N48	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015	0.000	0.022	0.019	0.011	0.011	0.004	0.000	0.000	0.000	0.004		
31	N. rosea N49	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015	0.000	0.022	0.019	0.011	0.011	0.004	0.000	0.000	0.000	0.004	0.000	
32	N. flavescens N57	0.023	0.022	0.019	0.030	0.015	0.030	0.030	0.019	0.000	0.000	0.038	0.019	0.022	0.000	0.026	0.019	0.026	0.015	0.015	0.000	0.022	0.019	0.011	0.011	0.004	0.000	0.000	0.000	0.004	0.000	0.000

Selvi & Bigazzi, *N. caspica*, *N. persica* Boiss., and *N. armeniaca* with 100 % and 99.93 % similarity, respectively (Table 2). The newly studied Caucasian *Nonea* taxa were placed at two different subclades (Figure 2-Clade Bv and Clade Bvi). Most of them (except *N. versicolor* and *N. daghestanica*) were clustered at Clade Bvi. The Clade VI also comprises *N. macrantha* (Riedl) A. Baytop endemic to Turkey and *N. stenosolen* Boiss. & Balansa distributed out of Caucasia.

Discussion

A monophyletic *Nonea* first shown by Selvi *et al.* (2006) is confirmed by the current study inferred from nrITS dataset with a broader sampling. Besides, the nrITS dataset also confirmed that the genus *Nonea* is sister to the genus *Pulmonaria* as already suggested by Selvi *et al.* (2006). On the other hand, members of *Pulmonaria* are deeply nested in *Nonea* based on the *trnL-F* dataset. Therefore, the *trnL-F* dataset do not support the monophyly of the genus *Nonea*. These results suggest to potential events of ancient and more recent reticulation or chloroplast capture in the evolution of *Nonea* and *Pulmonaria*. The genus *Nonea* was firstly split into sections by Candolle (1846) and later by Popov (1953). Although Popov (1953) mainly followed Candolle's (1846) view, he also described several new series and sections within the genus. Present molecular findings do not fully coincide with the subgeneric delimitation of Candolle (1846) (Figures 1 and 2) as indicated by Selvi *et al.* (2006). However, members of sect. *Orthocaryum* DC. (except *N. rosea* (M. Bieb.) Link) are aggregated within the same clade (Figure 1-Clade II) based on nrITS. On the other hand, members of sect. *Orthocaryum* and sect. *Cryptanthera* DC. formed a polytomy in "Clade Bv" and "Clade Bvi" (Figure 2) based on *trnL-F* sequence data.

Discussion of the phylogenetic trees (Figures 1 and 2) are given in the following clade by clade with priority on nrITS in accordance with traditional taxonomical treatment of Caucasian *Nonea*. Results obtained from *trnL-F* will be discussed alongside where relevant.

Clade I (Figure 1): It comprises 3 of the 11 newly studied Caucasian *Nonea*. One of them is *N. bakuensis*, endemic to Azerbaijan. It was first treated as a variety of *N. caspica* (Popov 1953) which has a wide distribution range in the Caucasus. However, Karimov (2016) changed its status to species level based solely on morphological differences. Later Karimov & Illarionova (2018) confirmed the separation of *N. bakuensis* from the closely related species *N. caspica, N. diffusa*, and *N. melanocarpa* Boiss. based on seed micro-morphological features. Present molecular findings based on nrITS also support the separation of *N. bakuensis* from the closely related species with moderate support values (JK = 55 and PP = 1). Although four accessions of *N. bakunesis* are clustered together, there is no resolution at infraspecific level. In contrast to nrITS, these four and further morphologically distinct taxa are polytomously clustered based on *trnL-F* (Figure 2-Clade Bvi). Therefore, *trnL-F* is insufficient to reveal phylogenetic relationships within these taxa. Although *N. caspica* has a wide distribution from Central Asia to Europe, *N. bakuensis* (=*N. caspica* var. *bakuensis*) has limited distribution only around Baku (Popov 1953).

N. diffusa Boiss. & Buhse treated as a variety of *N. caspica* by Popov (1953) was listed under the heterotypic synonym of *N. caspica* in database of Euro+Med (2006-). Present findings based on five newly generated accessions belonging to *N. diffusa* did not support the view of Popov (1953), but supported its synonymy to *N. caspica* (Figure 1-Clade 1 and Figure 2-Clade Bvi).

N. rosea (M. Bieb.) Link endemic to the Caucasus was considered a putative hybrid of members belonging to *Nonea* sect. *Orthocaryum* DC. (Popov 1953). *N. rosea*, evaluated for the first time based on both nuclear and plastid data in the present study, is nested in the members of *Nonea* sect. *Cryptanthera* DC. rather than members of sect. *Orthocaryum* (Figure 1). This means that the phylogenetic position of *N. rosea* contradicts Popov's (1953) view, but this needs further morphological and molecular studies to clarify its relationship and putative hybrid origin.

N. melanocarpa, evaluated for second time based on nuclear and for the first time based on plastid data in the present study, is distributed throughout Central Asia and Turkey (Popov 1953; Euro+Med 2006-). The species firstly studied by Cecchi *et al.* (2009) based on ITS1. Results of Cecchi *et al.* (2009) revealed that *N. melanocarpa* is closely related to *N. echioides* and *N. palmyrensis*. Our analyses based on nrITS (Figure 1), with addition their data to our dataset, mostly coincide with results of Cecchi *et al.* (2009). However our results contained three newly generated accessions belonging to *N. melanocarpa* suggest that it is sister to *N. pisidica* Selvi, Bigazzi & Hilger and a member of the *N. caspica* group (Figure 1- Clade I) as previously proposed by Riedl (1963). Furthermore *N. melanocarpa, N. diffusa* and *N. caspica* share similar fruit surface pattern (Karimov & Illarionova 2018). Parallel to Riedl (1963) and Karimov & Illarionova (2018), two newly generated *trnL-F* accessions of *N. melanocarpa* formed a polytomy with *N.*

caspica, N. pisidica and the rest in Clad B-vi (Figure 2) but were not close to *N. echioides*. This may suggest that *N. melanocarpa* is better placed in the *N. caspica* group. *N. pisidica* is closely related to *N. caspica* and *N. polychroma* Selvi & Bigazzi according to Bigazzi *et al.* (2004). However, our results suggest that *N. melanocarpa* is closer to *N. pisidica* than the others.
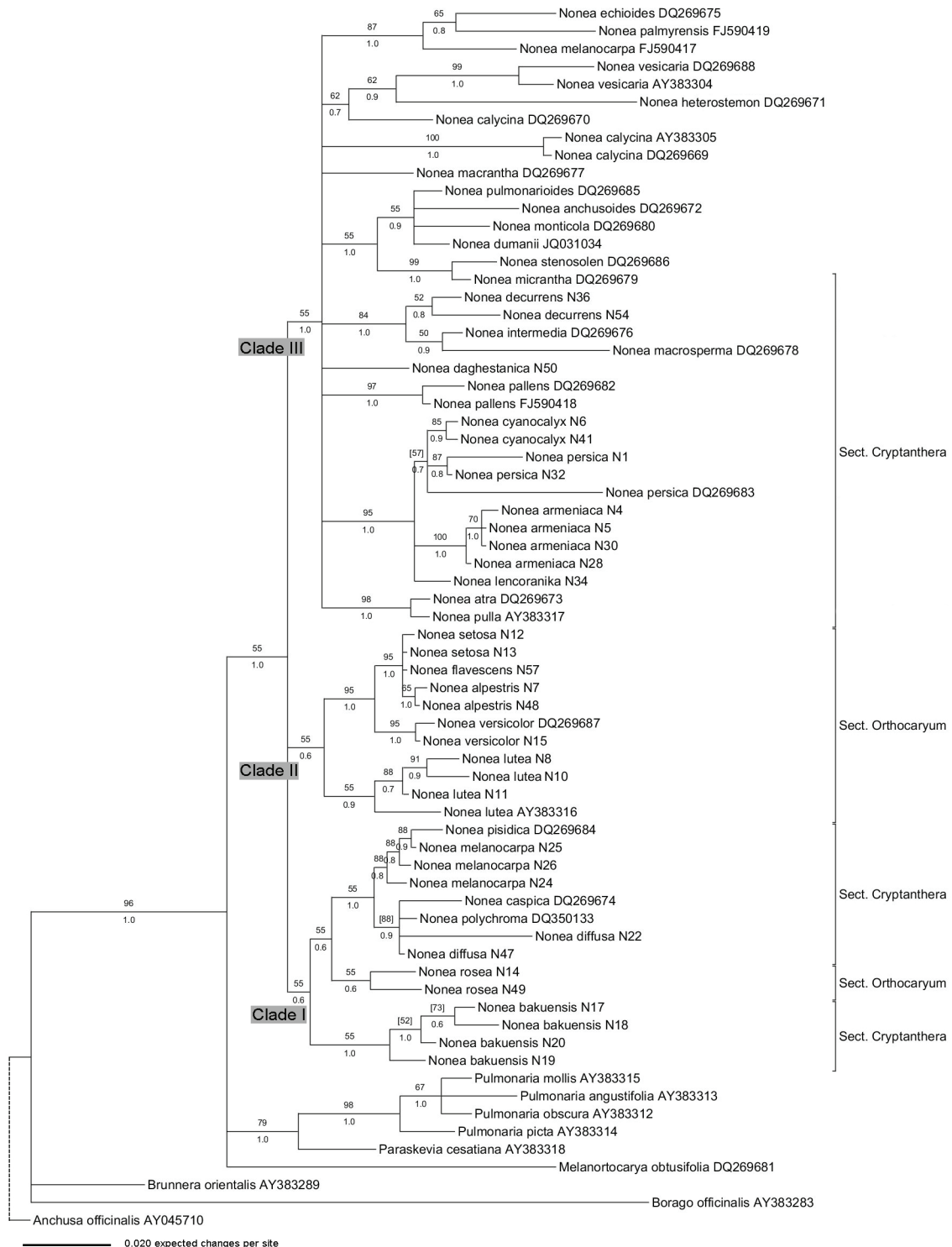

FIGURE 1. Majority rule consensus phylogram of *Nonea* from the Bayesian analysis based on the nrITS dataset. Support values: maximum parsimony jackknife above branches, bayesian posterior probability below branches. CI = 0.608 and RI = 0.737.
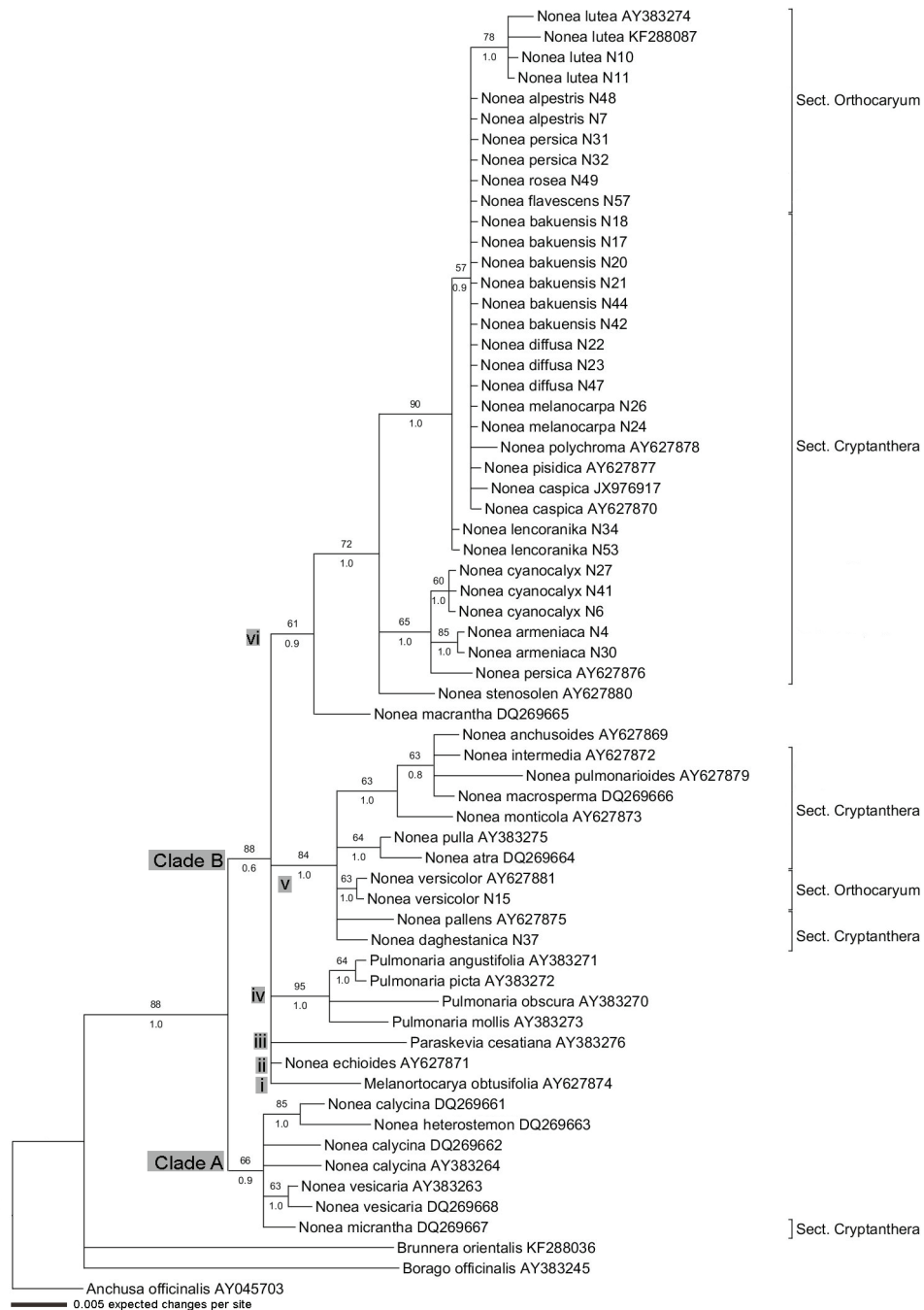
FIGURE 2. Majority rule consensus phylogram of *Nonea* from the Bayesian analysis based on the *trnL-F* dataset. Support values: maximum parsimony jackknife above branches, bayesian posterior probability below branches. CI = 0.879 and RI = 0.926.

Clade II (Figure 1): It comprises 3 of the 11 newly studied Caucasian *Nonea*. *N. lutea* (Desr.) DC. is chiefly distributed in the Caucasus but it was introduced to Europe (Popov 1953). This species was firstly included in molecular studies by Selvi *et al.* (2006) and then by Cohen (2014) with a unique sample, but it was sampled with five newly generated sequences belonging to three individuals in the current study. There is a contradiction between the results of present study and Selvi *et al.* (2006). Although *N. lutea* clustered with members of sect. *Cryptanthera* in Selvi *et al.* (2006), all samples (including GenBank sequence belonging to *N. lutea*) clustered with members of sect. *Orthocaryum* in the current study (Figure 1-Clade II). This contradiction basically resulted from the fact that although the two datasets were separately analyzed in this study, a combined dataset was analyzed in Selvi *et al.* (2006). *N. lutea* characterizes in both trees with moderate to good support values (Figure 1-Clade II and Figure-Clade Bvi). Furthermore Popov (1953) highlighted close relationships among yellow flowered taxa (*N. lutea, N. flavescens* and *N. setosa*) belonging to series

Flavescentes M. Pop. This is another good indication to consider why nrITS and *trnL-F* dataset are not to be combined in the present study. Pale violet flowered *N. versicolor* (Steven) Sweet is closely related to pale yellow flowered *N. alpestris* (Steven) G. Don based on nrITS. Although Popov (1953) reported that these two taxa are doubtful provisional species due to similar morphological features, they are listed as accepted taxa in the database of Euro+Med (2006-). In line with Euro+Med (2006-), accessions of *N. versicolor* and *N. alpestris* are clustered together in the nrITS tree (Figure 1-Clade II) with good to strong support values. In contrast to the nrITS tree, the *trnL-F* tree does not support their close relatedness (Figure 2-Clade Bv and Bvi). Therefore, the current molecular analyses support the segregation of *N. versicolor* and *N. alpestris* as separate species. *N. setosa* (Lehm.) Roem. & Schult. endemic to Caucasus and *N. flavescens* Fisch. & C.A.Mey. were evaluated for the first time in this study based on molecular data. These two species were affiliated with *N. lutea* (Popov 1953). Leaf moprhology of *N. setosa* and *N. flavescens* is more similar to each other than to *N. lutea*. *N. flavescens* differs from *N. setosa* in its dense inflorescence in fruiting and smaller flowers. The closeness of *N. setosa* and *N. flavescens* is confirmed by the current analysis based on nrITS in accordance with Popov (1953). Furthermore *N. setosa* and *N. flavescens* are more closely related to *N. alpestris* and *N. versicolor* than to *N. lutea*. However, there is no clear delimitation among *N. setosa*, *N. flavescens* and *N. alpestris,* which form a polytomy based on both nrITS and *trnL-F* (Figure 1-clade II and Figure 2-clade Bvi).

FIGURE 3. Images of *N. bakuensis* Popov ex V. Karimov (a), *N. caspica* (Willd.) G. Don. (b), *N. cyanocalyx* Popov ex V. Karimov (c) and *N. armeniaca* Kusn. from the habitat (d) (photographed by Karimov).

Clade III (Figure 1): It comprises 5 of the 11 newly studied Caucasian *Nonea*. *N. pulla* group was established and discussed by Selvi & Bigazzi (2001) with respect to morphology and taxonomy. Popov (1953) listed 5 varieties (var. *rossica* (Stev.) M. Pop., var. *taurica* (Ldb.) Kuzn., var. *armeniaca* Kusn., var. *persica* (Boiss.) M. Pop. and var. *lencoranica* Kusn.) and two races (*N. armeniaca-N. lencoranica-N. persica* race and *N. cyanocalyx-N. voronovii* race) under *N. pulla*. According to Selvi & Bigazzi (2001), *N. persica* Boiss. is a distinct species and *N. pulla* (L.) DC. var. *armeniaca* Kusn. is a synonym of *N. persica* within the *N. pulla* group. Contrary to Selvi & Bigazzi (2001), Popov (1953) kept var. *armeniaca* separated from var. *persica* based on its shorter and wider leaves and vertical root. Agreeing with Popov (1953), Grossheim (1967) elevated var. *armeniaca* to specific level based on morphological traits. In line with Popov (1953) and Grossheim (1967), the present molecular findings support to treat *N. armeniaca* (≡*N. pulla* var. *armeniaca*) as a separate species because four newly generated nrITS sequences belonging to *N. armeniaca* clustered together with full support values (Figure 1-Clade III). Furthermore, two newly generated *trnL-F* sequences belonging to *N. armeniaca* clustered together with strong support values (Figure 2-Clade Bvi). The current results support to Grossheim's (1967) view. *N. lencoranica*, which is another member of the *N. pulla* group, was listed as variety of *N. pulla* by Popov (1953). Contrary to Popov (1953), it was treated as a separate species by Grossheim (1967). However, the current molecular analyses do not present any resolution for these taxa in both trees (Figure 1-Clade III and Figure 2-Clade Bvi).

N. cyanocalyx was listed as a part of the *N. cyanocalyx-N. voronovii* race under *N. pulla* by Popov (1953). Afterwards, Karimov (2016) re-evaluated *N. cyanocalyx* as a separate species based on morphological data. Later Karimov & Illarionova (2018) confirmed the separation of *N. cyanocalyx* based on seed micro morphological features. *N. cyanocalyx* is represented with five newly generated sequences in the current study. Both trees support to treat *N. cyanocalyx* as a separate taxon with strong support values (Figure 1-Clade III and Figure 2-Clade Bvi). Furthermore Popov (1953) emphasized separation of mesic *N. cyanocalyx-N. voronovii* race with its high mountains distribution, larger calyces, and longer teeth in the *N. pulla* group. These ecological and morphological features might lead to separation of *N. cyanocalyx* from the closely related taxa. According to the trees, *N. cyanocalyx* is closely related to *N. persica* and *N. armeniaca*.

N. daghestanica Kusn. is evaluated for the first time in the present study with two newly generated sequences. It is morphologically closely related to *N. pulla* (Popov 1953) and listed as unresolved taxa in The Plant List (2010). The *trnL-F* tree (Figure 2-Clade Bv) supports Popov's view with strong support values (JK = 84 and PP = 1). Unfortunately, nrITS tree (Figure 1-Clade III) does not provide resolution with respect to its relatives. Thus, *N. daghestanica* needs further studies to clarify its placement.

N. decurrens (C.A.M.) G. Don, endemic to the Caucasus, is evaluated for the first time in the present study with two newly generated sequences belonging to nrITS. It was classified in series *Pullae* M. Pop. by Popov (1953). *N. decurrens* and *N. intermedia* Ledeb. are morphologically closely related taxa according to Popov (1953). The current results confirmed their close affinity based on nrITS data. In fact, *N. decurrens* is sister to *N. intermedia* Ledeb.

Conclusion

Monophyly of *Nonea* is confirmed based on nrITS data by the current study. On the other hand, results obtained from the *trnL-F* dataset do not support monophyly of the genus. The current findings indicated that traditional sub-generic classification of *Nonea* cannot be fully supported with molecular data. The current study also supports that recently re-evaluated *N. bakuensis* is a distinct species and closely related to *N. caspica, N. diffusa*, and *N. melanocarpa. N. cyanocalyx* is another recently re-evaluated species from Azerbaijan and sister to *N. persica*. The analyses support its separation as a distinct species in the *N. pulla* group. Taxa below species, such as varieties, became distinguishable from species when divergent natural selection led to gaps in the distribution of morphology due to geographical and ecological factors. The analyses revealed that the phylogenetic position based on nrITS and morphological classification of *N. rosea* are not in line with each other. On the other hand, our *trnL-F* tree did not provide any resolution for *N. rosea*. Thus, *N. rosea* needs further studies to clarify its phylogenetic position. Both trees support the synonymy of *N. diffusa* under *N. caspica* and the separation of *N. versicolor* from *N. alpestris*. The nrITS tree supports to treat *N. alpestris* and *N. decurrens* as separate species. The current analysis indicated that *N. setosa* and *N. flavescens* are more closely related to *N. alpestris* and *N. versicolor* than to *N. lutea*. The present molecular findings support to treat *N. armeniaca* (=*N. pulla* var. *armeniaca*) as a separate species.

Acknowledgments

References

Bigazzi, M., Selvi, F. & Hilger, H.H. (2004) *Nonea pisidica* (Boraginaceae-Boragineae), a new species from southwest Anatolia and its relationships inferred from karyology and cpDNA sequences. *Plant Biosystems - An International Journal Dealing with all Aspects of Plant Biology* 138 (2): 135–144.
https://doi.org/10.1080/11263500412331283744

Blattner, F.R. (1999) Direct amplification of the entire ITS region from poorly preserved plant material using recombinant PCR. *Biotechniques* 27: 1180–1186.

Candolle, A. de. (1846) *Prodromus Systematis Naturalis Regni Vegetabilis* 10. Masson, Paris, pp. 27–34.

Cecchi, L., Coppi, A. & Selvi, F. (2009) *Nonea palmyrensis* (Boraginaceae): morphology and phylogenetic affinities of a rare endemic of the Syro-Iraqui desert. *Nordic Journal of Botany* 27: 381–387.
https://doi.org/10.1111/j.1756-1051.2009.00418.x

Chacón, J., Luebert, F., Hilger, H.H., Ovchinnikova, S., Selvi, F., Cecchi, L., Guilliams, C.M., Hasenstab-Lehman, K.E., Sutorý, K., Simpson, M.G. & Weigend, M. (2016) The borage family (Boraginaceae s.str.): A revised infrafamilial classification based on new phylogenetic evidence, with emphasis on the placement of some enigmatic genera. *Taxon* 65: 523–546.
https://doi.org/10.12705/653.6

Cohen, J.I. (2014) A phylogenetic analysis of morphological and molecular characters of Boraginaceae: Evolutionary relationships, taxonomy, and patterns of character evolution. *Cladistics* 30: 139–169.
https://doi.org/10.1111/cla.12036

Cunningham, C.W. (1997) Can three incongruence tests predict when data should be combined? *Molecular Biology and Evolution* 14: 733–740.

Doyle, J.J. & Doyle, J.L. (1987) A Rapid DNA Isolation Procedure for Small Quantities of Fresh Leaf Tissue. *Phytochemical Bulletin* 19: 11–15.

Edgar, R.C. (2004) MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research* 32: 1792–1797.
https://doi.org/10.1093/nar/gkh340

Euro+Med (2006–Present) Euro+Med PlantBase - the information resource for Euro-Mediterranean plant diversity. Available from: http://ww2.bgbm.org/EuroPlusMed/ (accessed 2 March 2018)

Farris, J.S, Källersjö, M., Kluge, A.G. & Bult, C. (1994) Testing significance of congruence. *Cladistics* 10: 315–319.
https://doi.org/10.1111/j.1096-0031.1994.tb00181.x

Farris, J.S, Albert, V.A, Källersjö, M., Lipscomb, D. & Kluge, A.G. (1996) Parsimony jackknifing outperforms neighbor-joining. *Cladistics* 12: 99–124.

Galushko, A.I. (1980) *Flora Severnogo Kavkaza [Flora of the North Caucasus]*. Vol. 2. - Rostov-na-Donu: Rostov University Publ, 317 pp. [in Russian]

Grossheim, A.A. (1967) *Flora Kavkaza [Flora of the Caucasus]*, second ed. 7. Leningrad: Nauka, 893 pp. [in Russian]

Gültepe, M., Uzuner, U., Coşkunçelebi, K., Beldüz, A.O. & Terzioðlu, S. (2010) Internal transcribed spacer (ITS) polymorphism in the wild *Primula* (Primulaceae) taxa of Turkey. *Turkish Journal of Botany* 34: 147–157.
https://doi.org/10.3906/bot-0905-23

Kadyrov, G.M. (1957) *Nonnea* Medic. *In:* Karjagin, I.I. (Ed.) *Flora Azerbaijana [Flora of Azerbaijan]* 7. Baku: Academy of Sciences of Azerbaijan SSR Publ., pp. 175–184. [in Russian]

Karimov, V.N. (2016) Two new species of *Nonea* Medik (*Boraginaceae* Juss.) from Azerbaijan. *Journal of botany (Ботанический журнал)* 101 (4): 428–434.

Karimov, V.N. & Ali-zade, V.M. (2016) The Systematic Analysis of Species of the *Nonea* Medik Genus in Azerbaijan Flora. *Proceedings Azerbaijan National Academy of Sciences Biological and Medical Sciences* 72 (3): 49–58 [in Azerbaijanian]

Karimov, V.N. & Illarionova, I.D. (2018) Ultrasculpture of the fruits surface of Azerbaijan *Nonea* species (Boraginaceae Juss.). *Turczaninowia* 21 (1): 66–80. [in Russian]
https://doi.org/10.14258/turczaninowia.21.1.8

Kumar, S., Stecher, G. & Tamura, K. (2016) MEGA7: Molecular Evolutionary Genetics Analysis version 7.0. *Molecular Biology and Evolution* 33 (7): 1870–1874.

https://doi.org/10.1093/molbev/msw054

Kusnezow, N.I. (1915) *Nonea* Medik. *In:* Kusnezow, N.I., Busch, N.A. & Fomin, A.V. (Eds.) *Materiały dlya flory Kavkaza - Flora caucasica critica 4 (2).* Yuriyev, pp. 291–330. [in Russian]

Medicus, F.K. (1789) *Philosophische Botanik* 1. Schwan, Mannheim, pp. 31–32.

Mtzchvetadze, D.I. (1985) *Nonea* Medik. *In:* Gagnidze, R. & Kuthatheladze, S. (Eds.) *Sakartvelos ploris—Flora Georgiae.* 10. Tbilisi: Metsniereba, pp. 251–261. [in Georgian]

Müller, K. (2004) PRAP-computation of Bremer support for large data sets. *Molecular Phylogenetics and Evolution* 31: 780–782.

https://doi.org/10.1016/j.ympev.2003.12.006

Müller, K. (2005a) SeqState: Primer design and sequence statistics for phylogenetic DNA datasets. *Appl Bioinformatics* 4 (1): 65–69.

https://doi.org/10.2165/00822942-200504010-00008

Müller, K. (2005b) The efficiency of different search strategies in estimating parsimony jackknife, bootstrap, and Bremer support. *BMC Evolutionary Biology* 5: 58.

Müller, K., Müller, J. & Quandt, D. (2010) PhyDE: Phylogenetic Data Editor, version 0.9971. Available from: http://www.phyde.de/index.html (accessed 25 September 2019)

Nixon, K.C. (1999) The parsimony ratchet, a new method for rapid parsimony analysis. *Cladistics* 15: 407–414.

https://doi.org/10.1111/j.1096-0031.1999.tb00277.x

Nylander, J. (2004) MrModeltest, version 2. Evolutionary Biology Centre, Uppsala University. Available from: http://www.abc.se/~nylander/mrmodeltest2/mrmodeltest2.html (accessed 25 September 2019)

Popov, M.G. (1953) *Nonea* Medic. *In:* Komarov, V.L. (Ed.) *Flora SSSR, vol. 19.* Akademii Nauk SSSR, Moskva-Leningrad, pp. 237–257.

Riedl, H. (1963) Bemerkungen über neue oder kritische Boraginaceen der asiatischen Flora. *Österreichische botanische Zeitschrift* 110: 511–542.

Selvi, F. & Bigazzi, M. (2001) The *Nonea pulla* group (Boraginaceae) in Turkey. *Plant Systematics and Evolution* 227: 1–26.

https://doi.org/10.1007/s006060170053

Selvi, F., Papini, A. & Bigazzi, M. (2002) Systematics of *Nonea* (Boraginaceae-Boragineae): new insights from phenetic and cladistic analyses. *Taxon* 51: 719–730.

Selvi, F., Bigazzi, M., Hilger, H.H. & Papini, A. (2006) Molecular phylogeny, morphology and taxonomic re-circumscription of the generic complex *Nonea/Elizaldia/Pulmonaria/Paraskevia* (Boraginaceae-Boragineae). *Taxon* 55: 907–918.

Simmons, M.P. & Ochoterena, H. (2000) Gaps as characters in sequence-based phylogenetic analyses. *Systematic Biology* 49: 369–381.

https://doi.org/10.1093/sysbio/49.2.369

Sullivan, J. (1996) Combining data with different distributions of among-site variation. *Systematic Biology* 45: 375–380.

https://doi.org/10.1093/sysbio/45.3.375

Swofford, D.L. (2003) *PAUP*: Phylogenetic analysis using parsimony (*and other methods).* Version 4.0b 10. Sunderland, Massachusetts: Sinauer.

Taberlet, P., Gielly, L., Pautou, G. & Bouvet, J. (1991) Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* 17: 1105–1109.

https://doi.org/10.1007/BF00037152

The Plant List. (2010) Version 1. Available from: http://www.theplantlist.org/ (accessed 10 March 2018)

Thiers, B. (2008 [continuously updated]) *Index herbariorum*: *A global directory of public herbaria and associated staff.* New York Botanical Garden, Bronx, NY. Available from: http://sweetgum.nybg.org/ih/ (accessed 10 March 2018)

Appendix 1. Locality information and GenBank accession numbers of the studied taxa.

GenBank accession numbers in the following sequence: nrITS, *trnL-F*, missing sequences designated by a dash (—).
Nonea persica **Boiss.: N1**: Nakhchivan, Sadarak district, Chalkhangala village (Aznabyurt), mountain xerophyte stepped meadows, mainly in stony areas. 06.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839164,—); **N31:** Azerbaijan, Baku, Garadagh district, near to Duvanni area and Duvanni (Alat) near to volcano. 24.04.2015. *V.N.Karimov, G.Parolli* s.n. (BAK) (—, MK852185); **N32**: Nakhchivan AR, Sadarak district, Chalkhangala village (Aznabyurt), mountain xerophyte stepped meadows, mainly in stony areas 06.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839165, MK852186).

N. armeniaca **(Kusn.) Grossh.: N4**: Nakhchivan AR, Shahbuz district, Bichanak village. In village, xerophyte stony areas. ~1800 m height from sea level. 07.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839166, MK852205); **N5:** Nakhchivan AR, Shahbuz district, Bichanak village. In village, xerophyte stony areas. ~1800 m height from sea level. 24.05.2015. *V.N.Karimov* s.n. (BAK) (MK839167,—); **N28:** Nakhchivan AR, Shahbuz district, Bichanak village. In village, xerophyte stony areas. ~1800 m height from sea level. 12.07.2016. *V.N.Karimov* s.n. (BAK) (MK839169,—); **N30**: Nakhchivan AR, Shahbuz district, Bichanak village. In village, xerophyte stony areas. ~1800 m height from sea level. 07.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839168, MK852206).

N. cyanocalyx **Popov ex V. Karimov: N6**: Nakhchivan AR, Shahbuz district, area of Shahbuz State Reserve, Near to Batabat lake, Zorbulag area. ~2400 m height from sea level, alp meadows. 07.05.2016. *V.N.Karimov* s.n. (BAK) (MK839162, MK852204); **N27:** Nakhchivan MR, Þahbuz rayonu, Þahbuz Devlet Qoruðunun arazisi, Batabat gölünün yaný. 28.07.2016. *V.N.Karimov* s.n. (BAK) (—, MK852202); **N41**: Nakhchivan AR, Shahbuz district, area of Shahbuz State Reserve, Near to Batabat lake, Zorbulag area. ~2400 m height from sea level, alp meadows. 07.05.2016. *V.N.Karimov* s.n. (BAK) (MK839163, MK852203).

N. alpestris **G.Don: N7:** Azerbaijan, Gusar district, Laza village, near to border military forces outpost, ~2000-2100 m height from sea level, alp meadows. 14.07.2016. *V.N.Karimov* s.n. (BAK) (MK839174, MK852184); **N48**: Azerbaijan, Gusar district, Laza village, near to border military forces outpost, ~2000-2100 m height from sea level, alp meadows. 14.07.2016. *V.N.Karimov* s.n. (BAK) (MK839175, MK852183).

N. lutea **(Desr.) DC.: N8:** Azerbaijan, Shaki district, Kish village, on the road near to Gurdjana river. 15.05.2016. *V.N.Karimov* s.n. (BAK) (MK839177,—); **N10:** Azerbaijan, Lankaran city, on the road from Lankaran to Lerik, near to the Airport, near the road-30.04.2016 *V.N.Karimov s.n.* (BAK) (MK839178, MK852181); **N11:** Azerbaijan, Baku, area of the Botanical Garden. 16.05.2016. *V.N.Karimov, V.Farzaliyev* s.n. (BAK) (MK839158, MK852182).

N. setosa **Roem. & Schult.: N12:** Azerbaijan, Tovuz district, Ashaghi Goychali village, on the shore of Hasansu river, with the superiority of Artemisia fragrance, semi-desert areas. Near to the 398th km of BTC line. 20.04.2015. *V.N.Karimov, G.Parolli* s.n. (BAK) (MK839171,—); **N13:** Azerbaijan, Tovuz district, Ashaghi Goychali village, on the shore of Hasansu river, with the superiority of Artemisia fragrance, semi-desert areas. Near to the 398th km of BTC line. 10.05.2014. *V.N.Karimov, V.Farzaliyev* s.n. (BAK) (MK839172,—).

N. rosea **(M.Bieb.) Link: N14:** Azerbaijan, ~80th km of Baku-Guba road, area of Beshbarmag, xerophyte superb, xerophyte stepped mountain meadows. 17.04.2015. *V.N.Karimov, G.Parolli* s.n. (BAK) (MK839184,—); **N49:** Azerbaijan, Roadside of Baku-Khizi road, ~85-90 km from Baku (~10-15 km distance from Baku-Guba road to Baku-Khizi road). Semi-desert areas with dominant species Artemisia fragrans and Salsola nodulosa. 03.05.2015. *V.N.Karimov* s.n. (BAK) (MK839183, MK852187).

N. versicolor **(Steven) Sweet: N15:** Azerbaijan, Guba district, on the road to Khinalig village, on the left shore of Gudyalchay, ~40-45 km distance from Guba, stony mountain meadows. 1960 m height from sea level 15.06.2016. *V.N.Karimov, V.Farzaliyev* s.n. (BAK) (MK839176, MK852207).

N. bakuensis **Popov ex V. Karimov: N17:** Azerbaijan, Baku, Garadagh district, near to Duvanni area. 24.04.2015. *V.N.Karimov, G.Parolli* s.n. (BAK) (MK839185, MK852190); **N18:** Azerbaijan, Baku, Garadagh district, near to Duvanni area and Duvanni (Alat) near to volcano. 24.04.2015. *V.N.Karimov, G.Parolli* s.n. (BAK) (MK839186, MK852189); **N19:** Azerbaijan, Baku, Garadagh district, area of Gobustan State Reserve, bottom area of Kaniz mountain, semi-desert areas 24.04.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839188,—); **N20:** Azerbaijan, Sumgayit city, "Azerikimya" PU, area of EP factory's Polymer 120 production, xerophyte stepped and semi-desert areas. 06.04.2016. *V.N.Karimov* s.n. (BAK) (MK839187, MK852191); **N21:** Azerbaijan, Baku, Türkan qesebesi, Sovxoz Türkan arazisi. Serhad Qosunlarýnýn N saylý hissesinin etrafýnda, deniz sahili kumluklarda. 15.05.2016. V.N.Karimov (—, MK852192); **N42:** Azerbaijan, Baku, Türkan qesebesi. 28.02.2018. *V.N.Karimov* s.n. (BAK) (—, MK852194); **N44:** Azerbaijan, Baku, Türkan qesebesi, Sovxoz Türkan area. 15.05.2016. *V.N.Karimov* s.n. (BAK) (—, MK852193).

N. diffusa **Boiss. & Buhse: N22:** Azerbaijan, Tovuz district, Aşaghi Goychali village, on the shore of Hasansu

river, with the superiority of Artemisia fragrance, semi-desert areas. Near to the 398th km of BTJ line. 20.04.2015. *V.N.Karimov, G.Parolli* s.n. (BAK) (MK839159, MK852195); **N22:** Azerbaijan, Lerik, Zuvand- Kosmalyan ve Divaðac kendlerine yakýn, yol kenarý. 02.05.2016. *V.N.Kerimov, A.Sýtin* s.n. (BAK) (-, MK852195); **N47:** Azerbaijan, Lerik, near to Zuvand- Kosmalyan, Divaghaj villages, with the superiority of roadside Artemisia fragrance, xerophyte stepped mountain meadows. 01.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839182, MK852197).

N. melanocarpa **Boiss.: N24:** Nakhchivan AR, Babak district, Duzdagh area, near to around Television Tower, xerophyte stepped and semi-desert areas, stony areas. 07.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839181, MK852199); **N25:** Nakhchivan AR, Shahbuz district, on the road to Arinj village, xerophyte stepped mountain meadows, semi-desert areas. ~1800 m height from sea level. 15.06.2014. *V.N.Karimov* s.n. (BAK) (MK839179,—); **N26:** Nakhchivan AR, Culfa district, area of Milakh village. Semi-desert areas near river, stony areas. 08.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839180; MK852198).

N. lenkoranika **(Kusn.) Grossh.: N34:** Azerbaijan, Lerik, near to Zuvand- Kosmalyan və Divaghac villages, with the superiority of roadside Artemisia fragrance, xerophyte stepped mountain meadows. 02.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839170, MK852200); **N53:** Azerbaijan, Lerik, near to Zuvand- Kosmalyan, Pirosan. 03.05.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (—, MK852201).

N. decurrens **G.Don: N36:** Azerbaijan, Shamakhi, Chukhuryurd village, area of Pirgulu State Reserve, forest areas.12.04.2008. *V.Karimov* s.n. (BAK) (MK839157,—); **N54:** Azerbaijan, Shamakhi, Chukhuryurd village, area of Pirgulu State Reserve, forest areas.12.04.2008. *V.Karimov* s.n. (BAK) (MK839160,—).

N. daghestanica **Kusn.: N50:** Azerbaijan, Guba district, on the road to Khinalig village, on the left shore of Gudyalchay river, ~40-45 km distance from Guba, stony mountain meadows. 1960 m height from sea level 15.06.2016. *V.N.Karimov, V.Farzaliyev* s.n. (BAK) (MK839161,—); **N37:** Azerbaijan, Quba rayonu, Xýnalýq kendine giden yolun üstü, 15.06.2016. *V.N.Karimov, V.Farzaliyev* s.n. (BAK) (—, MK852208).

N. flavescens **Fisch. & C.A.Mey.: N57:** Azerbaijan, Baku, Garadagh district. Area of Gobustan State Reserve, bottom side of Kaniz mountain, semi-desert areas-24.04.2016. *V.N.Karimov, A.Sitin* s.n. (BAK) (MK839173, MK852188).

Appendix 2. Accession number of the taxa getting from Genbank

nrDNA ITS—*Nonea echioides* DQ269675, *N. palmyrensis* FJ590419, *N. vesicaria* DQ269688, *N. vesicaria* AY383304, *N. macrantha* DQ269677, *N. pulmonarioides* DQ269685, *N. anchusoides* DQ269672, *N. monticola* DQ269680, *N. stenosolen* DQ269686, *N. micrantha* DQ269679, *N. intermedia* DQ269676, *N. macrosperma* DQ269678, *N. pallens* DQ269682, *N. pallens* FJ590418, *N. persica* DQ269683, *N. atra* DQ269673, *N. pulla* AY383317, *N. versicolor* DQ269687, *N. lutea* AY383316, *N. pisidica* DQ269684, *N. caspica* DQ269674, *N. polychroma* DQ350133, *N. heterostemon* DQ269671, *N. calycina* AY383305, *N. calycina* DQ269669, *N. calycina* DQ269670, *Melanortocarya obtusifolia* DQ269681, *Pulmonaria picta* AY383314, *P. angustifolia* AY383313, *P. mollis* AY383315, *P. obscura* AY383312, *Paraskevia cesatiana* AY383318, *Borago officinalis* AY383283, *Brunnera orientalis* AY383289, *Anchusa officinalis* AY045710

cpDNA *trnL-F*—*Nonea vesicaria* AY383263, *N. lutea* AY383274, *N. lutea* KF288087, *N. pulla* AY383275, *N. anchusoides* AY627869, *N. caspica* AY627870, *N. echioides* AY627871, *N. intermedia* AY627872, *N. monticola* AY627873, *N. pallens* AY627875, *N. pisidica* AY627877, *N. polychroma* AY627878, *N. pulmonarioides* AY627879, *N. stenosolen* AY627880, *N. versicolor* AY627881, *N. caspica* JX976917, *N. atra* DQ269664, *N. macrantha* DQ269665, *N. macrosperma* DQ269666, *N. micrantha* DQ269667, *N. vesicaria* DQ269668, *N. persica* AY627876, *N. calycina* DQ269661, *N. calycina* DQ269662, *N. calycina* AY383264, *N. heterostemon* DQ269663, *Anchusa officinalis* AY045703, *Brunnera orientalis* KF288036, *Borago officinalis* AY383245, *Melanortocarya obtusifolia* AY627874, *Paraskevia cesatiana* AY383276, *Pulmonaria obscura* AY383270, *P. angustifolia* AY383271, *P. picta* AY383272, *P. mollis* AY383273