Phylogeny of subgenus *Vicia*

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Karyological and molecular characterization of subgenus *Vicia* (Fabaceae)

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Abstract

In the present report we have analysed the subgenus *Vicia* by karyological and molecular approaches with the aim to clarify the relationships among *Vicia* species included in this subgenus by previous evidenced morphological investigations. Multivariate analysis using several karyomorphological parameters in addition to symmetry indices has allowed the construction of a dendrogram of linkage distances very useful to compare and to include in a phylogenetic tree obtained by ITS rDNA sequences. Moreover a separate analysis was performed combining our molecular data on ITS sequences with those reported in the literature for the section *Vicilla*. Our analyses partly confirm the monophyletic status of the various sections in which the subgenus *Vicia* has been divided, however questioning, in some cases, the real need to maintain all the 9 sections so far accepted and the placement of some individual species in the two subgenera *Vicia* and *Vicilla*.

Key words

Automated karyotype analysis, ITS DNA sequences, Karyotype evolution, Phylogeny, *Vicia* species.

Introduction

The study of the taxonomic relationships between the crops and their wild relatives has been always important especially in connection with the use of the germplasm of wild (relatives) species as a source of new characteristics to be introduced into the crop plants by wide crosses (Hajjar and Hodgking 2007; Maxted et al., 2012).

In this connection the genus *Vicia* L., as a member of the legume tribe *Vicieae* Adans (*Fabaceae*) and the genus itself, including domesticated plants of considerable economic importance, has proved a popular group to study, then being 20 major classification of the genus since Linneus. The relationships between *Vicia faba* L. and its putative allies of the subgenus *Vicia* has always been controversial.

Ball (1968) divided the genus into four sections: *Vicia*, *Cracca* S.F. Gray, *Ervum* (L.) Tanb. and *Faba* (Miller) Ledeb.; afterwards Kupicha (1976) recognized two subgenera, *Vicilla* (Schur) Rouy and *Vicia,* with 17 and 5 sections respectively. This division in two subgenera is still convincing with the subgenus *Vicia* containing fewer species than *Vicilla*, but comprising the more important agronomical crops.

Maxted et al. (1991) included two newly discovered species in *Vicia* section *Faba* sensu Kupicha: *V. kalakhensis* Khattab, Maxted & Bisby and *V. eristalioides* Maxted.

Later Maxted (1993) proposed a new classification of subgenus *Vicia* where nine sections were presented, instead of five of Kupicha, with 38 species, 14 subspecies and 22 varieties and splitted, according to the data of 1991, the section *Faba* sensu Kupicha in three distinct sections: *Bithynicae* (B. Fedtsch.) Maxted and *Faba* monospecific units, and *Narbonensis* (B. Fedtsch.) Maxted which contained the seven species before referred to as the "Narbonensis" complex: *V. eristalioides* Maxted*, V. kalakhensis* Khattab, Maxted & Bisby*, V. johannis* Tamamschjan in Karyagin*, V. galilaea* Plitm. & Zoh. in Plitm.*, V. serratifolia* Jacq.*, V. hyaeniscyamus* Mout.*, V. narbonensis* L..

This classification is now widely accepted, however some issues remain obscure including not only the relationships among *V. faba* and its putative allies but also the sectional membership of some species.

Many aspects of the classification proposed by Maxted (1993) have been recently confirmed with different methodological approaches: by phenetic analysis of morphological characters (Maxted 1994, 1995; Endo and Ohashi 1995, 1997; Maxted and Douglas 1997; Jaaska and Leht 2007; Leht 2009), by isozymes (Jaaska 1997; Leht and Jaaska 2002; Jaaska 2005, Jaaska and Leht 2007), by electrophoretic seed globulin and albumin patterns (Zimniak-Przybylska and Przybylska 1995; Przybylska and Zimniak-Przybylska 1997) and by DNA sequences analyses (Fennell et al. 1998; Potokina et al. 1999; van de Wouw et al. 2001, 2003; Choi et al. 2006; Endo et al. 2008; Schaefer et al. 2012). In particular, the very recent contribution by Schaefer et al. (2012), which includes over 250 species of tribe Fabeae (among which *Lathyrus, Lens*, *Pisum*, *Vavilovia*, *Vicia*) and employs various DNA markers, showed that *Vicia* (and *Lathyrus* as well) is not monophyletic and that ancestors of Fabeae had an ancestral basic chromosome number p= 7 (for the use of p instead of x, see Peruzzi 2013).

In the nineties of the last century , karyological, cytological, cytophotometric and biochemical data on *Vicia faba* and on other species belonging to *Vicia* genus have been published by our team (De Pace et al. 1991; Maggini et al. 1991, 1995; Cremonini 1992; Cremonini et al. 1992a, 1992b, 1993, 1995, 1998a, 1998b; Frediani et al. 1992; Lipucci et al. 1992; Venora et al. 1999; Campeol et al. 2000; Kotseruba et al. 2000). Despite the great positive impact of the molecular phylogeny in recent years, the information on the chromosomal complement is still fundamental to assess the phyletic relationships and allow the processing of phylogenetic trees comparable to those obtained from molecular data.

Indeed the use of karyomorphological parameters as characters for the reconstruction of phylogenetic relationships is a relative recent matter, valid thanks to the most sophisticated and repeatable dedicated algorithms available today (Watanabe et al.

1999; Venora et al., 2002). These remarks strongly support the idea of studying the phylogenetic relationships among species by means of different approaches and using the number of parameters as large as possible. With this type of background, more than ten years ago our research team started analyses of the nine sections of the subgenus *Vicia* by cytological, karyological and molecular methods, sometimes accompanied by monographic reports on individual species of particular interest because rare taxon and/or poorly studied (Venora et al. 2000, 2008; Frediani et al. 2004, 2005; Caputo et al. 2006; Ruffini Castiglione et al. 2007, 2009, 2011 and 2012). Our interest over time was to assess the phylogenetic relationships among the species following a multidisciplinary approach to obtain valid complementary data-sets that can be integrated with traditional taxonomic studies. Therefore the aim of the present work is both to join together and process in a new cladistic elaboration our previously published data on the different sections, also implementing our molecular results on ITS rDNA sequences with those of the section *Vicilla,* and to obtain a complete picture of the relationships among *Vicia*, subgenus *Vicia.*

Materials and methods

Plant material

The name, source and accession numbers of the analysed species are listed in Table 1, according to the classification of Maxted (1993); unfortunately it has not been possible to consider all the species belonging to section *Atossa* (Alef) Asch. & Graebner, because of the impossibility of obtaining the seeds of *V. balansae* Boiss and *V. abbreviata* Fischer ex Sprengel from the international germplasm banks.

Karyomorphometry

Karyomorphometric data of the 36 *Vicia* species, obtained according to Venora et al. (1999), has been exploited to construct a single spatial representation of the following

symmetry indices: the TF% index (Total Form %) that is expressed by the ratio of the sum of the lengths of the short arms of individual chromosomes to the total haploid length of the complement (Huziwara 1962); the Rec index (Greilhuber and Speta 1976) that expresses the mean of the ratios of the length of each chromosomes (CL) to that of the longest one (LC); the Syi value (Greilhuber and Speta 1976) that indicates the ratio of the mean length of the short arms against the mean length of the long arms in a chromosome set. *Cicer arietinum* L. was used as an outgroup.

A hierarchical UPGMA cluster analysis using Euclidean distances (SPSS release 13 Inc. 1989-2003, statistic package) was also employed to compare all the karyological data reported in Table 3 and related to the chromosome complements of the species belonging to *Vicia* subgenus and to construct a dendrogram of linkage distance.

DNA sequence analysis

ITS1 and ITS2 rDNA sequences of all the species of the *Vicia* subgenus discussed in this paper (EMBL accession numbers reported in Table 4) were aligned by using the Clustal 1.83 software with default values (Thompson et al. 1994). To the 36 sequences indicated above, other sequences belonging to other species, as well as an appropriate set of sequences from other genera to be employed as outgroups were added, also in the light of the work by Schaefer et al. (2012). The sequences employed in an exploratory cladistic analysis (data not shown) were indeed 178; however, various sequences for the same taxa occurred more than once; when these sequences were identical, or formed a terminal clade, only one (in the latter case the

earliest-diverging one according to the exploratory analysis) was chosen. In few cases of multiple sequences for the same taxon, when only one of them was quite distant from the others and outside the clade formed by them, such sequence was excluded from further investigation. In various cases, however, when species belonged to subgenus *Vicia*, a small number of different sequences for the same taxon was included in the analysis to better represent the biodiversity of the taxon. After this selection, a total of 97 ITS sequences was taken into account (see Fig. 3 for accession details). Alignments were carried out as daughter processes of Bioedit (Hall 1999), which was also used for sequence editing and manipulation.

A cladistic analysis was carried out by employing the data handling environment Winclada (Nixon 1999), running Nona (Goloboff 1999) as a daughter process, using *Trifolium repens* and *Melilotus officinalis* as outgroups (see Fig. 3 for accessions). Setting included a maximum storage space of 10000 trees, a tree storage space per iteration of 100 and one hundred iterations of the default algorithm followed by branch swapping on the found trees. Cladograms were further manipulated with Winclada.

A further cladistic analysis was carried out adding also the karyological parameters listed in Table 3 on a reduced dataset, including only the species for which karyological data were collected. Only parameters from code n. 4 to code n. 37 were added. The cladistic analysis, therefore, also included 34 continuous characters for the taxa of subgenus *Vicia*. *V. lutea* was employed as an outgroup as a consequence of its more plesiomorphic karyological characters (it has the highest Rec value). The

investigation was carried out by using the TNT software (Goloboff et al. 2003), using the same parameters as above. Karyological characters were considered as additive, according to the software specifications. As one state of karyological character 8 (see Table 3) exceeded 65 (the upper limit for continuous characters in the software), the states of that character were all divided by 2. Bootstrap (1000 replicas) and Bremer (1994) support (up to trees 10 steps longer) for this investigation were computed with TNT.

Results

Karyotyping and karyomorphometry

The chromosome number, the nuclear DNA content, the karyotype formulas, the total length of haploid complement and the symmetry indices are summarized in Table 2. Most analysed species (18) possess 14 chromosomes (2n), while 14 species have 12 chromosomes and only four species have 10 chromosomes. These four species belong to sect. *Hypechusa* (Alef) Asch.& Graebner, ser. *Hypechusa* (Alef) Asch.& Graebner, the other species are randomly placed in all the sections. If we consider the satellites present in the karyotype of the analysed species we notice that the number ranges from one to two; only *V. assyriaca* Boiss presents three satellites. As regards as the chromosome morphology, the subtelocentric chromosomes are predominant in the subgenus *Vicia.*

In regard to 4C DNA content the value of section *Atossa* ranges from 7.10 to 18.70 pg and in this section *V. oroboides* Wulfen in Jacq. shows the lower DNA content; the DNA content of section *Hypechusa* ranges from 25.81 to 52.95 pg; the 4C DNA content of the species belonging to section *Peregrinae* Kupicha ranges from 30.61 to 36.22; in section *Wiggersia* (Alef) Maxted the DNA content ranges from 10.50 to 18.50 pg; in *Vicia* section it ranges from 9.00 to 16.60 pg and inside this section, *V. sativa* shows the shorter length of haploid set (16.04 μm); in the species of section *Narbonensis* the 4C DNA content ranges from 25.08 to 42.22 pg and *V. faba* shows both the greater DNA content (53.12 pg) and the major length of haploid set (58.11 μm).

As regards the symmetry indices, the greater value of Rec is that of *V. lutea* and the smallest is that of *V. faba*; the greater value of SYi is that of *V. hyaeniscyamus* and the smallest is that of *V. peregrine*; the greater value of TF% is that of *V. hyaeniscyamus* and the smallest is that of *V. aintabensis*.

The spatial representation of all the analysed species based on the symmetry indices is reported in Fig. 1. This representation points a partial mixing up of the species; only the three species of section *Peregrinae* are distinct but close to each other. *V. bithynica* and *V. faba,* which are enclosed in two monophyletic sections, are distinct and far from each other and at the same time distinct from the species belonging to section *Narbonensis*.

The two species belonging to section *Atossa* (*V. sepium* and *V. oroboides*) are characterized by a different position, indeed *V. sepium* is together with the species of

section *Narbonensis* while *V. oroboides* is together with the species of section *Hypechusa*. The species of section *Hypechusa* are grouped into three entities according to the diploid chromosome number; indeed all the species with 2n=10 form the first group, all the species with 2n=12 form the second group and *V. lutea* (2n=14) alone forms the third one. The species of section *Narbonensis* are grouped but not so strictly as section *Peregrinae*.

A cluster analysis, using Euclidean distances (Fig. 2), has been used to exploit all the karyological data listed in Table 3, determining each species cluster memberships.

The dendrogram partially confirm the grouping of the species according to the section; some differences evidenced by karyological parameters in Fig. 1 are confirmed as the distance between *V. bithynica* and *V. faba*.

The species belonging to section *Narbonensis* are split up into two different groups far from each other. The species belonging to section *Hypechusa* are clustered in two different groups according to the two series (*Hyrcanicae* B. Fedtsch ex Radzhi and *Hypechusa*). Only *V. assyriaca* is outside the two groups.

Moreover in the larger group (series *Hypechusa*) are present other two species: *V. dionysiensis* (sect. *Microcarinae*) and *V. sativa* (sect. *Vicia*). Furthermore the three species of section *Peregrinae* with 2n=14 are close each other, likewise the four species of section *Hypechusa* with 2n=10.

Sequence analysis

The length of nucleotide sequences of ITS1 and ITS2 rDNA obtained by us are reported in Table 4 with their accession numbers. As the 5.8S was invariably 164 bp in length in all the taxa for which it is available, it was removed from the alignment. In order not to overburden Table 4, we preferred not to indicate there the sequences obtained from the EMBL nucleotide sequence database which we added to our data; their accession numbers are however reported in Fig. 3.

The resulting molecular matrix had a consensus length of 482 character (154 of which informative). An overflow of 10,000 trees (L=600, C.I. = 0.57, R.I. =0.74; by removing uninformative characters, L=494, C.I. = 0.47, R.I. =0.74) was obtained, whose majority consensus is shown in Fig. 3.

The topology of the tree shows that our species of interest appear in a single clade, and therefore, within the limits of a majority consensus, subg. *Vicia* should be monophyletic. However, the only clade which occurs in all the most parsimonious trees which includes subg. *Vicia*, also includes *V. Americana* Muhl. Ex Willd., *V. bungei* Ohwi, and *V. dumetorum* L. as early branching lineages.

Several monophyletic groups of species, however, do appear within our section of interest. In particular, a clade includes *V. aintabensis*, *V. michauxii*, and *V. peregrina*, with *V. aintabensis* and *V. peregrina* which are sister to *V. michauxii* (the latter three belonging to sect. *Peregrinae)*. This clade is sister to *V. cuspidata* and *V. lathyroides* (both belonging to sect. *Wiggersia*). Another clade contains *V. eristalioides*, *V. galilaea*, *V. hyaeniscyamus*, *V. johannis*, *V. kalakhensis*, *V. narbonensis*, and *V. serratifolia* (all belonging to sect. *Narbonensis*); *V. oroboides* (a member of sect.

Atossa) is the earliest-diverging member of this clade. A further group is that of *V. assyriaca*, *V. mollis*, *V. hybrida* and *V. sericocarpa* (all belonging to sect. *Hypechusa*), with *V. hybrida* in a sister group relationship with *V. mollis* and *V. sericocarpa*. Another group includes *V. dionysiensis*, *V. esdraelonensis*, *V. galeata*, *V. hyrcanica*, *V. noeana*, *V. tigridis*, with *V. noeana* which is sister to *V. dionysiensis* and *V. esdraelonensis*, and the other three species which form a clade in which *V. galeata* is sister to *V. tigridis* and *V. hyrcanica* (all these species belong to sect. *Hypechusa*, but *V. dionysiensis* belongs to the monotypic sect. *Microcarinae*).

A last clade includes *V. anatolica*, *V. barbazitae*, *V. grandiflora*, *V. qatmensis*, *V. pyrenaica*, *V. sativa* and *V. sepium (*all belonging to sect. *Vicia*, with the exception of *V. sepium*, which belongs to sect. *Atossa*), with *V. grandiflora* and *V. qatmensis* as sister group and *V. anatolica* included in the variation of *V. sativa.* Bootstrap support (not shown) for the majority of the clade was very poor and this, together with vast collapses in several clades of the strict consensus tree (not shown: all the clades which do not occur 100% of the times in the tree in Figure 3) prompted us to add karyological information to the DNA dataset.

Adding karyological characters to the subset of species listed in Table 4, yielded a single most parsimonious cladogram, rooted with *V. lutea* (L=1915.783, C.I. = 0.36, R.I. =0.63), shown in Fig. 4. The ingroup is divided into two major clades. The first one includes *V. oroboides* (sect. *Atossa*) and *V. qatmensis* (sect. *Vicia*), in a sister group relationship with a clade including specie from sects. *Vicia*, *Atossa* and *Narbonensis*). In detail, this group includes two clades: one contains *V. pyrenaica*

(sect. *Vicia*), *V. bithynica* (sect. *Bithynicae*), *V. sativa* (sect. *Vicia*), *V. cuspidata* and *V. lathyroides* (the latter both belonging to sect. *Wiggersia*); the other includes a ladderized succession of *V. grandiflora*, *V. barbazitae* (both from sect. *Vicia*) and *V. sepium* (sect. *Atossa*), together with all the seven representatives of the monophyletic sect. *Narbonensis*. The other major clade includes sects. *Faba*, *Hypechusa*, *Microcarinae*, and *Peregrinae* and, in detail, an early-diverging clade, with *V. dionysiensis*, *V. esdraelonensis*, *V. galeata*, *V. hyrcanica*, *V. noeana*, *V. tigridis*, is in a sister group relationship with a clade including *V. assyriaca* and *V. pannonica* and then *V. hybrida* and *V. sericocarpa*, the latter two in a sister group relationship with a further clade. This contains in turn two groups, one with *V. anatolica*, *V. ciliatula*, *V. melanops* and *V. mollis* and another with *V. faba* and then the three species belonging to the monophyletic sect. *Peregrinae* (namely, *V. aintabensis*, *V. michauxii* and *V. peregrina*).

Bootstrap percentages (in Fig. 4 only those few above 50% are shown) are low; Bremer indices of some clades, on the contrary, are high (for example, those for sects. *Narbonensis*, *Peregrinae*, *Wiggersia*).

Discussion

In terms of both DNA content and chromosome complement our results are very heterogeneous and the DNA amount per 4C nucleus ranged from 7.10 to 53.12 pg without any correlation with the chromosome number (2n) that varies from 10 to 14.

This fact is not surprising and it confirms previous studies of subgenus *Vicia* (Ruffini Castiglione et al. 2011 and references therein) in which the genome size often seems to vary independently to the evolution and diversification of the species within the sections. The chromosome morphology can be a diagnostic feature to analyse karyological evolution: even if in some cases symmetric karyotype does not necessarily implies "primitivity" (Peruzzi and Eroğlu 2013), it is commonly assumed that a determinated group of angiosperms with a more asymmetrical karyotype can be derived from a more symmetrical group (Seijo and Fernandez 2003).

In the subgenus *Vicilla* (= *Cracca*) metacentric chromosomes are mainly predominant (Hanelt and Mettin 1989), while in the subgenus *Vicia* the subtelocentric chromosomes are predominant; this fact may have an evolutionary significance mostly confirming the presence of more evolved karyotypes in this subgenus. More precisely karyomorphological indices (TF%, Rec and SYi) are considered to be directly correlated with the evolution of the karyotypes, as reported not only for *Vicia* (Venora et al. 2000; Frediani et al. 2005; Caputo et al. 2006; Ruffini Castiglione et al. 2011) but also for *Cicer arietinum* (Venora et al. 1995), *Vigna* (Venora et al. 1998) and *Triticum* (Venora et al., 2002). Accurate determinations and elaborations of these indices provide insights into the fundamental aspects of chromosome evolution, indicating the level of asymmetry of the chromosome complement since, as reported above, the higher the asymmetry the more evolved is the karyotype of a species.

From the analyses of the indices of symmetry and of the spatial representation of the analysed species it is possible to notice that, according to karyotype evolution, each

section has overall a precise position in the spatial representation, section *Faba* being the most evolved and sect. *Atossa* the least evolved.

The multivariate analysis using 34 karyological parameters in addition to the three symmetry indices produces a dendrogram which confirm partially the grouping of the species according to the sections or to chromosome numbers.

The ITS sequence analysis only partly confirms the monophyletic status of the various sections in which the subgenus *Vicia* is divided. Sects. *Narbonensis*, *Peregrinae* and *Wiggersia* are indeed monophyletic (as also shown by Schaefer et al. 2012, Fig. 3); as far as sect. *Vicia* is involved, the clade to which it belongs includes also *V. anatolica* (sect. *Hypechusa*) and *V. sepium* (sect. *Atossa*); also Schaefer et al. (2012) do not find the section monophyletic. Sect. *Hypechusa*, on the contrary, is not necessarily monophyletic according to our ITS data. However, as the tree in Fig. 3 is a majority consensus tree, it is necessary to underline that a monophyletic sect. *Hypechusa* is included in our set of most parsimonious trees, although it does not occur in the totality of the available cladograms. Overall, for the species in common, our ITS results are quite similar to those by the above mentioned authors; the most relevant difference is that sect. *Narbonensis* does not belong, according to cladograms by Schaefer et al. (2012), to the same clade in which all the other species within the subg. *Vicia* are included.

The phylogenetic data generated from ITS rDNA sequences and karyomorphological parameters together (Fig. 4), for which *V. lutea* was employed as an outgroup, provide a somehow different hypothesis of relationships from both those depicted in

our consensus tree of Fig. 3 and those indicated by Schaefer et al (2012). One notable difference is that sect. *Hypechusa* is paraphyletic, but it belongs to a clade which includes also *V. dionysiensis* (belonging to the monotypic sect. *Microcarinae*), *V. faba* (sect. *Faba)* and a clade including all species of sect. *Peregrinae;* the close connection of *V. dionysiensis* with the species belonging to series *Hyrcanicae* of section *Hypechusa* is also confirmed by dendrogram constructed with karyological data (Fig. 2) and by molecular data (Fig. 3). The other major clade of the single most parsimonious tree of that investigation (Fig. 4) includes the monophyletic sects. *Narbonensis* and *Wiggersia*, but shows that sect. *Vicia* and sect. *Atossa* do not represent closely related units. All the monotypic sections of subg. *Vicia* (i.e., sects. *Bithynicae*, *Faba*, *Microcarinae*) appear as inequivocally placed within groups which themselves may be treated at sectional level and therefore should not be granted sectional status.

The remoteness indicated by Schaefer et al. (2012) of sect. *Narbonensis* from the other species of subg. *Vicia*, albeit not apparent in our ITS data (for the lack of additional taxa), finds correspondence in karyological information (Fig. 2), according to which at least part of the section (i.e., *V. galilaea*, *V. hiaenyscyamus*, *V. johannis*, *V. narbonensis*) is a closely related unit quite removed from the rest of the species in study.

Further studies including karyomorphological data for other species which are potentially to be included in subg. *Vicia* and/or closely related to sect. *Narbonensis,*

according to the above cited recent literature, will help completing the picture of the karyological evolution within the subgenus and related taxa.

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Legends to figures

Figure 1. Karyotype symmetry of the sections of subgenus *Vicia.*

Figure 2. Hierarchical cluster analysis, using linkage between groups, based on the 37 chromosome parameters listed in Table 3: dendrogram of *Vicia* species belonging to subgenus *Vicia.*

Figure 3. Strict consensus out of an overflow of 10000 equally parsimonious trees for the DNA sequence data (L=600, C.I. = 0.57, R.I. =0.74; by removing uninformative characters, L=494, C.I. = 0.47, R.I. =0.74). The accession number of the ITS sequences are reported only here and not in the text. Numbers above clades indicate the percentages of occurrence of the clades on the right out of the 10000 equally parsimonious cladograms.

Figure 4. Single most parsimonious tree for the combined matrix including DNA sequences and 34 karyological parameters of the species belonging to subgenus *Vicia* (L=1915.783, C.I. = 0.36, R.I. =0.63). Number above internodes are bootstrap percentages (only those above 50% were shown; numbers below nodes are the clade decaying indices up to trees 10 steps longer). The accession number of the ITS sequences are also reported.

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Table 1. Names, accession numbers and source of the analyzed *Vicia* species

Section	Series	Species	Accession number	Source
Atossa (Alef) Asch. & Graebner	*Pseudovicilla* Maxted	*V. oroboides* Wulfen in Jacq.	Botanical Garden	Ljubljana
	Atossa	*V. sepium* L.	PI 440768	A
Microcarinae Maxted		*V. dionysiensis* Mout.	IG 63377	B
Hypechusa (Alef) Asch.& Graebner	*Hyrcanicae* B. Fedtsch ex Radzhi	*V. assyriaca* Boiss.	IG 64098	B
		V. esdraelonensis Warb. & Eig.	Botanical Garden	Haifa
		V. tigridis Mout.	IG 63488	B
		V. galeata Boiss.	PI 602380	A
		V. hyrcanica Fischer & C. Meyer	PI 561419	A
		V. noeana (Reuter in Boiss.) Boiss.	IG 63757	B
	Hypechusa	*V. melanops* Sibth. & Smith	IG 64074	B
		V. ciliatula Lipsky	IG 63373	B
		V. anatolica Turrill	IG 64625	B
		V. mollis Boiss. & Hausskn. ex Boiss	IG 62649	B
		V. pannonica Crantz	PI 369156	A
		V. hybrida L.	IG 60008	B
		V. sericocarpa Fenzl	IG 64103	B
		V. lutea L.	201994	A
Peregrinae Kupicha		*V. michauxii* Sprengel	831	C
		V. aintabensis Boiss & Hausskn. ex Boiss	17514	A
		V. peregrina L.	12096	A
Wiggersia (Alef) Maxted		*V. cuspidata* Boiss.	IG 63208	B
		V. lathyroides L.	PI 422500	A
Vicia	*Vicia*	*V. pyrenaica* Pourret	Vic 69/43	C
		V. sativa L.	PI 253426	A
		V. barbazitae Ten. & Guss.	IG 64040	B
	Grandiflorae B. Fedtsch. ex Radzhi	*V. qatmensis* Gomb	IG 64141	B
		V. grandiflora Scop.	IG 64144	B

Bithynicae (B. Fedtsch.) Maxted		*V. bithynica* L.	VIC 303/79	C
Narbonensis (B. Fedtsch.) Maxted	*Rhombocarpae* Maxted	*V. eristalioides* Maxted	877321	D
	Narbonensis (B. Fedtsch.) Maxted	*V. kalakhensis* Khattab, Maxted & Bisby	867095	D
		V. johannis Tamamschjan in Karyagin	112019	E
		V. galilaea Plitm. & Zoh. in Plitm.	112018	E
		V. serratifolia Jacq.	NAR 121/77	C
		V. narbonensis L.	105786	E
		V. hyaeniscyamus Mout.	112008	E
Faba (Miller) Ledeb.		*V. faba* L.	113064	E

A=USDA United States Department of Agriculture, Pullman, WA, USA
B= ICARDA International Centre for Agricultural Research in the Dry Areas, Aleppo, Syria
C= IPK Institute of Plant Genetics and Crop Plant Research, Gatersleben, Federal Republic of Germany
D= Genebank, University of Southampton, U.K.
E= Istituto per il Germoplasma, CNR, Bari, Italy

Table 2. Chromosome number, DNA content, karyotype formulas, means set lengths and indices in *Vicia* samples

Species	Chromosome number (2n)	DNA amount 4C (pg)	Karyotype Formula	Total length of haploid set (µm)[a]	Rec index	SYi index	TF% index
V. oroboides[1]	14	7.10	sm^{sc}+sm+5st	32.46±5.20	82.97	34.06	24.58
V. sepium[1]	14	18.70	m^{sc}+m+5sm	20.27±0.28	78.50	50.35	32.36
V. dionysiensis[2]	12	27.60	sm^{sc}+3sm+2st	35.66±3.89	71.20	35.66	23.75
V. assyriaca[3]	12	26.31	$2m^{sc}$+2sm+st^{sc}+st	42.55±0.30	71.02	43.86	26.77
V. esdraelonensis[4]	12	30.89	sm^{sc}+5sm	38.79±1.96	77.73	38.63	28.52
V. tigridis[3]	12	25.81	m^{sc}+2sm+3st	35.07±0.30	71.07	34.07	23.00
V. galeata[3]	12	30.55	m^{sc}+2sm+3st	36.43±2.43	77.93	35.88	23.74
V. hyrcanica[3]	12	46.91	m^{sc}+2sm+3st	33.59±1.80	76.00	35.37	23.57
V. noeana[3]	12	52.95	sm^{sc}+2sm+3st	39.91±2.08	69.18	33.82	22.17
V. melanops[3]	10	27.52	m^{sc}+ sm^{sc}+3st	39-91±2.20	56.45	38.91	24.23
V. ciliatula[3]	10	26.84	m^{sc}+3sm+st^{sc}	35.12±2.89	60.00	39.54	24.00
V. anatolica[3]	10	30.18	$2m^{sc}$+sm+2st	35.07±3.04	59.47	50.99	30.08
V. mollis[3]	10	31.31	m^{sc}+4st	38.12±1.41	48.31	38.12	25.81
V. pannonica[3]	12	39.92	m^{sc}+3sm+st^{sc}+st	30.13±1.82	79.28	38.75	25.02
V. hybrida[3]	12	27.93	m^{sc}+st^{sc}+4st	40.73±3.01	80.44	36.60	24.28
V. sericocarpa[3]	12	48.52	m^{sc}+sm+st^{sc}+3st	39.44±3.00	74.47	36.32.	24.57
V. lutea[3]	14	35.90	2sm+st^{sc}+4st	49.37±5.83	91.46	32.73	23.98
V. michauxii[5]	14	31.86	m^{sc}+st^{sc}+3st+2t	35.96±2.68	66.69	22.45	16.49
V. aintabensis[3]	14	30.61	m^{sc}+st^{sc}+3st+2t	40.57±1.17	66.61	22.09	16.42
V. peregrina[5]	14	36.22	m^{sc}+st^{sc}+3st+2t	42.29±1.08	66.75	21.71	16.24
V. cuspidata[2]	12	18.50	2m+st^{sc}+3st	25.66±1.88	76.29	27.17	21.01
V. lathyroides[2]	12	10.50	m^{sc}+4st+t	21.19±2.88	74.39	21.77	17.41
V. pirenaica[2]	14	16.60	4sm+st^{sc}+2st	23.06±1.12	82.90	33.47	24.41
V. sativa[2]	12	9.00	m+sm^{sc}+sm+3st	16.14±0.69	78.72	34.79	24.16
V. barbazitae[6]	14	14.50	m+sm^{sc}+sm+3st	24.67±2.51	77.42	38.08	26.75
V. qatmensis[2]	14	14.00	m^{sc}+m+2sm+3st	34.67±2.50	68.94	39.80	27.69
V. grandiflora[2]	14	13.40	3sm+st^{sc}+3st	26.48±1.66	74.33	29.89	22.58
V. bithynica[7]	14	18.03	st^{sc}+6st	24.07±1.83	86.99	23.72	18.49

V. eristalioides[7]	14	38.58	sm^{sc}+3m+3sm	33.38±0.37	83.02	56.76	34.07
V. kalakhensis[7]	14	42.22	st^{sc}+3m+3sm	42.28±0.10	85.02	55.52	33.73
V. johannis[1]	14	25.08	sm^{sc}+6m	33.56±3.19	86.33	48.12	31.31
V. galilaea[7]	14	26.09	sm^{sc}+6sm	40.34±1.71	85.94	47.69	31.18
V. serratifolia[7]	14	39.59	sm^{sc}+5sm+m	38.16±2.06	89.69	52.44	32.68
V. narbonensis[1]	14	29.10	sm^{sc}+2m+4sm	27.15±2.28	84.60	56.05	34.57
V. hyaeniscyamus[7]	14	31.24	sm^{sc}+2m+4sm	29.21±2.85	65.99	57.64	35.32
V. faba[7]	12	53.12	sm^{sc}+3st+2t	58.11±5.04	46.05	23.08	17.70

[a] values are means with standard errors
m = metacentric; sm = submetacentric; st = subtelocentric; t = telocentric.

[1] Ruffini Castiglione et al. 2009
[2] Ruffini Castiglione et al. 2011
[3] Caputo et al. 2006
[4] Ruffini Castiglione et al. 2007
[5] Frediani et al. 2005
[6] Ruffini Castiglione et al. 2012
[7] Venora et al. 2000

Table 3. Parameters used in cluster analysis, recorded in any plate of every analysed accession.

Parameters	Code n.	Description
TF%	1	Total Form (%)
Rec	2	Resemblance among chromosomes
SYi	3	Symmetry index
Cl	4	Total length of haploid complement
2n	5	Chromosomes diploid number
N. cro. sat	6	Number of satellited couples
1st satellite	7	Length of the 1st satellite
1st Sat. Pos.	8	Satellite position, short or long arm
2nd satellite	9	Length of the 2nd satellite
2nd Sat. Pos.	10	Satellite position, short or long arm
3rd satellite	11	Length of the 3rd satellite
3rd Sat. Pos.	12	Satellite position, short or long arm
N. cro. m	13	Number of metacentric couples
N. cro. sm	14	Number of submetacentric couples
N. cro. st	15	Number of subtelocentric couples
N. cro. t	16	Number of telocentric couples
C1 L	17	Chromosome 1st length
C1 ar	18	Chromosome 1st arm ratio
C1 CI	19	Chromosome 1st Centromeric Index *
C2 L	20	Chromosome 2nd length
C2 ar	21	Chromosome 2nd arm ratio
C2 CI	22	Chromosome 2nd Centromeric Index
C3 L	23	Chromosome 3rd length
C3 ar	24	Chromosome 3rd arm ratio
C3 CI	25	Chromosome 3rd Centromeric Index
C4 L	26	Chromosome 4th length
C4 ar	27	Chromosome 4th arm ratio
C4 CI	28	Chromosome 4th Centromeric Index
C5 L	29	Chromosome 5th length
C5 ar	30	Chromosome 5th arm ratio
C5 CI	31	Chromosome 5th Centromeric Index
C6 L	32	Chromosome 6th length
C6 ar	33	Chromosome 6th arm ratio
C6 CI	34	Chromosome 6th Centromeric Index
C7 L	35	Chromosome 7th length
C7 ar	36	Chromosome 7th arm ratio
C7 CI	37	Chromosome 7th Centromeric Index

* The Centromeric index is the ratio of the short arm and chromosome length

Table 4. EMBL accession numbers and lengths of ITS1 and ITS2 sequences in *Vicia* species.

Species	EMBL accession nr.	ITS1 length (bp)	ITS2 length (bp)
V. oroboides	AM950283	236	209
V. sepium	AM087144	222	209
V. dionysiensis	AM087146	236	209
V. assyriaca	AJ851217	236	209
V. esdraelonensis	AM181810	236	209
V. tigridis	AJ851216	236	209
V. galeata	AJ851219	236	209
V. hyrcanica	AJ851221	236	209
V. noeana	AJ861224	236	209
V. melanops	AJ851223	236	209
V. ciliatula	AJ851218	236	209
V. anatolica	AJ851215	219	209
V. mollis	AJ566208	200	209
V. pannonica	AJ851225	236	205
V. hybrida	AJ851220	236	209
V. sericocarpa	AJ851226	200	209
V. lutea	AJ851222	236	209
V. michauxii	AJ414585	236	209
V. aintabensis	AJ566207	236	210
V. peregrina	AJ566206	236	201
V. cuspidata	AM087147	236	209
V. lathyroides	AM087148	236	209
V. pyrenaica	AM087150	219	209
V. sativa	AJ010804/5	218	209
V. barbazitae	FR856902	220	209
V. qatmensis	AM087149	220	208
V .grandiflora	AM087151	220	209
V. bithynica	AJ130831/2	235	209
V. eristalioides	AJ010808/9	235	209
V. kalakhensis	AJ131071/2	235	209
V. johannis	AJ131080/1	235	209
V. galilaea	AJ131082/3	235	209
V. serratifolia	AJ131075/6	235	209
V. narbonensis	AJ130034/3	235	209
V. hyaeniscyamus	AJ131073/4	235	209
V. faba	Yokota et al. 1989	235	208

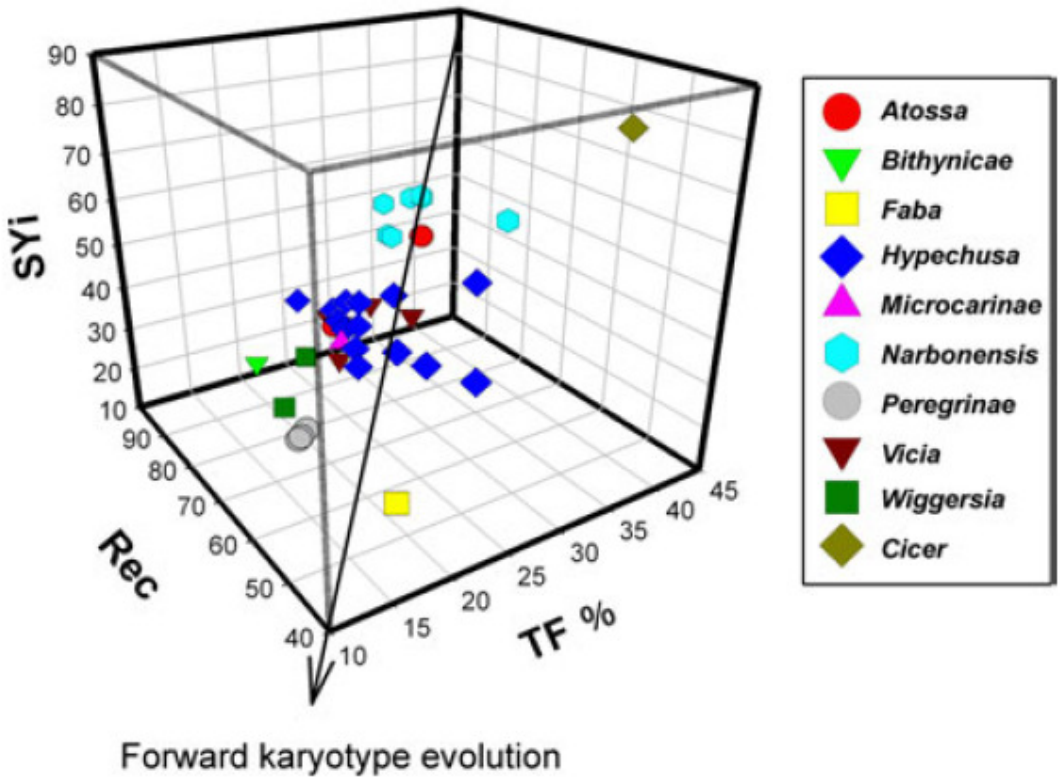
SYi
Rec
TF %
Forward karyotype evolution
Atossa
Bithynicae
Faba
Hypechusa
Microcarinae
Narbonensis
Peregrinae
Vicia
Wiggersia
Cicer

Figure 1.

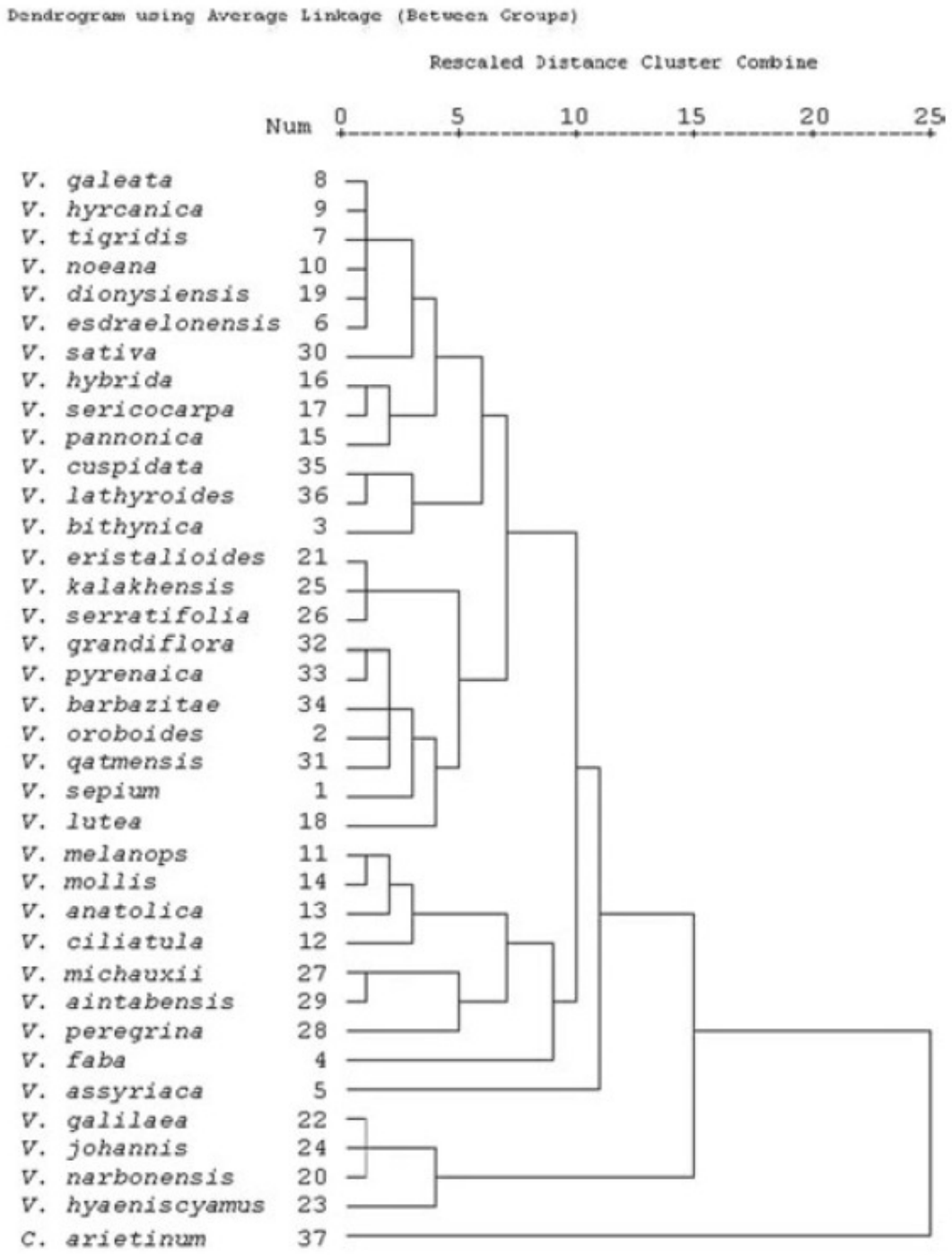
Dendrogram using Average Linkage (Between Groups)
Rescaled Distance Cluster Combine
Num
0
5
10
15
20
25
V. galeata 8
V. hyrcanica 9
V. tigridis 7
V. noeana 10
V. dionysiensis 19
V. esdraelonensis 6
V. sativa 30
V. hybrida 16
V. sericocarpa 17
V. pannonica 15
V. cuspidata 35
V. lathyroides 36
V. bithynica 3
V. eristalioides 21
V. kalakhensis 25
V. serratifolia 26
V. grandiflora 32
V. pyrenaica 33
V. barbazitae 34
V. oroboides 2
V. qatmensis 31
V. sepium 1
V. lutea 18
V. melanops 11
V. mollis 14
V. anatolica 13
V. ciliatula 12
V. michauxii 27
V. aintabensis 29
V. peregrina 28
V. faba 4
V. assyriaca 5
V. galilaea 22
V. johannis 24
V. narbonensis 20
V. hyaeniscyamus 23
C. arietinum 37

Figure 2.

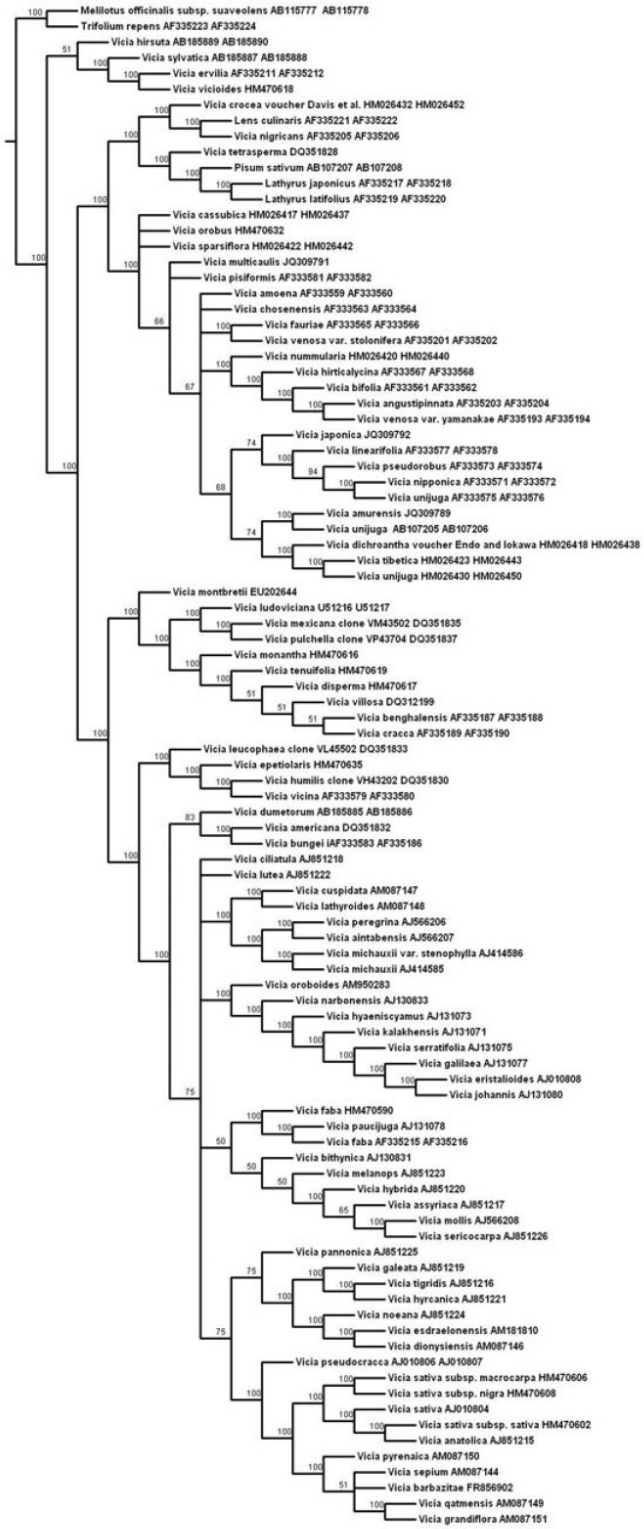
Melilotus officinalis subsp. suaveolens AB115777 AB115778
Trifolium repens AF335223 AF335224
Vicia hirsuta AB185889 AB185890
Vicia sylvatica AB185887 AB185888
Vicia ervilia AF335211 AF335212
Vicia vicioides HM470618
Vicia crocea voucher Davis et al. HM026432 HM026452
Lens culinaris AF335221 AF335222
Vicia nigricans AF335205 AF335206
Vicia tetrasperma DQ351828
Pisum sativum AB107207 AB107208
Lathyrus japonicus AF335217 AF335218
Lathyrus latifolius AF335219 AF335220
Vicia cassubica HM026417 HM026437
Vicia orobus HM470632
Vicia sparsiflora HM026422 HM026442
Vicia multicaulis JQ309791
Vicia pisiformis AF333581 AF333582
Vicia amoena AF333559 AF333560
Vicia chosenensis AF333563 AF333564
Vicia fauriae AF333565 AF333566
Vicia venosa var. stolonifera AF335201 AF335202
Vicia nummularia HM026420 HM026440
Vicia hirticalycina AF333567 AF333568
Vicia bifolia AF333561 AF333562
Vicia angustipinnata AF335203 AF335204
Vicia venosa var. yamanakae AF335193 AF335194
Vicia japonica JQ309792
Vicia linearifolia AF333577 AF333578
Vicia pseudorobus AF333573 AF333574
Vicia nipponica AF333571 AF333572
Vicia unijuga AF333575 AF333576
Vicia amurensis JQ309789
Vicia unijuga AB107205 AB107206
Vicia dichroantha voucher Endo and Iokawa HM026418 HM026438
Vicia tibetica HM026423 HM026443
Vicia unijuga HM026430 HM026450
Vicia montbretii EU202644
Vicia ludoviciana U51216 U51217
Vicia mexicana clone VM43502 DQ351835
Vicia pulchella clone VP43704 DQ351837
Vicia monantha HM470616
Vicia tenuifolia HM470619
Vicia disperma HM470617
Vicia villosa DQ312199
Vicia benghalensis AF335187 AF335188
Vicia cracca AF335189 AF335190
Vicia leucophaea clone VL45502 DQ351833
Vicia epetiolaris HM470635
Vicia humilis clone VH43202 DQ351830
Vicia vicina AF333579 AF333580
Vicia dumetorum AB185885 AB185886
Vicia americana DQ351832
Vicia bungei iAF333583 AF335186
Vicia ciliatula AJ851218
Vicia lutea AJ851222
Vicia cuspidata AM087147
Vicia lathyroides AM087148
Vicia peregrina AJ566206
Vicia aintabensis AJ566207
Vicia michauxii var. stenophylla AJ414586
Vicia michauxii AJ414585
Vicia oroboides AM950283
Vicia narbonensis AJ130833
Vicia hyaeniscyamus AJ131073
Vicia kalakhensis AJ131071
Vicia serratifolia AJ131075
Vicia galilaea AJ131077
Vicia eristalioides AJ010808
Vicia johannis AJ131080
Vicia faba HM470590
Vicia paucijuga AJ131078
Vicia faba AF335215 AF335216
Vicia bithynica AJ130831
Vicia melanops AJ851223
Vicia hybrida AJ851220
Vicia assyriaca AJ851217
Vicia mollis AJ566208
Vicia sericocarpa AJ851226
Vicia pannonica AJ851225
Vicia galeata AJ851219
Vicia tigridis AJ851216
Vicia hyrcanica AJ851221
Vicia noeana AJ851224
Vicia esdraelonensis AM181810
Vicia dionysiensis AM087146
Vicia pseudocracca AJ010806 AJ010807
Vicia sativa subsp. macrocarpa HM470606
Vicia sativa subsp. nigra HM470608
Vicia sativa AJ010804
Vicia sativa subsp. sativa HM470602
Vicia anatolica AJ851215
Vicia pyrenaica AM087150
Vicia sepium AM087144
Vicia barbazitae FR856902
Vicia qatmensis AM087149
Vicia grandiflora AM087151

Figure 3


Vicia lutea AJ851222
Vicia oroboides AM950283
Vicia qatmensis AM087149
Vicia pyrenaica AM087150
Vicia bithynica AJ130831
Vicia sativa AJ010804
Vicia cuspidata AM087147
Vicia lathyroides AM087148
Vicia grandiflora AM087151
Vicia barbazitae FR856902
Vicia sepium AM087144
Vicia serratifolia AJ131075
Vicia eristalioides AJ010808
Vicia kalakhensis AJ131071
Vicia hyaeniscyamus AJ131073
Vicia narbonensis AJ130833
Vicia galilaea AJ131077
Vicia johannis AJ131080
Vicia galeata AJ851219
Vicia tigridis AJ851216
Vicia hyrcanica AJ851221
Vicia esdraelonensis AM181810
Vicia noeana AJ851224
Vicia dionysiensis AM087146
Vicia pannonica AJ851225
Vicia assyriaca AJ851217
Vicia hybrida AJ851220
Vicia sericocarpa AJ851226
Vicia ciliatula AJ851218
Vicia anatolica AJ851215
Vicia melanops AJ851223
Vicia mollis AJ566208
Vicia faba AF335215 AF335216
Vicia peregrina AJ566206
Vicia michauxii AJ414585
Vicia aintabensis AJ566207

Figure 4.