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Colour polymorphism in common primrose (Primula vulgaris Huds.): many colours–many species?

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Abstract *Primula* vulgaris exhibits flower colour polymorphism in the eastern part of its range, especially pronounced on the NE coast of the Black Sea contains both the highest flower colour and haplotype diversities. The results suggest that common primroses colonized the NE coast of the Black Sea from this refugium, spreading along the coast westward. At the same time, the analysis of ITS haplotypes indicates that *P. vulgaris* colonized the Crimea from NW Anatolia. This makes it clear that no segregated species can be recognized within flower colour polymorphic *P. vulgaris* in the Caucasus region. However, its phylogeography needs further detailed study on a broader scale.
Keywords *Primula* ┐ Phylogeography ┐ Colchis refugium┐ Ponto-Caspian region

Introduction

Many plant species display flower colour polymorphism in natural populations (Hannan 1981; Rausher 2008). It was always believed that pollinators are the primary selective agents that influence flower colour, often playing an important role in plant speciation (Faegri and van der Pijl 1979). Recently this concept has been debated in many publications, demonstrating that many instances of variation in flower colour could be caused by non-pollinator agents of selection acting on various pleiotropic effects of flower colour genes and/or by genetic drift (Hannan 1981; Rausher 2008; de Jager et al. 2011 and references therein). Nevertheless, variation in flower colour is frequently associated with speciation. Flower colour polymorphism is often detected in hybrid zones (Wolf et al. 1997; Whibley et al. 2006) and it can facilitate speciation even if differently coloured flowers are visited by the same pollinators (Hopkins and Rausher 2012). Thus, the causes underlying flower polymorphism in each particular case cannot be determined without the use of a molecular phylogenetic methods (Whibley et al. 2006; Schemske and Bierzychudek 2007; Matsumura et al. 2009). Such an approach should be based on different types of molecular markers to estimate robust phylogenetic hypotheses (Wolf et al. 1997).

An interesting example of flower colour polymorphism of unknown nature has been described for the common primrose (*Primula* vulgaris Huds., *Primula*ceae), a perennial plant that is distributed in the outer regions of Western Europe, the Mediterranean (including North Africa), southwestern Ukraine, the Crimea, the Caucasus and the southern and western coast of the Caspian Sea (Jacquemyn et al. 2009). In many parts of Western Europe this species is rare or declining because its habitat is shrinking due to the negative effects of intensive agriculture and the continuous improvement of infrastructure (Endels et al. 2002). In most of the European part of its range, the common primrose is more or less monochromic (usually with yellow flowers), whereas in eastern regions (Caucasus, Turkey, Iran) plants are remarkably polychromic: apart from yellow- flowering plants, white, pink, violet and purple flowers of different shades are present in most of the populations (Richards 2003). North-western Transcaucasia is especially interesting for an investigation into the diversity of primroses, because the flower colour predominant in populations changes from yellow to purple along the Black Sea coastline from Novorossiysk to Pitsunda, a distance of 250 km (Richards 2003; Shipunov et al. 2011).
The flower colour in *P. vulgaris* has long been considered as an important taxonomical character. Some researchers divided Caucasian primroses into several (up to five) species according to the expression of different colours: P. komarovii Lozina-Lozinsk. with ivory white flowers, *P. vulgaris* s. str. with yellow flowers, P. woronowii Lozina-Lozinsk. with pink flowers, P. sibthorpii Hoffm. with pink-violet flowers and P. abchasica Sosn. with purple flowers (Lozina-Lozinskaya 1933; Kolakovsky 1985). Some of these species (namely P. woronowii and P. sibthorpii) are listed as rare or decreasing in number in the Red Books of several republics of South-Western Russia: Chechnya, Dagestan and Ingushetiya (Prisyazhnyuk 2009). Heritability of flower colour in common primrose (Marsden- Jones and Turrill 1944) establishes a genetic basis for such a delimitation. Some morphological characters, such as size of petiole, calyx and calyx teeth, corolla tube and petal limb were also believed to provide sufficient taxonomic resolution (Lozina-Lozinskaya 1933; Kolakovsky 1985). Nevertheless, most of these ‘‘species’’ remained polychromic as to their flower colour. Furthermore, a detailed study in variability of the Caucasian primroses showed no other significant morphological differences between the various coloured morphs (Shipunov et al. 2011) though, at the same time, it failed to develop any firm hypothesis explaining the nature of the observed colour polymorphism. To delimit species (or even subspecies), some characters other than flower colour should be used (Richards 2003). Despite the inheritability of the flower colour, there is no reason to accept different coloured morphs of common primrose even as subspecies, at least until some independent morphological or molecular characters are discovered (Shipunov et al. 2011).
The history of the region where different flower colour populations of *P. vulgaris* occur may also be of importance in understanding the nature of this polymorphism. It is well known that the speciation processes were affected by the climatic fluctuations that occurred during the Quaternary period. Many species experienced severe range contractions during the cold periods, surviving in climatically more favourable regions, so-called glacial refugia, from which subsequent range expansions took place (Hewitt 2004). The Ponto-Caspian region, which includes the Black and the Caspian Sea basins, has been shown to be among the most important refugia for freshwater fishes in Europe, where speciation processes took place (Kotlik et al. 2004). It is well established that the Ponto-Caspian region also included important refugia for terrestrial plants, Hyrcanian on the south-western coast of the Caspian Sea and Colchis on the eastern coast of the Black Sea (Tarkhnishvili et al. 2012), although the genetic contribution from these refugia to European plant populations still remains undetermined (Taberlet et al. 1998; Hewitt 2004; P. Taberlet and G. Hewitt, pers. comm.). At the same time, the refugia support most of the current genetic variation (Taberlet et al. 1998) and thus are critical to understanding taxonomic borders between closely related species or subspecies.

In the following, we explore the nature of colour polymorphism in *P. vulgaris* s.l., with special focus on northwestern Transcaucasia, using nuclear (ITS) and cpDNA sequences. Our study addresses the following questions: (1) which taxonomic treatment among those available

may be supported by the molecular data? (2) Could the refugia of the Ponto-Caspian region serve as sources of genetic diversity for other areas?

Materials and methods

Sampling

Between 2005 and 2012 we sampled 44 natural populations of *P. vulgaris*, usually collecting one plant per population. In six populations we sampled two plants per population, with different flower colours, if available, to check for intra-population genetic variability (Table 1). We determined flower colour in the field using a specially developed colour chart (Shipunov et al. 2011). Leaf samples from all the plants were dried in silica gel for DNA analysis, and one plant per population was pressed as a voucher. Voucher specimens were deposited in the Herbarium of

Table 1. Geographic origin, ITS and cpDNA haplotypes, and flower colour in the investigated populations of *Primula vulgaris* and the outgroup species *P. veris*. Usually we used silicagel-dried leaves of plants collected in nature. For plants sampled from herbarium material the herbarium acronym (MW – Moscow State University or MHA – Main Botanical Garden, Moscow) is given in parenthesis. ITS sequences for Turkish plants were taken from Gultepe et al., 2010. One plant per population was usually analyzed. In case of including two plants from one population, flower colour was indicated for each of them, separated by "+" symbol.

Pop. no.	Geographic origin (cf. Fig. 1)	Geographic coordinates		Haplotype		flower color
		long., N	lat., E	ITS	cp	
	Primula vulgaris L.					
NE cost of the Black Sea (between Anapa and Pitsunda): 46 plants from 42 populations						
91	S slope of Khuko mtn.	43° 56.10'	39° 48.22'	A	d	light yellow
92	S shore of lake Khuko	43° 55.80'	39° 49.15'	A	s	light yellow
301	vil. Mamedova Shchel', E shore of riv. Kuapse	43° 58.11'	39° 18.58'	C	a	white
302	1.5 km to the SW from vil. Mamedova Shchel	43° 57.85'	39° 18.13'	C	a	light yellow
303	vil. Tihonovka	43° 57.91'	39° 17.47'	C	a	white
305	0.2 km to the N from railway station Vodopadnyij	43° 57.97'	39° 15.43'	C	d	dark pink
306	0.5 km to the N from railway station Vodopadnyij	43° 58.02'	39° 15.56'	C	d	pink
308	1 km to the NE from railway station Vodopadnyij	43° 58.48'	39° 15.73'	C	a	white
309	1 km to the SE from railway station Soloniki, E shore of the unnamed stream	43° 52.69'	39° 22.34'	B	e	pink-violet
311	1.5 km to the SE from railway station Soloniki, E shore of the unnamed stream	43° 52.94'	39° 22.98'	A	e	purple
312	vil. Vardane	43° 44.17'	39° 33.63'	A	e	purple
314	1 km to the E from vil. Vardane	43° 44.26'	39° 34.06'	A	e	dark pink
320	1.5 km to the N from town Gagra, E shore of stream Zhvava-Kvara	43° 20.29'	40° 13.64'	B	b	white
322	1 km to the NW from town Gagra	43° 20.01'	40° 12.65'	B	c	white
323	8 km to the SE from town Pitsunda, mouth of stream Riapshi	43° 09.83'	40° 25.04'	A	–	purple
327	town Gagra	43° 17.63'	40° 01.00'	B	–	light yellow
401	2 km to the SE from vil. Kirpichnyij, S shore of riv. Alepsi	44° 09.15'	39° 13.73'	C	a	light yellow
404	2 km to the NE from railway station Matsesta, E shore of riv. Agura	43° 33.32'	39° 49.46'	B	d	dark pink

Pop. no.	Geographic origin (cf. Fig. 1)	Geographic coordinates		Haplotype		flower color
		long., N	lat., E	ITS	cp	
407	W border of city Tuapse (cape Kodosh)	44° 07.45'	39° 02.39'	A	a	light pink
418	5 km to the NE from vil. Verhneyakornaya Shchel	43° 49.22'	39° 33.77'	A	f	white
419	3 km to the NW from vil. Otradnoe, S shore of riv. Shakhe	43° 49.83'	39° 34.45'	A	d	dark pink
420	1 km to the W from vil. Otradnoe, S shore of riv. Shakhe	43° 48.54'	39° 35.38'	A	b	white
421	vil. Kudepsta	43° 29.66'	39° 54.23'	A	d	pink
603	vil. Arhipovo-Osipovka	44° 22.28'	38° 31.45'	A	a	light yellow
604	vil. Novomihaijlovskiij	44° 15.48'	38° 50.75'	A	a	white
606	2 km to the N from vil. Plyaho, shore of riv. Maloje Plyaho	44° 17.68'	38° 50.48'	A	a	white
607	vil. Verhnerazdolnoe	43° 37.93'	39° 46.88'	–	a	purple
608	2 km to the E from vil. Verhnematsestinskiij	43° 38.17'	39° 48.25'	A	d	white
609	1 km to the N from vil. Prigorodnyij	44° 07.9'	39° 06.97'	A	a	light yellow
610	1.5 km to the E from vil. Shepsi	44° 02.55'	39° 10.27'	C	a	white
703	1.5 km to the NE from vil. Golubaya Dacha, E shore of riv. Neozhidannaya	43° 59' 37.8"	39° 15' 06.4"	C	a	white + pink
704	1 km to the E from vil. Verkhneyakornaya Schel'	43° 47' 02.7"	39° 31' 41.1"	A+C	e	white + pink
705	2 km to the NE from vil. Baranovka, E shore of riv. Vostochnyj Dagomys	43° 43' 58.3"	39° 42' 16.8"	A	a	dark pink + white
706	0.5 km to the SE from vil. Mar'ino, E shore of riv. Psezuapse	43° 56' 11.3"	39° 28' 57.6"	C	e	light yellow
708	SE shore of lake Ritsa	43° 28' 38"	40° 32' 54"	A	d	white
709	2 km to the S from lake Ritsa, shore of river Yupshara	43° 27' 01"	40° 32' 45"	A	d	white
710	14 km to the NE from vil. Mar'ino, pass Grachevskij	44° 00' 03"	39° 39' 38"	C	e	light yellow
711	10 km to the N from vil. Pshada, mt. Moldovanskaya	44° 33' 39"	38° 24' 48"	A	a	white + light yellow
717	6 km to the NE from city Gagra, southern slope of mnt. Mamzdyshkha	43° 18' 19"	40° 19' 55"	B	b	yellow
718	vil.Ankhashtuk	43° 14' 13"	40° 30' 08"	M	d	purple
728	Under pass Aishkho (MW)	43° 39.82'	40° 27.00'	N	d	–
729	cape Malyj Utrish, ridge Navagirskij (MW)	44° 43.14'	37° 27.93'	A	a	–
The Crimea: five plants from five populations						
719	near town Yalta (MHA)	44° 31.00'	34° 08.63'	H	k	violet

Pop. no.	Geographic origin (cf. Fig. 1)	Geographic coordinates		Haplotype		flower color
		long., N	lat., E	ITS	cp	
721	Belogorsk region, upper part of riv. Burulcha (MHA)	44° 49.58'	34° 24.92'	H	j	yellow
737	Bajdarskie vorota (MHA)	44° 52.00'	34° 36.45'	H	k	violet
738	shore of riv. Ulu-Uzen (MHA)	44° 48.53'	34° 26.71'	H	k	–
739	Chatyrdag (MHA)	44° 39.15'	34° 19.07'	H	k	light yellow
Turkey: six plants from six populations						
Pvv01	Trabzon, Yeni Camii village (Gultepe et al., 2010)	41° 01.44'	39° 33.82'	J	–	yellow
Pvv02	Trabzon, Yeni Camii village (Gultepe et al., 2010)	41° 01.44'	39° 33.82'	L	–	white
Pvs01	Trabzon, Yeni Camii village (Gultepe et al., 2010)	41° 01.44'	39° 33.82'	K	–	purple
Pvv03	Kastamonu, Bozkurt (Gultepe et al., 2010)	41° 57.81'	34° 00.74'	H	–	white
Pvv04	Kastamonu, Bozkurt (Gultepe et al., 2010)	41° 57.81'	34° 00.74'	I	–	yellow
Pvs02	Kastamonu, Bozkurt (Gultepe et al., 2010)	41° 57.81'	34° 00.74'	H	–	purple
Other parts of Ponto-Caspian region: 8 plants from seven populations						
707	Dagestan, Derbent region, mtn. Dzhalgan	42° 01.68'	48° 15.69'	D	m	light pink + light pink
725	Georgia, Chakvinsky region, vil. Khalo (MHA)	41° 43.33'	41° 47.85'	D	o	violet
726	Chechnya republic, Achkhoj-Martanovskij region, vil. Valerik (MHA)	43° 10.84'	45° 24.48'	E	p	violet
734	Georgia, shore of stream Gldanis-Khevi (MHA)	41° 58.12'	44° 56.21'	E	n	violet
735	Azerbajdzhan, near vil. Lerik (MHA)	38° 38.64'	48° 41.00'	G	q	white
713	Azerbajdzhan, park Girkanskij	38° 39' 02"	48° 46' 36"	–	t	light yellow
715	Azerbajdzhan, park Girkanskij	38° 38' 50"	48° 47' 04.8"	G	t	light yellow
Middle Russia: three plants from two populations						
716	Smolensk region, Shumyachskij district, 50 m to the south from vil. Glumyanka	53° 12.00'	32° 17.00'	F	g	white + white
724	Bryansk region, Ludinovo district, 1 km to the west from bay "173 km" (MHA)	53° 43.00'	34° 23.30'	F	h	–
	Primula veris L.: two plants from two populations					
723	Lipetsk region, Dankov district, vil. Polibino (MHA)	53° 30.53'	38° 58.61'	Z	q	–

Pop. no.	Geographic origin (cf. Fig. 1)	Geographic coordinates		Haplotype		flower color
		long., N	lat., E	ITS	cp	
731	Tula region, Schekinsk district, 2.5 km to the north from vil. Krapivna (MHA)	53° 57.88'	37° 09.88'	Z	r	–

Moscow State University (MW), Russia. We also included in this study 12 additional herbarium specimens of *P. vulgaris*, collected from various regions between 1960 and 2007 (Table 1). In accord with the aims of our study, the sampling was focused on the NE coast of the Black Sea (between Novorossijsk and Pitsunda) where geographically structured flower colour polymorphism was detected (Shipunov et al. 2011). Samples from other parts of the Ponto-Caspian region were also included. We analysed plants from the two known localities of *P. vulgaris* in Middle Russia, Bryansk and Smolensk regions, (Majorov in press) to reveal the possible geographic origins of these populations. Two herbarium samples of *P. veris* L., collected in 2006/2007 in Middle Russia (Tula and Lipetsk regions), were included in the study as an outgroup (Mast et al. 2006).

DNA isolation and sequencing

DNA was extracted from dehydrated leaf material using the CTAB method (Doyle and Doyle 1987). Four noncoding and potentially highly variable cpDNA regions were initially amplified and sequenced from eight samples of *P. vulgaris* with different flower colour and from different geographical regions: intergenic spacers rpl132–trnL (Shaw et al. 2007), trnL–trnF (Taberlet et al. 1991) and trnH–psbA and intron rps16 (Shaw et al. 2005). We found some variation in all these regions and selected the intergenic spacers trnL–trnF and rpl32–trnL for the final analysis as they were the most variable. The complete ITS region (ITS1, 5.8S and ITS2) was amplified using the primers NNC-18S10 and C26A (Wen and Zimmer 1996).
Polymerase chain reactions (PCR) were conducted in 20 ll reaction volumes containing 4 mcl of Ready-to-Use PCR MaGMix (200 mcM of each dNTP, 1.5 mM MgCl2, 1.5 U SmarTaqDNA Polymerase and reaction buffer; Dialat Ltd., Moscow, Russia), 15 mcl deionised water, 3.4 pmol of each primer and 1 mcl of template DNA of unknown concentration. PCR cycling was performed with a MJ Research PTC-220 DNA Engine Dyad Thermal Cycler (BioRad Laboratories, USA) with the following parameters: initial denaturation for 5 min at 95 oC followed by 35 cycles of 30 s at 95 oC, 30 s at 50 oC and 2 min at 72 oC, ending with 10 min extension at 72 oC. Double-stranded PCR products were checked on agarose gels and purified using centrifugation with a solution of ammonium acetate in ethanol. Sequencing was performed in both directions using ABI PRISM BigDye┘ Terminator v 3.1 Kit (Applied Biosystems) according to the manufacturer's manual, except for using 10 ll reaction volumes, and further analysed on 3130 and 3500 Genetic Analyzers (Applied Biosystems).
GenBank accession numbers of the cpDNA and ITS sequences are JQ927012–JQ927212. We also used in the analyses several ITS sequences for Turkish *P. vulgaris* (Gultepe et al. 2010) retrieved from GenBank (cf. Table 1, accession numbers EU643642–EU643647).

DNA sequences were aligned using BioEdit 7.0.5.3. (Hall 1999). The sequences of the two intergenic spacers were combined into a single data set. When DNA sequences differ only by a few substitutions (as in our case, see ''Results''), conventional phylogenetic methods may perform poorly (Crandall 1996). Therefore, we carried out statistical parsimony analysis using the network algorithm described in Templeton et al. (1992) and implemented in the TCS v. 1.21 program (Clement et al. 2000). This method estimates the unrooted haplotype network and a 95 % plausible set of all haplotype lineages in that network. We analysed gaps as the fifth state and considered gaps with a length greater then one as a single mutation.

The Nested Clade Analysis (NCA) was performed manually according to the rules described in Templeton et al. (1987) and Templeton and Sing (1993). Despite a lot of criticism (Knowles and Maddison 2002; Petit and Grivet 2002), a reliable performance of this approach was shown in a large number of studies where it was compared with other methods used in statistical phylogeography (Stehlik 2002; Templeton 2004 and references therein; Geml et al. 2006; Wu et al. 2006; Bartish et al. 2006). At least, it enables a consistent and reasonable way to

delimit clades or lineages of more closely related haplotypes within a network to further compare their geographic distributions in that or another way. The statistical phylogeographic analysis was performed using the GeoDis v. 2.6 program (Posada et al. 2000). The details of terminology used are given in Templeton et al. (1995). The results were interpreted with the revised inference key (version of 28 April 2009) of Templeton (2004) included in the program package.

The gene diversity (h) and nucleotide diversity (p) were calculated in Arlequin v. 3.11 (Excoffier 2007) for groups of populations from NW Caucasus. These groups (hereafter referred as nested clade areas) are determined by combinations of first step level nested clades revealed by NCA, for ITS and cpDNA haplotypes. They are designated in the text and figures as numbers of the first step level nested clades for ITS and for cpDNA separated by a slash character (e.g. 1/7). Explanation of the observed patterns of genetic diversity was made after Grant and Bowen (1998) and Gussarova et al. (2012) basing on combinations of high and low values of h and p (Table 2). Analyses of hierarchical molecular variance (AMOVA) as implemented in Arlequin v. 3.11 were used to assess the degree of differentiation between and within these pooled population samples.

Table 2. Interpreting haplotype (h) and nucleotide (π) diversities.

		π	
		<0.5%	>0.5%
h	<0.5	recent bottleneck or founder effect by a single or a few cp/ITS lineages	divergence between geographically subdivided populations or a brief bottleneck
	>0.5	population bottleneck followed by rapid population growth	stable population with large historical effective size or secondary contact between differentiated lineages

Correspondence between flower colour and cpDNA or ITS haplotype for the populations from NW Caucasus was tested with Pearson's Chi-square test with simulated p value, appropriate for correspondence tables with many zeroes, as in our case (Table 3). p value was calculated by Monte-Carlo simulation based on 2000 replicates. The test was performed in statistical environment R 2.9.2 (R Development Core Team 2009).

Results

cpDNA sequences

The concatenated trnL–trnF and rpl32–trnL matrix consisted of 790 aligned positions, 18 of which were parsimony informative. Highly variable poly-T and poly-A sites were excluded from the analyses because of possible homoplasy. Analysis in TCS revealed 39 haplotypes, 21 of which were not in the data and represented missing haplotypes. The TCS program calculated the 95 % limit of parsimony of 12 mutational steps and connected all the haplotypes into a single network (Fig. 1a). Four haplotypes, a, d, e and f, formed a closed loop caused by homoplasy in 678 and 679 positions of the alignment. This loop, however, caused no problem to the NCA, which grouped the 18 haplotypes present in the sample into twelve 1-step level clades, four 2-step level clades and two 3-step level clades.

At the 3-step level the NCA divides the network into two clades. Clade 3-1 is distributed throughout the Caucasus except for the NE coast of the Black Sea (Fig. 2a) and consists of seven

haplotypes (m, n, o, p, q, s, t), of which the haplotype s represents the only distributional exception, being found in a single individual (population 92) from the lake Khuko vicinity in the NW Caucasus. The same clade includes the haplotype r from *P. veris*. The haplotype q is shared between *P. vulgaris* and *P. veris*. Clade 3-2 includes haplotypes a, b, c, d, e, f, g, h, j, k and is distributed from the NE coast of the Black Sea to the Crimea and Central Russia. Statistical analysis in GeoDis (Suppl. table 1) inferred allopatric fragmentation for these two clades.

At the 2-step level the NCA unites the haplotypes into four clades, two of which belong to clade 3-1, and two others belong to 3-2. Clade 2-1 unites the haplotypes t, q, s, r, and thus seems to be the root clade, since it includes both of the *P. veris* haplotypes (q, r). As to the haplotypes of *P. vulgaris*, two of them are confined in their distribution to SE Transcaucasia (t, q), and one (s), as has been mentioned above, to the NW Transcaucasia. No significant pattern was inferred with GeoDis. Clade 2-2 unites haplotypes m, n, o, p distributed in the N, E and Central Caucasus. No significant pattern was inferred either. However, GeoDis infers contiguous range expansion for clade 3-1 as a whole.

Clade 2-3 includes haplotypes a, b, c, d, e f, all confined to the NE coast of the Black Sea (Fig. 3a). Their distribution is, however, not even. The haplotypes b, c, d mostly occur in the eastern part of the coast, to the E of Sochi; a occurs predominantly in the western part, to the W of Tuapse, while e and f occupy a much smaller area between them. This part of the network includes the loop, so the exact relationships of the haplotypes cannot be established firmly. Analysis in GeoDis infers restricted gene flow with

Table 3. Correspodence of cpDNA and ITS haplotypes to flowers colours (and taxa within *Primula vulgaris* s.l.) on the NE coast of the Black Sea (the focus area): the number of plants with each combination of cpDNA and ITS haplotype and flower colour is given. Results of chi-square test of correspondence between haplotypes and flower colour are also shown.

		Flower colour and taxa within *P. vulgaris* s.l.							
		woronowii			*vulgaris* s.str.		*abchasica*	*komarovii*	*sibthorpii*
Haplotype		light pink	pink	dark pink	light yellow	yellow	purple	white	pink-violet
cpDNA (N=42) p=0.512 χ^2= 37.472	a	1	1	1	5	0	1	9	0
	b	0	0	0	0	1	0	2	0
	c	0	0	0	0	0	0	1	0
	d	0	2	3	1	0	1	3	0
	e	0	1	1	2	0	2	1	1
	f	0	0	0	0	0	0	1	0
	s	0	0	0	1	0	0	0	0
ITS (N=43) p=0.096 χ^2= 29.760	A	1	1	3	5	0	3	10	0
	B	0	0	1	1	1	0	2	1
	C	0	3	1	4	0	0	5	0
	M	0	0	0	0	0	1	0	0

isolation by distance. However, since the westernmost part of this area is occupied by haplotype a exclusively, it seems plausible that some range expansion took place as well. Clade 2-4 consists of four haplotypes, which occur in the Crimea (j, k) and in the two known localities in Central Russia (g, h). No significant inference can be made with GeoDis, however, for the whole of clade 3-2 allopatric fragmentation is inferred.

The 1-step clades enable even finer geographical resolution, though GeoDis infers no statistically significant patterns at this level. They are described below together with the ITS network 1-step clades. The haplotypes did not correspond to flower colour (Tables 1, 3): plants with different flower colours had identical haplotypes and, vice versa, plants with flowers of the same colour may possess different haplotypes. No intra-population sequence variability was detected in the cases when two plants per population were sampled. The Chi-square test

performed for the focus area of the NE coast of the Black Sea revealed no significant ($p<0.05$) correspondence between cpDNA haplotypes and flower colours.

ITS sequences

The ITS matrix consisted of 713 aligned positions, 22 of which were parsimony informative. Analysis in TCS revealed 18 haplotypes, 4 of which were not in the data and

Fig. 1 The 95 % plausible parsimony network of chloroplast DNA and ITS haplotypes in Primula vulgaris and outgroup species Primula veris. Lines represent the mutational pathway interconnecting the haplotypes; dots represent inferred intermediate haplotypes not observed in the data. Internal haplotypes are represented by circles and tip haplotypes are represented by rectangles. The size of each node symbol is proportional to the sample size. Nested Clades are outlined with dotted lines and numbered (the first number is the order of step, the second—the number of clade). Combinations of colours and symbols denote different first-step clades. a The combined (tworegion) cpDNA haplotypes. b The ITS haplotypes

represented missing haplotypes. The TCS program calculated the 95 % limit of parsimony of 11 mutational steps and connected all the haplotypes from *P. vulgaris* into a single network. However, an additional haplotype from *P. veris* (Z) remained disconnected from the network of *P. vulgaris* haplotypes (not shown). Manual increase of the connection limit to 12 steps enabled integration of all haplotypes into a single network (Fig. 1b). Haplotype Z differed from the closest haplotype D of *P. vulgaris* by 11 steps. Despite 11 steps exceeding the calculated limit of parsimony, this enabled the network to be rooted in the central haplotype D. *P. vulgaris* and *P. veris* share no common ITS haplotypes.

The NCA recovered nine 1-step clades and four 2-step clades within the network of *P. vulgaris* haplotypes.

At the 2-step clade level, the haplotypes included in clade 2-1 (A, B, C, M, N) are strictly confined to the NE coast of the Black Sea (Figs. 2b, 3b). The analysis in GeoDis infers restricted gene flow with isolation by distance (Suppl. table 1). The haplotypes of the central clade, 2-2, (D, E, F) are distributed through the rest of the Caucasus (D, E) except for the most SE part of it (Talysh, Girkan), while the haplotype F is characteristic of the populations of *P. vulgaris* isolated in Central Russia. The haplotypes of clade 2-3 (J, L, K) are known from plants attributed to both *P. vulgaris* subs *P. vulgaris* and *P. vulgaris* subsp. sibthorpii (Hoffm.) W.W.Sm. & Forrest originating from a single locality in N Turkey (Trabzon: Gultepe et al. 2010). Clade 2-4 includes three haplotypes (G, H, I) that are attributed to plants of both subspecies from another Turkish locality (H, I: Kastamonu) and also to *P. vulgaris* from the Crimea (H). Haplotype G is known from two localities in Talysh of SE Azerbajdzhan (‘‘Girkanskij Park’’). At the whole cladogram level the analysis in GeoDis infers past fragmentation of the *P. vulgaris* populations probably with some long-distance colonization.

The spatial distribution of the ITS haplotypes on the NE coast of the Black Sea does not correspond perfectly to the geographical structure of the cpDNA haplotypes (Fig. 3). However, the correspondence increases if we take into account the 1-step clades formed by these haplotypes. Thus, the easternmost part of the area studied around Gagra (Fig. 3: area 1/7) is occupied by populations of *P. vulgaris* bearing ITS haplotypes of clade 1-1 (B, M) and cp haplotypes of clade 1-7 (b, c, d). However, in the most eastern population in this area the ITS A haplotype of clade 1-2 is found. The cp haplotype from this population remains unknown. The area between the towns of Sochi and Gagra (Fig. 3: area 2/7) is occupied by populations of ITS haplotypes of clade 1-2 (A, N) and mainly by cp haplotypes of

Fig. 2 Geographical pattern of the distribution of ITS and cpDNA haplotypes of Primula vulgaris in the whole area of investigation. The symbols on the map correspond to those in the

networks (reproduced in the inset). We have no information on cpDNA haplotypes for the Turkish populations. The NE coast of the Black Sea, on which the sampling was focused (Fig. 3), is delineated by the rectangle. a The combined (two-region) cpDNA haplotypes. b The ITS haplotypes

clade 1-7 (d) with the exception of two populations with cp haplotype a of clade 1-8. Yet another plant from population 92 at the lake Khuko possesses the phylogenetically ancient cp haplotype s from clade 1-2, closely related to the outgroup. In the eastern half of the coastal area between Sochi and Tuapse (area 2/9) grow populations bearing ITS haplotype A of clade 1-2 (with haplotype B from the tip clade 1-1 found in one population), while cp haplotypes are represented by e and f of clade 1-9. Inland (area 1/9) from this area two populations with the ITS haplotype C (clade 1-1) were sampled, which, however, did not differ from the latter area in cp haplotype (e, clade 1-9). To the west of this area, up to the town of Tuapse, plants with the ITS haplotype C (clade 1-1) and cp haplotype a (clade 1-8) occur (area 1/8). The cp haplotype d (clade 1-7) is, however, found in one population. Finally, the area 2/8 to the W of Tuapse is all occupied with plants bearing the ITS haplotype A (clade 1-2) and the cp haplotype a (clade 1-8). Thus, certain combinations of ITS and cp haplotypes seem to cooccur together in six areas bordering each other on the NE coast of the Black Sea.

The AMOVA analyses of chloroplast and ITS data sets (Table 4) resulted in 50.93 and 79.66 % variation among nested clade areas correspondingly. The highest haplotype diversity based on both cpDNA (h = 0.83) and ITS (0.60) data was observed in the easternmost area 1/7 (Table 5). The area 2/9 had a rather high haplotype diversity based on the ITS (0.52), but not the cpDNA (0.29) data. The other areas had medium to low haplotype diversity. The highest cpDNA nucleotide diversity was observed in the areas 1/7 (p = 1.17) and 2/7 (1.50), and the highest ITS nucleotide diversity was observed in the areas 1/7 (0.67) and 2/9 (0.76). The other areas had medium to low nucleotide diversity. The observed patterns of haplotype and nucleotide diversities based on cpDNA and ITS data coincided for areas 1/7, 1/9, 1/8 and 2/8 (Table 5) and fitted into two categories of Grant and Bowen (1998) and Gussarova et al. (2012) based on combinations of high and low values of

Fig. 3 The NE coast of the Black Sea. River system of the southern slope of the Greater Caucasus ridge is shown. The symbols on the map correspond to those in the partial networks (reproduced in the inset). Grey dashed lines indicate distribution areas of first step level nested clades for ITS and cpDNA haplotypes (nested clade areas). The areas are numbered according to the 1st step level ITS/cpDNA nested clades the haplotypes belong to. a The combined (two-region) cpDNA haplotypes. b The ITS haplotypes

Table 4. Analyses of molecular variance (AMOVA) based on cpDNA and ITS markers: differentiation within and among NW Caucasian population groups of *Primula vulgaris* (1st step level nested clade areas, Fig. 3). p-values were lower then 0.001 in all cases.

	Source of variation	d.f.	Percentage of variation
cpDNA	Among populations	5	50.93
	Within populations	39	49.07
ITS	Among populations	5	79.66
	Within populations	40	20.34

h and pi. This suggested that populations of the area 1/7 were historically stable with large effective population size, while those of the areas 1/9, 1/8 and 2/8 survived prolonged bottlenecks and/or experienced founder effect. The haplotype and nucleotide diversities in the areas 2/7 and 2/9 based on cpDNA and ITS data sets were not congruent and suggested somewhat controversial interpretations but did not completely contradict each other (Table 5).

The ITS haplotypes also did not correspond to flower colour (Tables 1, 3). There was no sequence variability within populations with one exception (pop. 704), which was located in the contact zone between the different ITS haplotypes (Fig. 3b). The Chi-square test (Table 3) revealed no significant ($p<0.05$) correspondence between ITS haplotypes and flower colours.

Discussion

Taxonomic implications

We did not find any molecular basis for splitting *P. vulgaris* into several species or subspecies according to flower colour, supporting the published data (Gultepe et al. 2010; Schmidt-Lebuhn et al. 2012). Thus we argue for a broad concept of *P. vulgaris* in line with the earlier conclusions based on extensive morphological data (Shipunov et al. 2011).

All the samples of *P. vulgaris* are clearly separated from the samples of the closely related species *P. veris*, based on ITS sequences. However, the cpDNA data delimit these species poorly. One of the cpDNA haplotypes (q) is shared among the two species. Another cpDNA haplotype of *P. vulgaris* from the NE coast of the Black Sea (s, pop. 92) differs by only one substitution from haplotype q. At the same time, this *P. vulgaris* haplotype is very different from the haplotypes that were detected for all the other plants from the NE coast of the Black Sea. Perhaps the picture would be even more complex if more samples of *P. veris* were included. This phenomenon of cpDNA haplotypes being shared among closely related species has been described for many different plant taxa (e.g. Wolf et al. 1997; Matsumura et al. 2009). There can be two explanations for such a shared haplotype presence (Schmidt-Lebuhn et al. 2012). On the one hand, this may be the result of a chloroplast capture due to hybridization, since *P. veris* and *P. vulgaris* can hybridize freely, even in natural habitats (Richards 2003; Jacquemyn et al. 2009), and both occur in the studied area. On the other hand, the presence of shared haplotypes may be due to incomplete lineage sorting, when common haplotypes and their derivatives are inherited from the nearest common ancestor. In the latter case, common haplotypes are usually internal in the network and are close to its root. Though this seems to be in agreement with our results, we think much more extensive sampling, in particular of *P. veris*, is needed to discriminate between these two explanations for the two species of Caucasian primroses. Even the recent extensive study of the phylogeny of section *Primula* (including *P. veris* and *P. vulgaris*) state that more data are needed to address the issue more confidently (Schmidt-Lebuhn et al. 2012).

Nature of the flower colour polymorphism

Neither cpDNA nor ITS haplotypes correlate with flower colour. Thus, it is unclear whether the variability of flower colour in *P. vulgaris* is caused by natural selection or

Table 5. Haplotype (h) and nucleotide (π) diversities and their interpretations for the group of populations (1st step nested clade areas, Fig. 3) of NW Caucasian populations of *Primula vulgaris* based on cpDNA and ITS haplotypes. *N*, number of individuals in a group of populations

	Group of populations	*N*	*h*	π	Inference (see Table 3)
cp haplotypes	2/8	7	0.00	0.00	recent bottleneck or founder effect
	1/8	11	0.33	0.33	recent bottleneck or founder effect
	1/9	2	0.00	0.00	recent bottleneck or founder effect
	2/9	7	0.29	0.29	recent bottleneck or founder effect

	2/7	14	0.38	1.50	divergence between geographically subdivided populations or a brief bottleneck
	1/7	4	0.83	1.17	stable population with large historical effective size
ITS haplotypes	2/8	7	0.00	0.00	recent bottleneck or founder effect
	1/8	11	0.00	0.00	recent bottleneck or founder effect
	1/9	2	0.00	0.00	recent bottleneck or founder effect
	2/9	7	0.52	0.76	stable population with large historical effective size or secondary contact between differentiated lineages
	2/7	13	0.15	0.15	recent bottleneck or founder effect
	1/7	6	0.60	0.67	stable population with large historical effective size

stochastic processes. It is often difficult to discriminate between these two evolutionary forces, especially if their intensity varies both in time and in space (Schemske and Bierzychudek 2007). However, if the spatial pattern of flower colour polymorphism were due primarily to random genetic drift one would expect a similar pattern of differentiation for neutral genetic markers that are not associated with flower colour (Whibley et al. 2006; Schemske and Bierzychudek 2007). From this point of view, the flower colour polymorphism in *P. vulgaris* is more probably caused by natural selection. However, it remains unclear whether the flower colour itself is the target of selection, or the colour polymorphism is rather caused by some pleiotropic effects (Schemske and Bierzychudek 2007 and references therein). Many insect species have been observed visiting *P. vulgaris* flowers, but the extent to which they serve as actual pollinators still remains poorly known (Jacquemyn et al. 2009). We also lack any information as to whether pollinators can discriminate between different flower morphs of this species.

The forces that maintain flower colour polymorphism within one population are similarly unclear. In Linanthus parryae, it was suggested that fluctuating selection along with a long-lived seed bank was key to understanding the maintenance of the flower colour polymorphism (Schemske and Bierzychudek 2007). However, such an explanation is hardly applicable to our case of *P. vulgaris* as the latter mostly has short-lived seeds and thus no seed bank (Jacquemyn et al. 2009). We can also suppose that colour polymorphism may be caused by diversity of sympatric pollinators, preferring different flower colours (Shipunov et al. 2011). Finally, high intra-population flower colour polymorphism can be explained by historical gene flow between populations (see below).

Phylogeography

Clear spatial pattern of *P. vulgaris* cpDNA and ITS haplotype distributions can be observed on the NE coast of the Black Sea. The distribution of the haplotypes of maternally inherited cpDNA and biparentally inherited ITS is not perfectly congruent; however, a much concordant picture can be retrieved when comparing the two datasets (Fig. 3), especially the pattern of distribution of nested clades. The results of AMOVA analyses (Table 4) confirm the consistent delimitation of these nested clade distribution areas by the high values of variation between these groups of populations.

The internal root haplotype D of the ITS network, as well as the chloroplast root haplotype q and their nearest derivatives are all found in the Caucasus outside the NE coast of the Black Sea. This implies that common primroses probably colonized the NE coast of the Black Sea from the eastern (Colchis) refugium, spreading along the coast westward. The plants with ancestral (closer to the outgroup) haplotype (ITS/cpDNA: A/d) grow currently mainly around the city of Sochi (in the area shown as 2/7 in Fig. 3) and also near the town of Gagra (Fig.

3, area 1/7). Then they apparently spread mainly westwards (in between the cities of Sochi and Tuapse) in a series of short-distance colonization events. This spreading was not fast, because enough time elapsed between the successive colonization steps to accumulate one or two mutations in ITS and cpDNA regions. This result is statistically confirmed by NCA geographical analysis of the cp haplotypes of clade 2-3 which infers a restricted gene flow with isolation by distance. Interestingly, the coastal area (areas 1/8, 1/9, 2/9, 2/7 in Fig. 3) between the cities of Tuapse and Sochi corresponds to the western border of the Colchis refugium (Tarkhnishvili et al. 2012). Indeed, there the highest diversity of both ITS and cp haplotypes was observed. The analyses of haplotype and nucleotide diversity based both on cpDNA and ITS data sets (Table 5) strongly indicate to the existence of a historically stable population of *P. vulgaris* in the refugial area 1/7. At the same time, the populations outside the Colchis refugium seem all to survive short to prolonged bottlenecks. Slow spreading of *P. vulgaris* to the west of Sochi could be caused by the gradual improvement of habitat conditions outside the Colchis refugium after the last glacial maximum (LGM). Our data also suggest contacts and restricted gene flow between different populations in this area (Fig. 3). Such inter-population crosses could increase intra-population genetic diversity and thus cause higher variability of the flower colour as an inherited character. This hypothesis is supported by the results of Shipunov et al. (2011), demonstrating the highest levels of flower colour variability between the cities of Tuapse and Sochi.

All the western part of the coast (from Tuapse to Novorossijsk: Fig. 3 and Anapa: Fig 2) was colonized by *P. vulgaris* with an identical haplotype (ITS/cpDNA: A/a, their range corresponds to the area 2/8 in Fig. 3), probably as a result of long-distance dispersal events from the area 2/7 followed by a fast spread in the new area. The redistribution of this lineage of *P. vulgaris* to the west from Tuapse could have been facilitated by the flatter relief of the land in this area.

Reconstruction of the relationships between *P. vulgaris* populations in the whole Ponto-Caspian region was beyond the scope of this study, but some additional observations should be noted. Unfortunately, we lack any information on cpDNA haplotypes of *P. vulgaris* from Turkish populations. Nevertheless, analysis of the ITS haplotypes suggests that *P. vulgaris* colonized the northern coast of the Black sea from at least two different sources. Common primroses spread along the eastern part of the coast (to the

east from the Kerchensky strait, connecting the Black Sea and the Sea of Azov) from the eastern (Colchis) refugium. At the same time the central part of the northern coast of the Black Sea (the Crimean peninsula) was colonized from populations of the S coast of the Black Sea (Turkey, vicinity of Kastamonu) but not from eastern Turkey (vicinity of Trabzon) which was most likely colonized by *P. vulgaris* from the Colchis refugium. The NCA infers contiguous range expansion for the populations of Kastamonu and the Crimea, and restricted gene flow for the rest of the populations to the E of those cities from the ITS haplotype distribution, which further confirms this conclusion. This finding agrees perfectly with the changing climate during the LGM indicating that a broad gap with an unfavorable climate existed between the Colchis and the western Turkey refugia (Tarkhnishvili et al. 2012). This circumstance prevented gene flow between these two refugia in the Pleistocene era. Currently, a dry segment of the southern Black Sea coast between Ordu and Sinop in Turkey (separating the cities, Kastamonu and Trabzon) hinders the dispersal of the Caucasian species into northwestern Turkey (Tarkhnishvili et al. 2012). Thus, our study provides the first direct evidence of the role of the Colchis refugium as an independent source of genetic variability of terrestrial plant species.

Our data suggest that the two isolated populations of *P. vulgaris* in Middle Russia originated from the Ponto- Caspian region. The observed differences between their cpDNA haplotypes indicate that these populations spread northward more or less independently, although their modern isolated position is due to allopatric fragmentation of the previously contiguous area. However, the exact geographic origin of these isolated populations remains

uncertain, because of the incongruence between ITS and cpDNA datasets, which appears to be a common phenomenon for plants (e.g. Wolf et al. 1997).

Further extensive sampling is required to determine the role of the Colchis refugiumand other poorly studied refugia of the Ponto-Caspian region (Tarkhnishvili et al. 2012) in shaping the genetic variability of *P. vulgaris* compared with the well-known Mediterranean refugia (Taberlet et al. 1998; Hewitt 2004). This task is especially relevant for Western Europe, where this species is rare or declining (Endels et al. 2002). Such a study could be hampered because colonization pathways of commercially important species, such as widely cultivated *P. vulgaris* (Jacquemyn et al. 2009), might be completely confounded by high levels of anthropogenic translocation (Provan and Bennett 2008). Furthermore, although *P. vulgaris* is characterized as a thermophilous species, it can survive in harsh alpine habitats (Jacquemyn et al. 2009), suggesting that cryptic northern refugia (Provan and Bennett 2008) could also be important sources of the genetic variability for this species.

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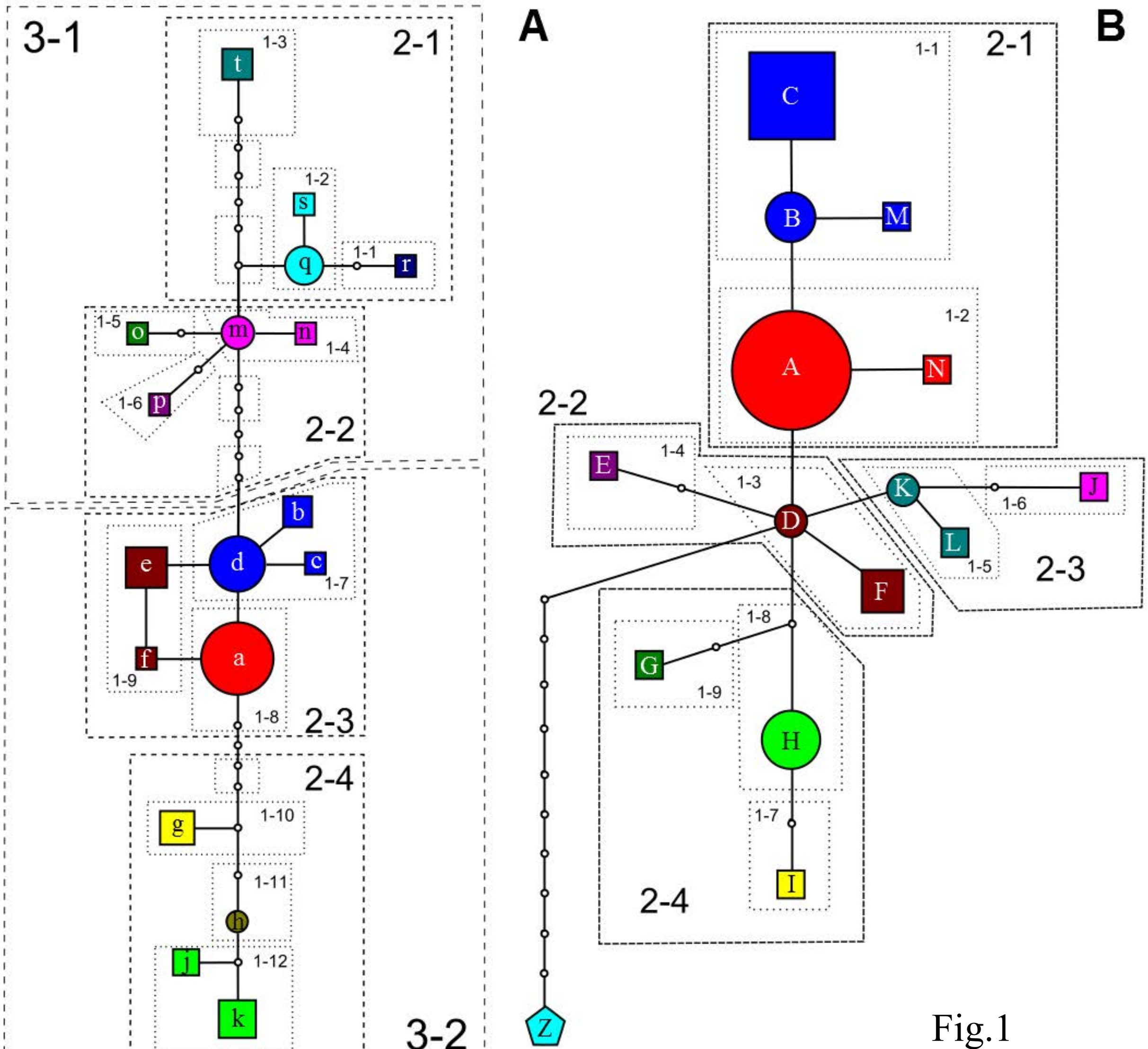
A
3-1
2-1
1-3
t
1-2
s
q
1-1
r
1-5
o
m
n
1-4
1-6
p
2-2
b
e
d
c
1-7
f
a
1-9
1-8
2-3
2-4
g
1-10
1-11
h
j
1-12
k
3-2
B
2-1
1-1
C
B
M
1-2
A
N
2-2
E
1-4
1-3
D
K
J
1-6
L
1-5
2-3
F
1-8
G
1-9
H
1-7
I
2-4
Z

Fig.1

A

Moskva
Volga
Smolensk
Bryansk
Don
Dnepr
Volga
Sea of Azov
Sevastopol
Anapa
Tuapse
Sochi
Gagra
Black sea
Kastamonu
Batumi
Trabzon
Tbilisi
Caspian sea
Baku
500 km

3-1
2-1
2-2
2-3
2-4
3-2

B

Moskva
Volga
Smolensk
Bryansk
Don
Dnepr
Volga
Sea of Azov
Sevastopol
Anapa
Tuapse
Sochi
Gagra
Black sea
Kastamonu
Batumi
Trabzon
Tbilisi
Caspian sea
Baku
500 km

2-1
2-2
2-3
2-4

Fig. 2

N

cp haplotypes

2/8

1/8

1/9

2/7

1/7

2/9

1-9

1-8

1-7

2-3

Tuapse

Sochi

Gagra

Novorossijsk

E 39° 30`

N 43° 40`

10 km

A

N

ITS haplotypes

1-1

2-1

1-2

Tuapse

Sochi

Gagra

Novorossijsk

E 39° 30`

N 43° 40`

10 km

B

Black Sea

Fig. 3.