

## Preliminary report on taxonomical position of *Vavilovia formosa* (Stev.) Fed. evidenced from morphological and molecular data

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The Fabaeae Rchb. (syn. Viciaeae Adans.) tribe includes numerous representatives of special agricultural value and has; therefore, received considerable attention. Despite (or because of) this, there are certain problems concerning its systematics and phylogeny. one of the most problematic groups within the tribe is the monotypic genus *Vavilovia* Fed. It includes very small perennial plants inhabiting the highlands of the Caucasus, Turkey, Lebanon and Iran. Although almost two centuries have passed since the first description of this plant was reported by C. Steven (more detailed information on the history of its taxonomical status is presented in (1)), its status remains unresolved. Previously it was included in the genera *Orobus*, *Lathyrus*, *Pisum*, or treated as a separate genus, *Alophotropis* Grossh. or *Vavilovia* Fed. The most recent monographs on the family tend to describe its status as a monotypic genus including a single species, *Vavilovia formosa* (Stev.) Fed. (2).

*Vavilovia* inhabits very specific areas and all efforts to maintain the plant beyond its natural habitat failed or presented significant difficulties (3, 4). Moreover, it is an endangered species with disrupted distribution areas, some of which are in danger of being overgrazed (5). Probably due to paucity of material, the genus remains poorly studied and until now few efforts have been made to use molecular data to resolve its taxonomical status. The only available work in which some features of the chloroplast genome were studied was by Jansen et al. (6), but this sheds no light on the position of *V. formosa* in the tribe. Strong relationship between *Vavilovia* and *Pisum* was demonstrated basing on sequences of both cpDNA and nrDNA regions and discussed in (7) and (8). Some other similarities such as susceptibility to pea-specialized fungal infections, chromosome number and successful crosses between *Pisum* and *Vavilovia*, etc., also indicated a strong relationship. It should be noted; however, that the possibility of mutual hybridization between listed genera (4) can hardly be interpreted as a feature of relationship. For example, some reports on crosses between *P. sativum* and *Vicia faba* exist (9, see also 10), while no success was reached in crosses between *Pisum* and *Lathyrus*. The same can be concluded based on chromosome number, where  $2n=14$  remains constant for all genera within Fabaeae (with rare exceptions) together with numerous related legumes (such as tribe Loteae DC.). Some differences in chromosome morphology between these genera were revealed in (11).

This work was aimed at clarification of the status of *Vavilovia* within the tribe Fabaeae with special reference to its relation with *Pisum*. This paper represents the preliminary results of the first stages of our investigation.

### Materials and Methods

#### Plant material

Herbarium specimens of *Vavilovia formosa* from Moscow State university Herbarium (MW) and the Institute of Botany of the National Academy of Sciences (Republic of Armenia) were used as the material for this study. These samples represent material from different parts of the area of habitat of studied specimens in the Caucasus (Fig. 1); a total of 14 accessions were studied. Six *Pisum sativum* L. ssp. *sativum* cultivars from different world regions, four wild *P. sativum* subspecies and one *P. fulvum*

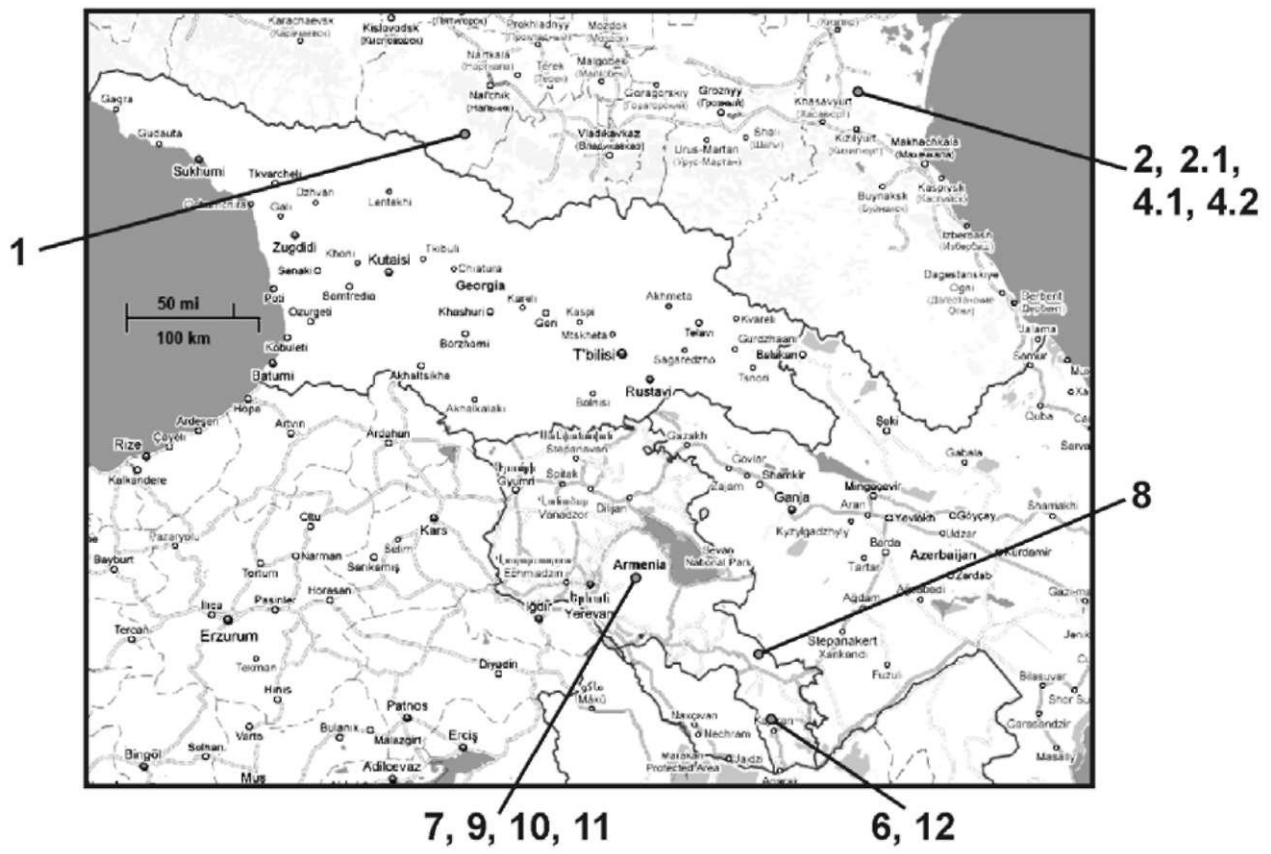


Figure 1. Map depicting locations of sample collection. The site of collection of accession number 5

Sibth. & Smith accession from the Genetics Department of Moscow State University and John Innes Center (Norwich, United Kingdom) *Pisum* germplasm collections were used for comparison. Eleven morphologically contrasting *Lathyrus* species were analyzed; *L. sativus* L., *L. tingitanus* L., *L. odoratus* L., *L. belinensis* N. Maxted & Goyder, *L. chloranthus* Boiss. & Balansa, *L. ochrus* DC., *L. aleuticus* (Greene) Pobed., *L. nissolia* L., *L. vernus* Bernh., *L. venetus* Rouy, and *L. aureus* (Steven) Bornm. (the three latter species were formerly placed in genus *Orobis* but are now treated as part of *Lathyrus*). Seeds of the first six species were kindly provided by Dr. G.D. Levko (All-Russian Research Institute of Breeding and Seed Production of Vegetables, Lesnoi Gorodok, Russian Federation). All listed material was used for both morphological and molecular analysis.

#### DNA extraction, PCR conditions and sequencing

DNA was extracted from freshly collected leaflets and herbarium specimens using a modified CTAB procedure (12). For homogenization of herbarium material, glass powder was added to the plant sample. PCR was carried out in a MC2+ Thermal Cycler (DNA Technology, Russia) according to a protocol described in earlier work (13). At this stage, only RAPD (Randomly Amplified Polymorphic DNA) markers were used to characterize polymorphism while usage of other marker types is in progress. The following primers were used: V (5'-gacagtagca-3'), VI (5'-cttggatgga-3'), V03 (5'-ctccctcaa-3'), B474 (5'-aggcgggaac-3'), D6 (5'-acctgaacgg-3'), F12 (5'-acgctaccag-3'), R11 (5'-gtagccgtct-3'), Q06 (5'-gagcgccttc-3'), QR2 (5'-cggccactgt-3'), AE07 (5'-gtgtcagtgg-3'), AE13 (5'-tgtggactgg-3'), Leb10 (5'-agccgcagct-3') (Syntol, Russian Federation). A total of 194 polymorphic bands were obtained.

The internal transcribed spacer region (ITS1-5.8 rRNA-ITS2) was amplified using primers flanking the ITS1-5.8 rRNA-ITS2 region of the nuclear genome. Primer sequences are available from (14) and (15) for forward and reverse primers, respectively. The fragment obtained from the sample designated Vf2.1 (Fig. 1) was sequenced using an automatic sequencer (ABI Prism 3100-Avant Genetic Analyzer) in the "Genome" Center (Institute of Molecular Biology of Russian Academy of Sciences) and used for further study.

#### Tree construction

Taxonomical analysis was performed by comparing obtained sequences with the corresponding region of all genera of the Fabaeae tribe (*Pisum*, *Lathyrus*, *Vicia*, *Lens*) arbitrarily chosen from GenBank (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>) database. Two *Cicer* (Cicereae) sequences were chosen as an outgroup. Phylogenetic analyses were conducted using the MEGA4 software (16). Data obtained from RAPD analysis was processed with usage of SplitsTree 4.10 software. The distances were calculated according to formula of L. Dice (17).

### **Results and Discussion**

#### Morphological features of *Vavilovia*

The *Vavilovia* plants are characterized with very specific morphology (Fig. 2). Some features were found which distinguish them from all studied *Pisum* accessions. These features include: perennial life form (*Pisum* annual) with long creeping rhizomes enabling vegetative propagation; narrow sepals covered with simple trichomes (glabrous and broad with overlapping margins in *Pisum*); leaf rachis ending with cusp (always tendrils in *Pisum*); small stipules (of the same size as leaflets or larger in *Pisum*); fluted leaf rachis cross-section (rounded in *Pisum*); supervolute ptyxis (plicate in *Pisum*, this trait is reported as one of high significance by F.

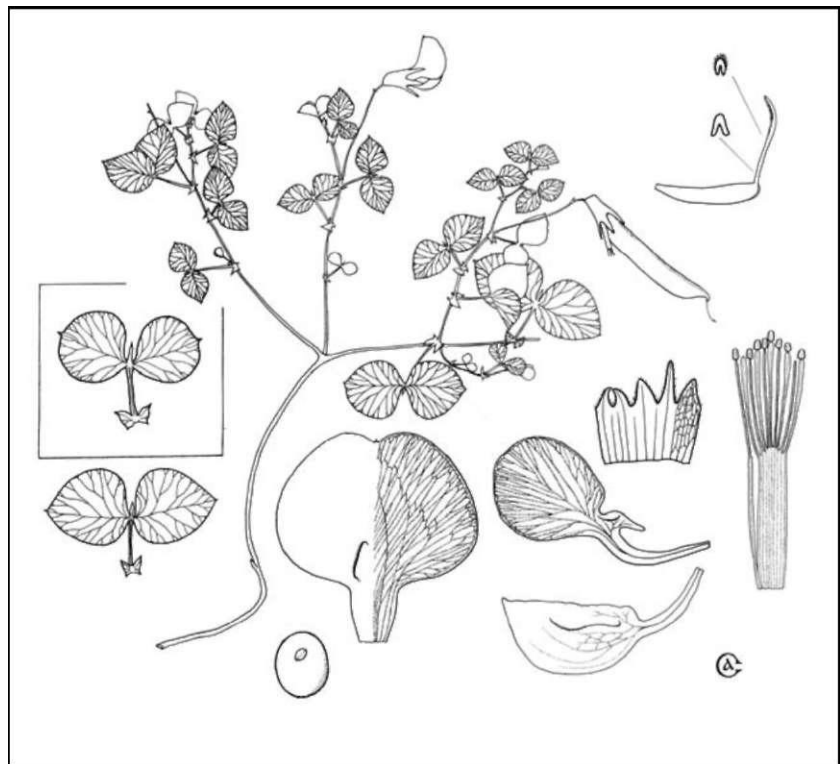


Figure 2. Morphology of *Vavilovia formosa*. Frame indicates the leaf of Armenian samples.

Kupicha (18)). All the listed characters can be found in *Lathyrus* species, but their interpretation is ambiguous. The life form (annual or perennial) is not used even to separate sections in *Lathyrus*. Leaf features are known to be very variable in ontogeny of legumes, and the first pea leaves are always characterized with inversely-ovate leaflets, fluted rachises ending with short process or even lacking any specialized terminus, and even small stipules (*P. fulvum*). Actually, *Vavilovia* leaves combine some features which can be found in juvenile forms of *Pisum* species. The calyx of the latter also

possess ciliate margins before anthesis. The only feature of special significance is ptyxis which was reported to be altered in mutational variation of *Vicia faba* (18).

The ovary of the *Pisum* flower is glabrous (C. Gunn and J. Kluge report glandular surface for some accessions (10)), while *Lathyrus* species exhibit both glabrous and pubescent (simple trichomes, nectariferous glands or combination of two types (10, original observations)) ovaries. *Vavilovia* samples we studied possessed a combination of simple trichomes and few-celled glands on the surface of the ovaries.

Some characters which distinguish *Vavilovia* from all studied *Lathyrus* species (and hence drawing with peas together) include; elliptic seed hilum (linear or rarely elliptic in *Lathyrus*); absence of pubescence on all organs except the calyx; and dilated filaments of outer whorl stamens (as they are in *Pisum*; somewhat dilated filaments are also found in *L. maritimus*). It should be noted that these characters had never been found altered even in mutational variation of *Pisum* and hence can be referred to as strongly constant for genus identity. Standard (vexillum) petal shape of *Vavilovia* also resembles that of *Pisum* rather than *Lathyrus*.

Two traits were cited to separate *Vavilovia* from *Pisum*, viz. pistil groove gaping only at the base (at base and apex in *Pisum*) and non-cristate keel (cristate in pea) (see (10); these authors reject these differences). Pistils appear to be grooved in the same way in both genera, as do other pistil features such as hair pattern (adaxial). As for keel crest, this feature is difficult to analyze on herbarium material and needs more detailed investigations.

At least two features distinguish *Vavilovia* from all other studied species. These are leathery leaflets and specific leaflet shape. In all studied plants, proximal leaflet half (i.e. one directed to leaf base) is wider than the distal (directed to leaf rachis tip) or has almost the same width. In all *Vavilovia* plants distal half is notably wider than proximal.

The *Vavilovia* specimens are not completely uniform in the sample studied. All Armenian plants differed from the rest in having leaflets with keel-like bases (rounded base in other specimens, Fig. 2). This feature was used by A.A. Grossheim (19) to separate two different species within *Alophotropis* (= *Vavilovia*), namely *A. formosa* and *A. aucheri*, but in our opinion the whole genus should be treated as monotypic (at least basing on available material), probably with the contrast in forms interpreted as subspecies. The morphologically distinct forms of *Pisum formosum* (= *V. formosa*) were treated as separate varieties by L. Govorov (20), viz. var. *typicum* Gov. and var. *microphyllum* Ser.

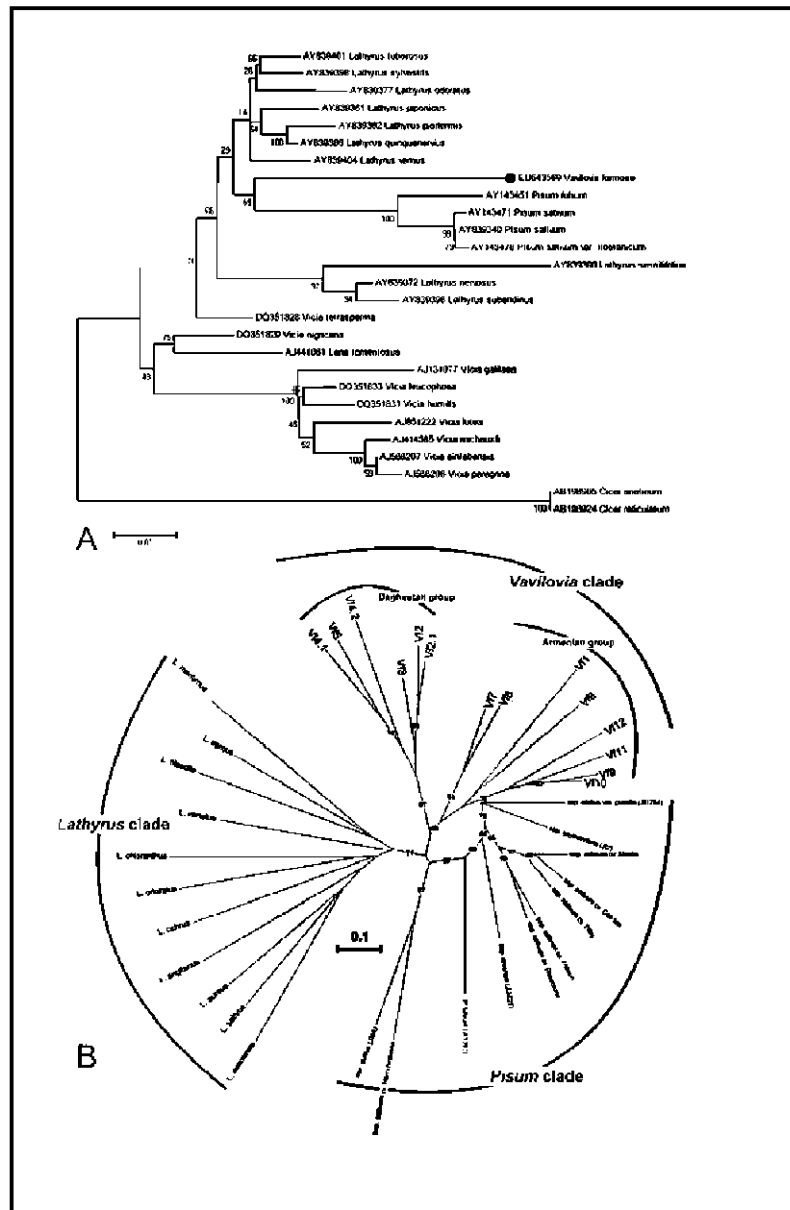
#### DNA analysis

Analysis of the RAPD data revealed three clades (Fig. 3B). The *Lathyrus* accessions were strongly different from other accessions and formed a single clade, but differences between them provide no opportunity to uncover any interrelations within the genus. Even "oroboid" species did not form a single group thus providing evidence that *Lathyrus* sections cannot be keyed out with this method (or at least with such resolution). Notably, variation between representatives of single genus *Lathyrus* are much more expressed than between forms interpreted as separate genera, *Pisum* and *Vavilovia*. *Vavilovia* accessions were distinct from other samples and were subdivided into two groups according to geographical position of sites of collection, Daghestan and Armenian groups.

The comparison of nucleotide sequences of ITS region of representatives of all genera comprising Fabaeae tribe exhibited some controversy to morphological features and revealed that *Vavilovia* demonstrates relation to *Pisum* rather than to other genera (Fig. 3A). However, it can be concluded that *V. formosa* is more distant from type pea subspecies (*P. sativum* ssp. *sativum*) than even *P. fulvum*. The differences between Armenian and Daghestan specimens can be interpreted as corresponding to subspecies rather to distinct species or varieties.

obviously, more detailed investigations on this point are needed but preliminary data presented here supports the similarity of *Pisum* and *Vavilovia*. In our opinion, the latter genus can be even treated as part of *Pisum sativum* L., i.e. as *Pisum formosum* (Stev.) Alef. The individuality of discussed species can probably serve as a basis for separation of *P. formosum* into a monotypic section. This idea was earlier proposed by some investigators (see (2)); for example, *Pisum sativum* L. was treated as subdivided into sections *Alophotropis* Jaub. et Spach (including *Vavilovia*) and *Lophotropis* Jaub. et Spach (including *Pisum sativum* Str.).

**Figure 3.** Dendrograms of *Vavilovia* samples and related species based on sequences of ITS1-5.8 rRNA-ITS2 region of the nuclear genome (A) and RAPD analysis (B). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates for A, 1000 for B) is shown next to the branches. Numbers of *V. formosa* specimens ("Vf series) correspond to those from Figure 1. *Vavilovia* accessions are denoted with a decimal point in A.



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