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Genetic diversity and natural hybridization in populations of clonal plants of *Mentha aquatica* L. (Lamiaceae)

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Summary: Analyses of genetic diversity in three populations of *Mentha aquatica* from Abkhazia, W Ukraine and S Russia with the use of ISSR markers reveal that these plants growing along river banks can form vegetative clones with ramets several meters distant from each other. Members of different clones (different genets) may be interspersed within a single growth patch. However, in the case of a putative long-distance dispersal, e.g. when a plant grown from a single seed establishes itself in a new unoccupied riverside habitat, a single clone may distribute itself for several kilometers along the river course by means of vegetative propagation only. In the absence of a mate, the self-incompatible *M. aquatica* cannot set seeds. Hybridization of *M. aquatica* with *M. arvensis* is confirmed both by field observations and molecular data.

Keywords: Lamiaceae, *Mentha aquatica*, *Mentha arvensis*, ISSR, hybridization, population

The size and structure of clones in vegetatively mobile plants as well as their impact on local population structure are still insufficiently known. In many ecological and population studies, separate ramets or even separate aerial shoots are used as units of investigation, while the size and number of genets remain unknown. In a few studies, the number of genets and spreading of separate clones in local plant populations were assessed by using molecular markers (Montalvo et al. 1997; Fischer et al. 2000; Reusch et al. 2000; Pluess & Stöcklin 2004; Ally et al. 2008). It appears, that clone size may vary from tens of centimeters in *Ranunculus reptans* L. (Fischer et al. 2000) to few meters in *Quercus chrysolepis* Liebm. (Montalvo et al. 1997) to tens of meters in *Populus tremuloides* Michx. (Ally et al. 2008). In particular, aquatic plants appear to form rather large clones (Charpentier et al. 2000), so that depending on the physical dimensions of a habitat, local populations are represented by a mixture of plants belonging to a few or even a single clone. This fact may have a great impact on population genetic structure since the probability of geitonogamy increases in self-compatible species, which in turn leads to a decrease in number and quality of fruit set (Charpentier et al. 2000; Eckert 2000; Pappert et al. 2000; Dorken & Eckert 2001). In self-incompatible species, this may potentially lead to a complete lack of seed set. However, in physically larger habitats the genetic diversity usually increases in terms of a number of clones or genets.

According to its very name, *Mentha aquatica* L. is an aquatic plant. It grows on muddy banks and in shallow water of small rivers forming dense growth patches and sometimes even floating mats and spreading up to two meters apart from the bank and 1–3 m along it. Here, we have studied three local populations of this species inhabiting similar habitats on banks of small rivulets in S Russia, Abkhazia and W Ukraine. The aim of the study was to assess the size of clones, the number of genets and their distribution pattern along river banks and in wet habitats nearby.

Table 1. Sampled localities and specimens composition.

Locality	Population designation	Geographical coordinates	Sample composition
Abkhazia, N of Novy Afon, bank of Psyrtzkha River	A	43°5'31.15"N 40°48'57.91"E	*M. aquatica* A-01a, A-01b, A-01c, A-01d A-02a, A-02b, A-02c, A-02d A-03a, A-03b, A-03c, A-03d *Mentha* sp. A-04a
Ukraine, Lviv Prov., Pustomytovsky distr., near Gamalievka vill., Yarychavka Riv.	U	49°54'13.03"N 24°8'52.15"E	*M. aquatica* U-12a, U-12b, U-12c
Ukraine, Lviv, Belogorshcha, bank of a drainage channel	U	49°50'53.17"N 23°56'29.91"E	*Mentha* sp. U-08a, U-08b
Russia, Voronezh Prov., 10–15 km NE of Voronezh, Usmanka Riv.	V	51°48'33.97"N 39°22'55.87"E	*M.* cf. *aquatica* V-01a, V-01b V-02a, V-02b, V-02c V-03a, V-03b *M. aquatica* V-04a, V-04b, V-04c, V-04d, V-04e V-05a V-06a V-07a V-08a V-09a V-10a V-11a V-12a V-13a V-15a V-16a V-17a V-18a V-19a V-20a
Russia, Vladimir Prov., Petushinsky distr., near Filimonovo vill., wet forest road	F	55°58'28.16"N 39°15'48.70"E	*M. arvensis* F-04a F-04b

Materials and methods

Population sampling: Plants of *M. aquatica* were sampled from three populations several hundred kilometers distant from each other. The first one is situated in W Ukraine (population U), the second one in Abkhazia in NW Caucasus (population A) and the third one in Voronezh Province of S Russia (population V). The list of localities and specimens studied is given in Table 1. In all cases, plants were collected from shallow water and muddy banks of small rivers. The sampling design was as follows: In population A, four shoots were collected 1 m away from each other from three sites (A-01, A-02 and A-03) ranging 50–200 m along the river. Additionally, a plant with unusually elongated leaves was collected about 2 m far from the water edge (A-04) near the site A-03. In the population U, only three plants were collected 3–4 m distant from each other along the river bank (U-12). Two additional plants, morphologically intermediate between *M. aquatica* and *M. arvensis* L. were collected on a bank of a drainage channel at another locality (U-08) about 15 km SW of the first. Here, both localities are regarded as a single population. In

the population V, usually one shoot was sampled from each growth patch, which initially was supposed to represent a single vegetative clone. One of such a patch (specimen V-04), about 3 × 7 m in size, was more extensively sampled by taking 5 aerial shoots from its marginal parts and the middle part. Specimens V-05–V-13 were collected from the mint growth patches on both banks of the river nearby the specimen V-04 in a distance of 5–10 m to each other. The specimen V-16 was collected about 300 m upstream. The specimen V-15 was located about 1.2 km upstream of V-16, and the specimens V-17–V-20 were taken from growth patches located about more than 1.2 km upstream. Altogether 17 supposed clones were sampled from 4 locations for 2.7 km along the river course. Three more samples (V-01–V-03) with 2–3 shoots from each potential clone were sampled from swampy banks of a bayou in an alder swamp reaching about 100 m to the flood plain from the river bank. Two plants of *M. arvensis* from Vladimir Prov. (population F) were taken as an outgroup. Voucher specimens of all the plants are kept at the Herbarium of Main Botanical Garden of Russian Academy of Sciences [MHA].

Morphological comparisons: The plants collected for this study were determined by use of the key in 'Flora partis Europaeae URSS' (Menitzky 1978) and by comparisons with the specimens available at MHA. Most of them were doubtlessly determined as *Mentha aquatica* or *Mentha arvensis* (2 outgroup specimens). Two specimens from W Ukraine (U-08) and one from Abkhazia (A-04a), however, were morphologically intermediate between *M. aquatica* and *M. arvensis* and were supposed to be putative hybrids between them. Three specimens (V-01–V-03) from the alder swamp of Usmanka River flood plain were evidently represented by shadow morphs and differed from the plants of the river banks by leaf shape. However, they were also preliminary determined as *M. aquatica*. No further detailed morphological comparisons were conducted since the plants from the population V were collected in June lacking in developed inflorescences.

DNA extraction and PCR conditions: DNA was extracted from dry leaves taken from herbarium specimens using CTAB method (Doyle & Doyle 1987). We used ISSR (Inter Simple Sequence Repeat) markers to study DNA polymorphisms, since they proved to be adequate and useful for these purposes in our previous studies (Schanzer & Vagina 2007; Schanzer & Voilokova 2008; Schanzer & Kutlunina 2010; Kramina & Schanzer 2010; Fedorova et al. 2010). Other types of DNA dominant markers (RAPD, AFLP) have previously been successfully applied to infer hybridization among species of the genus *Mentha*, including *M. aquatica* and *M. arvensis* considered here (Gobert et al. 2002; Shasany et al. 2005). Primers used for PCR were synthesized and purified in PAAG by Syntol Ltd. (Moscow, Russia). Eight primers were selected after preliminary screening. They are listed in Table 2. The details of ISSR PCR conditions were the same as described by Fedorova et al. (2010). Annealing temperature for all the primers was invariably 50°C.

Analyses of molecular data: Banding profiles of ISSR fragments were visualized in agarose gels and compared by eye. Only bright and clear bands were taken into consideration for further analyses. Each fragment that was amplified using ISSR primers and visualized as a band in an electrophoretic gel, then was treated as a unit character and scored in terms of a binary code (1/0 = +/-). Ambiguous bands were counted as missing data. Initially, the matrix of band presence/absence was analyzed using cluster analysis (Unweighted Pair Group Method with Arithmetic Mean, UPGMA) procedure with Jaccard similarity measure as implemented in PAST 2.07 (Hammer et al. 2001). Bootstrap procedure with 1000 replicates was used to test the stability of the resulting dendrogram.

Table 2. ISSR primers used for PCR.

Primer	Sequence
M12	$(CA)_6$(A/G)(C/T)
M2	$(AC)_8$(C/T)G
M3	$(GA)_8$(C/T)C
M7	$(CAG)_5$
M8	$(GTG)_5$
M9	$(GACAC)_4$
UBC 840	$(GA)_8$AYT
UBC855	$(AC)_8$CYT

Two Bayesian approaches implemented in Structure 2.3.1 (Pritchard et al. 2000; Falush et al. 2007) and NewHybrids 1.1 (Anderson & Thompson 2002) were used to analyze the data under two different genetic models. In Structure 2.3.1, the admixture model correlated with gene frequences was used, since most of the specimens analyzed were supposed to belong to the same species with only a few putative interspecific hybrids with the outgroup species. The numbers of K=1–9 were tested with 3 replicates per K and 1 million Markov chain Monte Carlo repetitions.

In NewHybrids 1.1, the default model of two hybridizing diploids was used. Despite both ingroup and outgroup species of *Mentha* are known to be polyploids from literature (Pogan et al. 1986; Chambers & Hammer 1994; Gobert et al. 2002), this model seems to be a rather universal one and usually gives very reasonable results when it is used to analyze hybridizing populations notwithstanding their ploidy level (Schanzer & Kutlunina 2010; Kramina & Schanzer 2010; Fedorova et al. 2010). Like the model used by Structure 2.3.1, this model also implies Hardy-Weinberg equilibrium and linkage equilibrium for the markers being analyzed. The analysis was run for 50000 repetitions in several replicates for assessing stability of the results.

Results

90 bands were generated for 47 individual shoots with 8 ISSR primers. The banding profiles for all samples of *M. aquatica* from Usmanka River (population V) appeared to be identical with all of the 8 primers. However, evident differences in banding profiles were found among samples from the river and among those from the bayou (specimens V-1–V-3) as well as from the other localities, including the outgroup specimens of *M. arvensis*.

Cluster analysis grouped together all the samples of *M. aquatica* into a single cluster with Jackard similarity of 0.65 and high bootstrap support of 98 (Fig. 1). Within this cluster, the specimen U-12c took the basal position, while two other specimens from W Ukraine, U-12a and U-12b, appeared to be indistinguishable from each other and therefore were grouped together with three specimens from Abkhazia to the subcluster B. All the specimens from the Usmanka River banks formed a separate subcluster A, sister to the rest of *M. aquatica* specimens, with all zero branch lengths within this subcluster. The cluster B unites the specimens from Abkhazia and W Ukraine. In turn, it is subdivided into two subclusters with low bootstrap support. Terminal

clusters grouping together separate specimens receive high bootstrap support (97–100%). All of them, however, have zero-length branches indicating the absence of differences between the grouped specimens in their corresponding ISSR profiles.

The specimens from W Ukraine (U-08) and Abkhazia (A-04a) determined as intermediate between *M. aquatica* and *M arvensis* on morphological grounds formed separate clusters C and D basal to the 'aquatica' cluster with lower similarity and bootstrap support.

Another cluster with support of 99 grouped all the specimens from the bayou (V-01–V-03) of Usmanka Riv. (cluster E). This cluster, however, grouped together with the outgroup (cluster F), i.e. the specimens from the bayou turned out to belong to *M. arvensis* instead of *M. aquatica* as it was supposed at first. Though these specimens were collected from three separate patches supposedly representing separate clones, 5 different genotypes would be identified among them.

The Bayesian analyses of these data in Structure 2.3.1 reveal that the highest LnP values are always achieved for K=5. As it can be seen from the graph in Fig. 2, the LnP(D) value grows rapidly with the increase of the K number (number of groups) from 2 to 5, and then slowly decreases with a drastic increase in variance between the runs. This may be interpreted as the most probable subdivision of the sample into 5 groups.

The bar plot in Fig. 3 (lower part) shows posterior probabilities of assigning particular specimens to one of the groups (K) for K=5. Here the majority of specimens are correctly assigned to their corresponding populations with posterior probabilities close to 100%. There are a few exceptions, however. The specimen A-04a from Abkhazia, which had initially been determined as a putative hybrid between *M. aquatica* and *M. arvensis*, was assigned to the same population as the outgroup (population F) with the posterior probability of 0.93. Among the specimens from

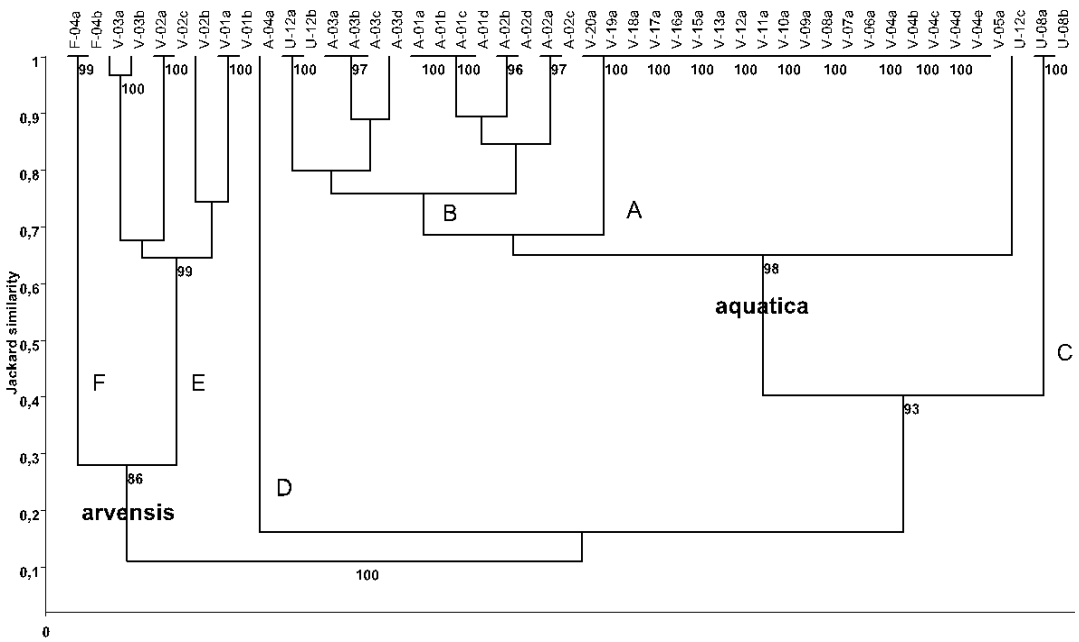

Figure 1. Results of Cluster Analysis of 90 ISSR markers for 47 specimens, Jaccard similarity measure. Specimen designation as in Table 1. A – population from Abkhazia; U – populations from W Ukraine; V – population from Voronezh Prov. of Russia; F – outgroup population of *Mentha arvensis*.

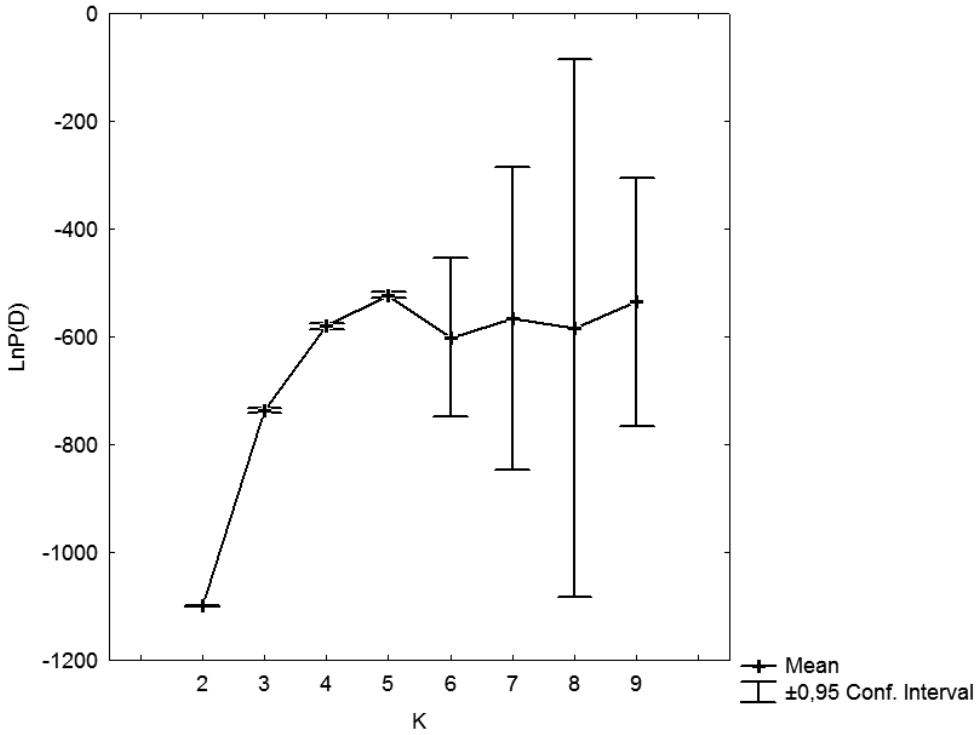

Figure 2. Plot of correspondence between the mean Ln probability of data (LnP(D)) and the number of groups (K) for the analyses of ISSR markers of *Mentha* in Structure 2.3.1.

population U, those from a bank of drainage channel in Lviv (U-08) had first been supposed to be hybrids between *M. arvensis* and *M. aquatica* as well. They, however, were assigned to a separate population with posterior probabilities of 100%. On the contrary, specimens of *M. aquatica* from banks of Yarychavka River (U-12) quite typical from a morphological point of view were revealed as admixed individuals.

The analysis in NewHybrids 1.1 (Fig. 3, upper part) divided the samples into three groups with posterior probabilities of 100%. All *M. aquatica* specimens from all the localities were determined

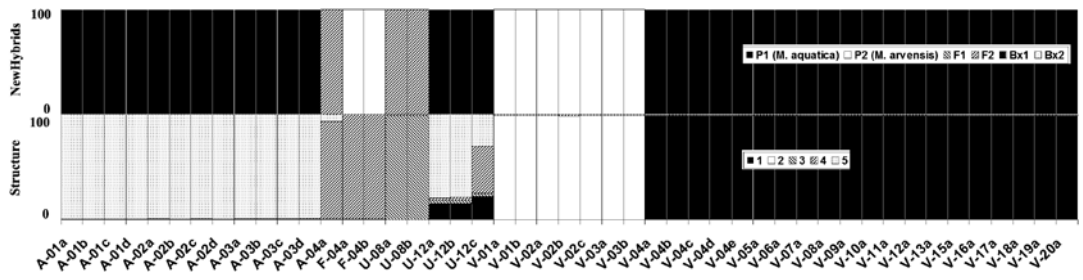

Figure 3. Results of Bayesian analysis in Structure 2.3.1 (lower bar plot): posterior probabilities of clusterization of 47 *Mentha* specimens into K=5 groups by ISSR marker composition. Specimen numbers are shown below the plot. Numbers in the legend correspond to the cluster numbers.

Results of Bayesian analysis in NewHybrids 1.1 (upper bar plot): posterior probabilities of clusterization of 47 *Mentha* specimens into genotype classes by ISSR marker composition. Designations in the legend correspond to genotype classes: P1 – first parental species; P2 – second parental species; F1 – first generation hybrids; F2 – second generation hybrids; Bx0, Bx1 – backcrosses.

as the first parental species (P1). All *M. arvensis* specimens, including those from the bayou of Usmanka River (V-01–V-03), were determined as the second parental species (P2). The three putative hybrid plants (A-04a from Abkhazia and U-08a and U-08b from W Ukraine) were determined as F2 hybrids with the same posterior probability of 100%.

Discussion

Our study revealed the genetic variability in populations of *M. aquatica*, even though just a few plants have been sampled from populations in Abkhazia and W Ukraine. It appears, that several (two in our case) different genotypes may occur within the same growth patch of mint. In the Abkhazian population A, the sample A-01 was represented by four genetically indistinguishable plants of the same clone, whereas two other samples (A-02 and A-03) both contained plants belonging to two different clones. In W Ukraine only three plants were sampled from the bank of Yarychavka River, and they appeared to belong to two different clones (U-12a,b and U-12c). Distances between plants of the same clone in all the cases varied between 1–3 meters, and plants sampled further downstream the river course invariably belonged to different genotypes. This pattern corresponds well to knowledge about populations of clonal plants from the literature.

The most unexpected result was achieved for the population V from the banks of Usmanka River in Voronezh Province of Russia. It appears that though plants from this population undoubtedly belong to *M. aquatica*, they at the same time belong to only one single genotype. Thus, all the growth patches of *M. aquatica* dispersed on the banks of Usmanka River 2.7 km along the stream are ramets of a single genet. This means that seed propagation is completely absent in this locality of *M. aquatica*. The most probable explanation is that *M. aquatica* is self-incompatible. Isolation of inflorescences of a plant collected from Usmanka River and grown in a nursery of the Main Botanical Garden's department of physiology resulted in complete lack of seed setting. At the same time, other inflorescences of the same plant set seeds under open pollination, despite all the other mint plants in the nursery belonged to other species of *Mentha*. Probably only one haphazard introduction of this species had occurred at Usmanka River in the past due to a long-distance dispersal from an unknown source. Though that introduction appeared to be successful, the single plant was able to propagate only vegetatively due to the absence of mates in the neighbourhood. Though plants of *M. arvensis* bearing different genotypes occur in close vicinity at the flood plain, no hybridization events occurred so far between them and the *M. aquatica* clone distributed along the river banks. However, this might happen in near future, because both ISSR data from Abkhazian and W Ukrainian populations and observations on seed setting in the nursery confirm the ability of these species to hybridize with each other. The analysis in NewHybrids 1.1 did not reveal a sign of backcrosses or specimens with low probabilities of assigning them to different classes of hybrids, which are usual when introgression takes place. This may be partly due to small sample sizes in our study, but this may also reflect the restricted nature of interspecific hybridization between these species of mint. Somewhat contradictory results of analyses were achieved in Structure 2.3.1 and NewHybrids 1.1 (Fig. 3). The first revealed the specimens U-12a, U-12b and U-12c from Yarychvka River in W Ukraine as genetically admixed individuals, the latter revealed the same specimens as pure representatives of *M. aquatica*. We cannot confidently explain this result, therefore we suppose it to be an artifact due to insufficient number of specimens sampled from this locality and/or markers used to assess the genetic nature of the plants.

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