

Application of Molecular Markers to Study the Systematics, Phylogeny, Biogeography, Genetic Diversity and Population Genetics of *Hippophae* L.

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ABSTRACT

Systematics and phylogeny of seabuckthorn (*Hippophae* L.) are controversial, when based on the morphological characters. Recent studies of these subjects by the application of molecular markers, such as isozymes, random amplified polymorphism of DNA (RAPD) and restriction fragment length polymorphism in chloroplast DNA (cpDNA RFLP), supported one of taxonomies of the genus. The number of taxa, for which a specific status can be justified, is higher than analysis of morphological characters could reveal. Therefore, a modification for previous systematics was suggested. New hypotheses on phylogeny and historical biogeography of *Hippophae* are discussed. Genetic diversity within different taxonomical hierarchies and its partitioning among them, as calculated from molecular data sets, is also discussed.

Key words: Seabuckthorn (*Hippophae* L.), RAPD, systematics, taxonomy, phylogeny and biogeography.

SYSTEMATICS AND TAXONOMY

Two main directions were followed in the systematics and taxonomy of *Hippophae*, during the last century. Starting from the first concise treatment of the genus by Servettaz (1909), one group of authors has been advocating the classification, in which the genus is monospecific (Yeliseyev 1974 a; Avdeyev, 1983), or has only two species (Hyvonen, 1996). Rousi (1971) initiated a revisional treatment of the genus and recognized three species: *H. rhamnoides*, *H. salicifolia* Don and *H. tibetana* Schlecht and nine subspecies within *H. rhamnoides* (*carpatica*, *caucasica*, *fluviatilis*, *gyantsensis*, *mongolica*, *rhamnoides*, *sinensis*, *turkestanica* and *yunnanensis*). Lian et al. (1998) developed this revision further by distinguishing two sections: sect. *Hippophae* (*H. rhamnoides* and *H. salicifolia*) and sect. *Gyantsensis* Lian (*H. goniocarpa*, *H. gyantsensis*, *H. neurocarpa* and *H. tibetana*). According to Lian et al. (1998), there are eight subspecies within *H. rhamnoides* (they attributed a specific status to *H. gyantsensis*, previously recognized as a subspecies within *H. rhamnoides*), two subspecies within *H. goniocarpa* (*goniocarpa* and *litangensis*) and two subspecies within *H. neurocarpa* (*neurocarpa* and *stellatopilosa*).

When analysis of morphological characters drives to controversial conclusions, more sophisticated approaches, such as analysis of molecular markers, can be used to solve a taxonomic problem (Doyle, 1992). Unfortunately, only few studies have been carried out so far to investigate the taxonomy and phylogeny in *Hippophae* by application of molecular analyses. Moreover, to the best of our knowledge, there still have been no publications in international literature on the analysis of molecular markers on a representative plant sample of all recognized taxa within *Hippophae*. Therefore, reviewing the literature on the subject, in many cases, we have to refer to our recent unpublished results.

Yao and Tigerstedt (1993) were probably the first who applied molecular markers to study the genetic diversity and phylogeny in *Hippophae*. Their results, although based on the small sizes of plant samples for two species (*H. tibetana* and *H. neurocarpa*) and not complete representation of subspecies for the third species (in *H. rhamnoides*, only wild populations of ssp. *rhamnoides*, *sinensis* and *turkestanica*, and a progeny derived from a cultivar of ssp. *mongolica*, were sampled), justified the division of subspecies under *H. rhamnoides* and specific status of *H. neurocarpa* and *H. tibetana*.

Within *H. rhamnoides*, populations from different geographical regions represented monophyletic groups and were clearly differentiated in accordance with their taxonomic status (Yao and Tigerstedt, 1993). RAPD analysis of wild populations representing all taxa but one (*H. goniocarpa* ssp. *litangensis*), according to the systematic classification suggested by Lian et al. (1998), provided further evidence of considerable genetic differentiation in *Hippophae* (Bartish et al., 1999 a). Corroborating the results of Yao and Tigerstedt (1993) and systematics of Rousi (1971), all populations of *H. rhamnoides* from different geographical regions represented clearly differentiated monophyletic groups in accordance with their taxonomic status (ssp. *carpatica*,

caucasica, fluviatilis, mongolica, rhamnoides, turkestanica). In agreement with the suggestion of Lian (1988), the specific status of *H. gyantsensis* was justified.

However, the analysis of nuclear DNA markers did not support systematic position of *H. rhamnoides* ssp. *sinensis* and ssp. *yunnanensis*. In contrast to all previous taxonomies of *Hippophae*, these two subspecies could not be attributed to any of the recognized species. In fact, Lian et al. (1993) have tried to separate these taxa into a species with two corresponding subspecies, but retreated from such idea in their latest classification (Lian et al., 1998). With the exception of unclear status of these two taxa, the analysis of nuclear DNA markers supported in general the systematic classification of *Hippophae*, suggested by Lian et al. (1993, 1998).

Parsimony analysis of cpDNA RFLP data on the same populations, which have been studied by RAPD markers of nuclear DNA, but with addition of several species from *Elaeagnus* and *Shepherdia* (Bartish et al. unpublished), revealed: i) strongly supported monophyly for all species within *Hippophae* and *Elaeagnus*; ii) monophyly for all subspecies of *H. rhamnoides*, except ssp. *sinensis*; iii) weak support for phylogenetic relationships among species of *Hippophae*; iv) relatively recent diversification within monophyletic group of *H. rhamnoides* subspecies (all except *sinensis*) from one common ancestor. This analysis provided no support for division of the genus into sections (suggestion of Lian, 1988). Comparative analysis of nuclear and chloroplast DNA phylogenies showed good correspondence between them in general. However, some reticulation events in evolution of *Hippophae* could not be ruled out.

HISTORICAL BIOGEOGRAPHY OF HIPPOPHAE L.

Several hypotheses have been suggested regarding historical biogeography and evolution of *Hippophae* on the basis of analysis of its morphological characters, fossil records and present distribution. Rousi (1971) followed the idea of Bobrov (1967) and suggested "a common origin for *H. salicifolia*, *H. tibetana* and the Central Asiatic subspecies of *H. rhamnoides*. As regards the other subspecies of *H. rhamnoides*, they seem to have attained their present areas by migration westwards from Central Asia". According to Bobrov (1967), Rousi (1971) and Yeliseyev (1974 b), the migration could take place in Miocene, which must have been a period of active evolution of the genus in Asia.

On the basis of parsimony analysis of 36 morphological characters and diploid chromosome number, which was applied to all presently recognized taxa within *Hippophae*, but two (*H. goniocarpa* ssp. *goniocarpa* and ssp. *litangensis*), and to five species from *Elaeagnus* and *Shepherdia* as an outgroup, Hyvonen (1996) came to conclusion about wide range of ancestral *Hippophae*. Although there is not yet any record of *Elaeagnaceae* among xerophytic plants in the early Tertiary in Southern Europe, he believes that "the ancestor of *Hippophae* might have been present in these communities" (Hyvonen, 1996). Therefore, the "process of gradual fragmentation of the former, wider range and subsequent isolation and differentiation of the local populations" could better explain the present geographical distribution of *Hippophae*. Following his argumentation, *Hippophae* must have been present in Southern Europe as early as in Palaeogene. Consequently, either differentiation between Asian and European taxa of this genus had occurred at least 25-30 million years ago (Ma) as in fact has been suggested by Bobrov (1967), or all Asian taxa of *Hippophae* were dispersed there from Southern Europe later.

The common drawback of all these hypotheses is the lack of fossil records, which would support the presence of *Hippophae* in Europe among Tertiary floras. Besides, it is difficult to explain on the basis of these hypotheses, why morphological differentiation between Asian and European subspecies of *H. rhamnoides* is so weak. If, according to the idea of Miocene dispersal of *H. rhamnoides* to Europe, they were differentiated for such a long time period that their morphological diversification would be much more pronounced, than can be seen from published reports.

Contrary to these hypotheses, results from parsimony analysis of cpDNA RFLP data set (Bartish et al., in preparation) indicate that both Central Asian and European subspecies of *H. rhamnoides* diverged from a common ancestor not earlier than in Quaternary, some 0.2-0.3 Ma, if estimated from a molecular clock under assumption that 1 Ma corresponds to 1.2% of differentiation in DNA sequences (Sang et al., 1995). The ancestor of present taxa of *H. rhamnoides* must have been growing in Central Asia before the ice ages of Pleistocene. During climatic oscillations of Quaternary, areas of open land after retreat of a glacier might make it possible for *H. rhamnoides* to disperse northward (and, correspondingly, in both north eastward and north westward directions) by following the retreating glacier and occupying its new habitats at the coasts of pluvial lakes. Thus, not a chain of fused mountains to the north of the Iranian Plateau in Miocene, as suggested by Rousi (1971), but a chain of unstable pluvial lakes, created by Siberian Ice Sheet during Quaternary, could be the main factor responsible for westward dispersal of *H. rhamnoides*. This would be in a good agreement with ecology of the species, which is known to be "a widespread pioneer species in the open Late-glacial plant communities of Europe" (Rousi, 1971). The populations of this species could survive the advance of the glaciers in southern refugia in Alps, Caucasus and Pamir. The putative dispersal of *H. rhamnoides* to north west of Central Asia must have been rapid, since no evidence of asymmetry in the phylogenetic tree of

subspecies of *H. rhamnoides* has been found. It is quite probable that this species had been widely (from Siberia to Atlantic coast of Europe) and even continuously distributed during some of interglacials of ice ages. Lately, however, the scenario of its evolution may have followed the prediction of Hyvonen (1996), i.e. the fragmentation of this wide range with the subsequent isolation and differentiation of local populations in geographically well separated southern refugia in mountains (Alps, Carpathian Mountains, Caucasus, Pamir-Tian Shan, Altai).

Three European subspecies of *H. rhamnoides* (*carpatica*, *fluviatilis* and *rhamnoides*) represent a monophyletic group differentiated from other subspecies, according to the cpDNA phylogeny (Bartish et al., in preparation). Both phylogenetic analyses (nuclear and cpDNA) indicate very close relationships between subspecies *rhamnoides* and *carpatica*. In accordance with palynological data on the fossils of *Hippophae* in Europe (Sandegren, 1943, Grichuk, 1972, Zoller et al., 1998), the ranges of these subspecies must have been separated by advance of boreal flora as late as only approximately 7000-9000 B.P. It is noteworthy that some occasional stands of *H. rhamnoides* (probably, ssp. *rhamnoides*) could be found in Central Russia even 200 years ago (Yeliseyev, 1974 b). However, it is unlikely that any continuous range of this subspecies existed in Europe for the last several thousand years.

Regarding differentiation between species, the cpDNA RFLP data suggest that all species of *Hippophae* diverged from one common ancestor at approximately the same time, some 0.7-1.2 Ma, according to estimates from a molecular clock (Bartish et al., in preparation). Such time scale corresponds well with the certain geological event - acceleration of uplift of Himalayas in Pleistocene (Skinner and Porter, 1987). If, in accordance with the previous suggestion of Lian et al. (1998), we assume the connection between evolution of *Hippophae* and the rise of Himalayas, then differentiation between species of this genus must be a result of the fragmentation of its ancestral range. Eastern, south western and north western parts of this range, where the extant populations of, respectively, *H. rhamnoides* ssp. *sinensis*, *H. salicifolia* and *H. rhamnoides* ssp. *turkestanica* presently grow, must have been separated by the rising mountains and their ice sheets during the first ice ages of Pleistocene. Northern part of this area has been occupied by Gobi desert at least since Pliocene (Sinitin, 1961), therefore it may be supposed that populations of the ancestor of *Hippophae* could grow in mountains closely to the southern edge of this desert at that geological period. These populations to the north of Himalayas could acclimatize to conditions of higher altitudes of Tibetan Plateau, as the ranges of their stands were constantly uplifted. *H. gyantsensis*, *H. neurocarpa* and *H. tibetana* could survive in mountainous refugia during cold periods and differentiate in their respective areas in such way.

RETICULATE EVOLUTION IN HIPPOPHAE L.

Two recently discovered taxa, *H. goniocarpa* ssp. *goniocarpa* and *H. goniocarpa* ssp. *litangensis*, were supposed to have been originated as interspecific hybrids (Lian et al., 1995). Subspecies *goniocarpa* is a putative hybrid of *H. rhamnoides* ssp. *sinensis* and *H. neurocarpa* ssp. *neurocarpa*, and ssp. *litangensis* may be a hybrid resulting from a cross between *H. rhamnoides* ssp. *yunnanensis* and *H. neurocarpa* ssp. *stellatopilosa*. A population of one of these subspecies, *goniocarpa*, has been analysed in the studies of molecular markers in *Hippophae*. The results of RAPD analysis corroborated the hypothesis of Lian et al. (1995). Several of RAPD markers, differentially fixed for presence or absence in populations of *H. rhamnoides* ssp. *sinensis* and *H. neurocarpa* ssp. *neurocarpa*, had frequencies between 0 and 1 within a population of *H. goniocarpa* ssp. *goniocarpa* (Bartish et al., 1999 a). This population was also phylogenetically intermediate between the putative parent taxa, as the neighbour-joining cluster analysis indicated. The results of cpDNA RFLP study suggest that *H. rhamnoides* ssp. *sinensis* was the mother parent of this hybrid.

Somewhat surprisingly, the molecular studies also suggest that *H. rhamnoides* ssp. *yunnanensis* is a putative interspecific hybrid between *H. rhamnoides* ssp. *turkestanica* (mother parent) and *H. salicifolia* (Bartish et al., in preparation and in review). On the other hand, however, such a result might be expected, since this taxon was included into *H. rhamnoides* by Rousi (1971), but into *H. salicifolia* by Hyvonen (1996). Besides, as it has been mentioned previously, Lian et al. (1993, 1998) were inconsistent regarding its position in their systematic classification of *Hippophae*. Further more, all three taxa grow sympatrically in Xizang province in China at present (Lian et al., 1998). On the basis of all this evidence, Bartish et al. (in review) hypothesised that *H. rhamnoides* ssp. *yunnanensis* is an interspecific hybrid and suggested to rise its taxonomic status to the level of a species. Since *H. goniocarpa* ssp. *litangensis* is a putative interspecific hybrid, and Lian et al. (1995) suggested ssp. *yunnanensis* to be one of its parents, the status of this hybrid taxon might also be revised. In this case, however, more evidence is needed.

H. rhamnoides ssp. *sinensis* (or *H. sinensis*, as has been suggested by Bartish et al., in review) is well diversified from all other taxa in *Hippophae* according to cpDNA phylogeny (Bartish et al., in preparation), but is relatively close to *H. rhamnoides* according to RAPD analysis. It may be an indication of a secondary contact and introgression of nuclear genes between these species, perhaps in the late Quaternary.

One more case of reticulate evolution may involve two subspecies of *H. rhamnoides*, ssp. *caucasica* and *fluviatilis*. These taxa were found to be grouped in phylogenetic analysis of RAPD markers (Bartish et al., 1999 a), but did not reveal any more close relationships comparing to other subspecies of *H. rhamnoides* in cpDNA phylogeny. The possible explanation for these results could be a secondary contact and introgression of nuclear DNA between these taxa in the late Pleistocene, subsequent to a previous differentiation by the advance of a glacier.

GENETIC DIVERSITY AND ITS PARTITIONING WITHIN HIPPOPHAE

Information on genetic variation is of great significance not only for evolutionary aspects of speciation, but also for applied research, as for instance, for breeding of new cultivars. The heterozygosity of isozyme loci is a frequently used measure of genetic variability within a population or a taxon of higher level. Yao and Tigerstedt (1993) reported the results of analysis of several isozyme loci in a group of populations of *H. neurocarpa*, *H. rhamnoides* and *H. tibetana*. They found that observed heterozygosity and Nei's unbiased estimates of expected heterozygosity varied between 0.022-0.265 and 0.024-0.245 among the 24 populations, respectively. The mean heterozygosity per population per locus was 0.168 in ssp. *rhamnoides*, 0.123 in ssp. *turkestanica*, 0.117 in ssp. *sinensis* and 0.140 in the total material. Partitioning of gene diversity into its components showed that 53.9% of the gene diversity within *H. rhamnoides* was allocated within populations, and gene diversity among geographical populations within subspecies was only 4.5%, indicating that there were only slight differences among the populations of the same subspecies. The gene diversity between subspecies was 41.6%, suggesting that significant differences exist among these taxa. Finally, at specific level, the gene diversity between species was 55.6% of the total variance, showing that a further differentiation appeared at the specific level.

The corresponding estimates of the genetic variability, gene diversity and its partitioning into components were quite similar in the RAPD study of *Hippophae* (Bartish et al., 1999 a, b). Although direct comparisons across studies of the actual values of expected heterozygosity obtained by isozyme and RAPD analyses may not be valid due to several reasons (discussed in Bartish et al., 1999 a), the relative order of estimated values obtained within a study should still be open to comparisons. As in the isozyme study, RAPD markers revealed higher expected mean across population's heterozygosity within *H. rhamnoides* ssp. *rhamnoides* than within ssp. *mongolica* and *sinensis*. *H. goniocarpa* and *H. tibetana* were the most diverse, and *H. salicifolia* was the least diverse species in the genus. Genetic variability within populations, among populations within subspecies and between subspecies of *H. rhamnoides* accounted for 57.6%, 6.1% and 36.3% of the total variance respectively. Considerably less variation among species within the genus (30.6%) was found in the RAPD analysis comparatively to isozyme study (55.6%). The difference in the structure of plant samples analysed by isozyme and RAPD markers could account for this discrepancy. It should also be noted; that in many cases a taxon was represented by only one population with relatively small sample of individual plants (8-15). Therefore, the reported estimates should be considered as preliminary.

CONCLUSIONS

Molecular markers, both isozyme and different kinds of DNA markers (RAPDs, cpDNA RFLPs) appeared to be very useful to study the phylogeny, systematics, historical biogeography and population genetic structure in *Hippophae*. In general, isozyme and DNA markers revealed similar patterns of genetic structure in different taxa of this genus. These methods justified systematic classification of *Hippophae*, first suggested by Rousi (1971) and developed further by Lian et al. (1993, 1998). However, the molecular analyses revealed also several important discrepancies with the morphological studies. They did not support division of the genus into two sections, and suggested to rise the taxonomic status for *H. rhamnoides* ssp. *sinensis* and *yunnanensis* to the level of a species.

Phylogenetic reconstructions of cpDNA of almost all recognized taxa within the genus were not in agreement with previous biogeographic hypotheses about differentiation between Asian and European taxa in Miocene. Instead, the connection between acceleration in upheaval of Himalayas in Pliocene and diversification among species of *Hippophae*, and between climatic oscillations of Quaternary and differentiation among subspecies of *H. rhamnoides*, was suggested on the basis of a molecular clock.

Analysis of genetic variability within and among taxa at different levels of taxonomical hierarchy, based on molecular markers, revealed considerable genetic variation within populations of all species and subspecies, which is in accordance with dioecious mating system of *Hippophae* and wide range of geographic distribution of *H. rhamnoides*. Within this widely distributed species, most of the genetic variation was found either within populations, or among subspecies. Genetic differentiation among populations from the same subspecies was relatively weak.

According to Lian et al. (1998), field collections of *Hippophae* are still very limited, and the taxonomic resources of the genus have not been clarified. We believe that collaborative work between botanists, collecting new unidentified taxa from this genus, breeders, producing new varieties and cultivars, and molecular biologists would provide a basis for productive studies in systematics, historical biogeography, genetic diversity and breeding of *Hippophae*. The main directions of further molecular studies in *Hippophae* would be represented by:

1. Combined analysis by isozyme and RAPD markers of more complete population samples within each taxon.
2. Sequencing and RFLP analyses of chloroplast DNA from the same plant samples to make possible comparisons between phylogenies of nuclear and organelle genomes of these taxa for detailed analysis of their historical biogeography and events of reticulate evolution.
3. Genomic mapping by application of RAPD, AFLP, SSR and isozyme markers to progenies of interspecific crosses and to families in which characters important for crop productivity segregate, to facilitate the search of molecular markers for such characters and the carrying out the markers-assisted selection.

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