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Repetitive DNA sequences as probes for phylogenetic analysis in *Vicia* genus

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Abstract — In the process of further characterizing phylogenetic relationships among *Vicia* species of the subgenus *Vicia*, four different molecular DNA markers were used: a tandemly repeated DNA sequences about 60 bp in length (FokI), a 336 bp element (p*Vf*7) homologous to the IGS repeats (but that does not reside in the policistronic rDNA units); a family of repeated DNA sequences (*Vf*B) of about 1200 bp in length (that might be derived from a mobile DNA element) and a family of repeated DNA sequences (*Vf*M) of about 60 bp that can be considered a minisatellite-like sequence (EMBL accession nr. AJ242773). The comparison of the obtained results has enabled the definition of the phylogenetic relationships among the analysed species confirming that *V. faba*, *V. bithynica* and the species of *Narbonensis* section, represent three distinct taxonomic groups according to the Maxted's classification (1993). *Vf*B and *Vf*M marker discriminate inside the sect. *Narbonensis*, too, evidencing a greater affinity between some species.

Key words: DNA sequences, phylogenetic relationships, repeated DNA, *Vicia*.

INTRODUCTION

The genus *Vicia* is a member of the legume tribe *Vicieae* of the *Papilionoideae* and the genus itself has proved a popular group to study, then being 20 major classifications of the genus since Linneus. BALL (1968) divided the genus into four sections: *Vicia, Cracca, Ervum* and *Faba*. KUPICHA (1976) recognized 22 sections in the two subgenera *Vicia* and *Vicilla*. MAXTED *et al.* (1991) included two newly discovered species in *Vicia* sect. *Faba*: *V. kalakhensis* Khatt., Maxt. et Bisby (KHATTAB *et al*. 1988) and *V. eristalioides* Maxt. (MAXTED 1988); as a consequence, the *Vicia* sect. *Faba* comprised three distinct units, two of which were monospecific: *V. faba* L. and *V. bithynica* L., while the third was larger, containing the seven species referred to as the Narbonensis complex: *V. narbonensis* L., *V. serratifolia* Jacq., *V. galilaea* Plitm. et Zoh., *V. hyaeniscyamus* Mout., *V. johannis* Tamasch., *V. kalakhensis* Khatt., Maxt. et Bisby and *V. eristalioides* Maxt. Later MAXTED (1993) proposed a new classification of subgenus *Vicia* where nine sections were present and the old *Faba* section was substituted by three distinct sections: *Bithynicae* and *Faba* monospecific and *Narbonensis* which contained the seven species before referred to as the Narbonensis complex.

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In previous papers, we reported cytological, karyological and biochemical data in order to elucidate the relationships inside *Vicia* subgenus (CREMONINI *et al*. 1998 a, b; FREDIANI *et al*. 1992, 1999; MAGGINI *et al*. 1991, 1995; VENORA *et al*. 2000).

In phylogenetic studies, it is important to use as markers a broad set of DNA sequences since more regions of the genome become involved and, consequently, more detailed information can be obtained. A way to gain knowledge about the structure and evolution of plant genomes is to study the organization of repetitive DNA sequences and to examine their conservation or divergence within related species. In this paper we report new experimental evidence based on the use of four different molecular markers belonging to the heterogeneous class of repeated DNA sequences; three of these molecular markers have been previously used to study the structure of the genome of *V. faba*. The first marker we used was FokI, a family of tandemly repeated DNA sequences about 60 bp in length, which represents a considerable portion of the genome of *V. faba* (KATO *et al*. 1984; Maggini *et al.* 1995); the second, named p*Vf*7 repeats, represents another family of repetitive DNA related to sequences founded in Intergenic Spacer (IGS) of ribosomal DNA and spread in centromeric and telomeric regions of *V. faba* (KATO *et al.* 1985; MAGGINI *et al*. 1991); *Vf*B represents a family of repeated DNA sequences of about 1200 bp in length which are dispersed in the genome of broad bean and absent or rare only at the hetero-

chromatic chromosomal regions (FREDIANI *et al.* 1999). The last marker, termed *Vf*M, is a minisatellite-like sequence isolated in a *V. faba* library.

Minisatellites are relatively short DNA sequences (10-100 bp) repeated in tandem, dispersed throughout the genome. Extreme variability in the tandem repeat copy number of minisatellite loci is considered to be the source of the observed polymorphisms in human (JEFFREYS *et al.* 1985; NAKAMURA *et al.* 1987) and in a variety of animal and plant species (JEFFREYS and MORTON 1987; BROUN and TANKSLEY 1993; WINBERG *et al.* 1993; TOURMENTE *et al.* 1994). HEATH *et al.* (1993) reported a new technique, named Direct Amplification of Minisatellite-region DNA (DAMD), which uses PCR to direct the amplification of minisatellite-rich regions by using as a single primer the common motif (core sequence) present in the repeats. Indeed it has been speculated that if a portion of a minisatellite array is involved in an inversion this would make PCR possible using a single minisatellite core sequence as a primer. As a consequence, this PCR application is capable of producing RAPD-like results in order to detect polymorphisms among species and cultivars, too.

The reported experimental data have allowed us to get further clarification of the phylogenetic relationships among the *Vicia* species belonging to the sections *Faba*, *Bithynicae* and *Narbonensis* (MAXTED 1993).

MATERIALS AND METHODS

Plant materials - The names, source and accession number of *Vicia* species are listed in Table 1. *V. sativa* has been used as outgroup.

DNA extraction - Nuclear DNAs were extracted and purified from secondary roots, produced after decapitation of the primary one, as described in MAGGINI *et al.* (1978).

*Preparation of probes FokI, p*Vf*7,* Vf*B and Southern blot hybridization* - The isolation and the preparation of the probes have been described previously (MAGGINI *et al.* 1991; 1995; FREDIANI *et al.* 1999).

Genomic DNAs of all the species reported in Table 1 were digested to completion with Sau3AI (for FokI and p*Vf7* hybridizations) and EcoRI (for *Vf*B hybridization) restriction endonucleases (Roche), electrophoresed in 0,8 % agarose gel and blotted on nitrocellulose filters (BA85; Schleicher and Schuell) according to SOUTHERN (1975).

The blots were hybridized with the DNA probes labelled with digoxigenin-11-dUTP (Roche), using a random primed DNA labelling kit (Promega).

Isolation of Vf*M elements* - From a genomic library of *Vicia faba* (FREDIANI *et al.* 1999), a clone containing a tandem array of repeated elements of 60 bp (termed *Vf*M), was isolated. The tandem array of repeated elements was separated by cutting with BamHI and HpaI restriction endonucleases, the band recovered and cloned in pUC18 BamHI/SmaI using a Sureclone ligation kit (Pharmacia).

Southern blot hybridizations - Genomic DNA of *Vicia faba* was digested with different restriction endonucleases (BamHI, TaqI, AluI, Sau3AI, EcoRI, RsaI, and HinfI) fractioned by electrophoresis on 1% agarose, transferred to nitrocellulose membrane (BA85; Schleicher & Schuell) and hybridized with *Vf*M labelled with digoxigenin-11-dUTP (Roche) using a random primed DNA labelling kit (Promega).

Table 1 — Accession, source, chromosome number, mean nuclear DNA amount in species.

Species	Accession	Source	Chromosome number (2n)	DNA amount 4C (pg)
V. narbonensis L.	105786	IGV	14	29,10± 0,3*
V. eristalioides Maxt.	877321	B	14	38,58± 0,5*
V. galilaea Plitm.et Zoh.	112018	IGV	14	26,09±0,2*
V. hyaeniscyamus Mout.	112008	IGV	14	31,24±0,1*
V. johannis Tamash.	112019	IGV	14	25,08±0,3*
V. kalakhensis Khatt.& al.	867095	B	14	42,22±0,4*
V. serratifolia Jacq.	NAR 121/77	IPK	14	39,59±0,4*
V. bithynica L.	VIC 303/79	IPK	14	18,03±0,2*
V. faba major L.	113064	IGV	12	53,12+
V. sativa L.	VIC 556/73	IPK	12	9,00°

IGV=Istituto Genetica Vegetale, CNR, Bari, Italy;
B=Genebank, University of Southampton, U.K.;
IPK=Institute of Plant Genetics and Crop Plant Research, Gatersleben, Germany;
* VENORA *et al.* (2000) and references therein
+ CECCARELLI *et al.* (1995),
° REES *et al.* (1966).

Genomic DNAs of all the species of Table 1 were digested with HinfI, fractioned by electrophoresis on 1% agarose, transferred to nitrocellulose membrane (BA85; Schleicher & Schuell) and hybridized with *Vf*M labelled probe.

Clone selection - The genomic DNA library was plated and three clones containing DNA sequences homologous to *Vf*M elements were identified by plaque hybridization with the digoxigenin-labelled probe. DNA fragments of positive clones (*Vf*R3, *Vf*R9, and *Vf*R10) were amplified by PCR with T7 and Sp6 primers. PCR products were recovered and subcloned in pUC18 Sma/Bap for sequencing.

DAMD-PCR - Genomic DNAs of the analysed *Vicia* species were amplified using as a single primer the initial sequence (5'-CCTGTGTAGGGTTGCTTC-3') of the 60 bp repeat units. After initial denaturation (5 min at 94° C) PCR was run for 35 cycles (1 min at 94°C, 1 min at 55°C and 2 min at 72°C). PCR reaction was performed using 40 pmole of primer, 100 ng of DNA in the standard conditions. The reaction products were then fractionated on 1.2% (w/v) agarose gel for 3-4 h at 80V in 1xTBE and visualized with ethidium bromide.

RESULTS

Fig. 1 shows the restriction patterns of genomic DNA of *Vicia* species after digestion with Sau3AI restriction endonuclease and hybridization with digoxigenin-labelled tandem arrays of FokI repeats. Substantial hybridization occurs only to the DNA of *V. faba.* FokI sequences hybridized to a minimum extent to the DNA of *V. narbonensis* and even less to the DNA of *V. sativa*; no hybridization signal is detected in similar blots of the genomic DNAs of all the other analysed species.

The restriction patterns of genomic DNA of *Vicia* species after digestion with Sau3AI restriction endonuclease and hybridization with digoxigenin-labelled p*Vf7* elements are shown in Fig. 2. A strong hybridization signal is present only on *V. faba* DNA. The analysed repeated elements hybridize only on *V. sativa* and, to a minimum extent, on *V. narbonensis* DNA, but again no hybridization signal is present in the DNAs of the remaining seven species.

Fig. 3 shows the restriction patterns of genomic DNA of all the analysed species after digestion with EcoRI restriction endonuclease and hybridization with digoxigenin-labelled *Vf*B elements. In addition to *V. faba* DNA, signal hybridization is evident in all

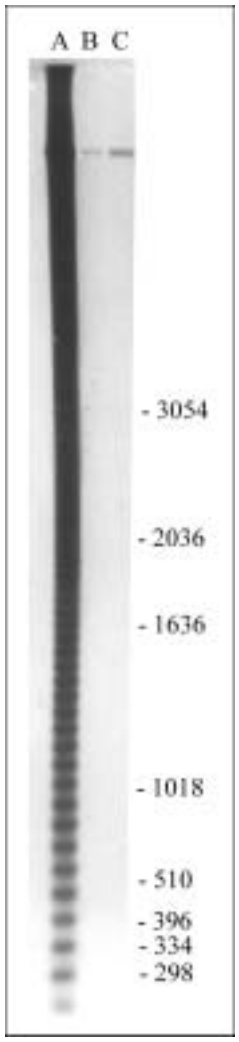

Fig. 1 — Band patterns of genomic DNAs of *V. faba* (A), *V. sativa* (B) and *V. narbonensis* (C) digested with Sau3AI restriction endonuclease after Southern blot hybridization with digoxigenin-labelled FokI repeats. Numerals indicate molecular sizes in base pairs as determined using a 1-kb DNA ladder as a marker.

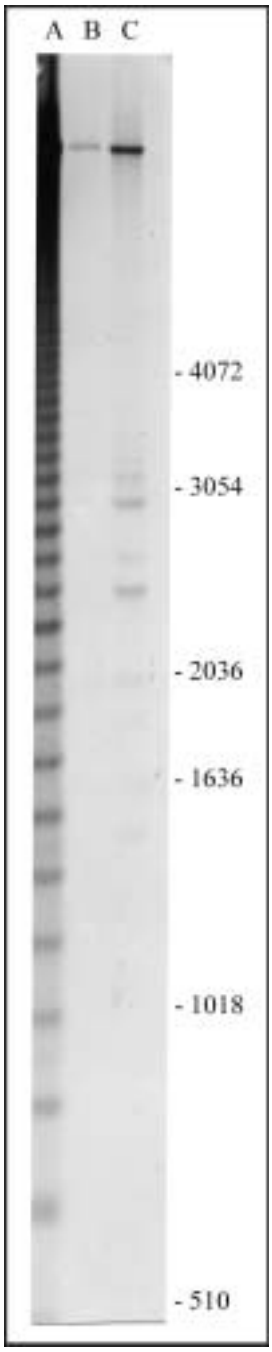

Fig. 2 — Band patterns of genomic DNAs of *V. faba* (A), *V. narbonensis* (B) and *V. sativa* (C) digested with Sau3AI restriction endonuclease after Southern blot hybridization with digoxigenin-labelled p*Vf7* probe. Numerals indicate molecular sizes in base pairs as determined using a 1-kb DNA ladder as a marker.

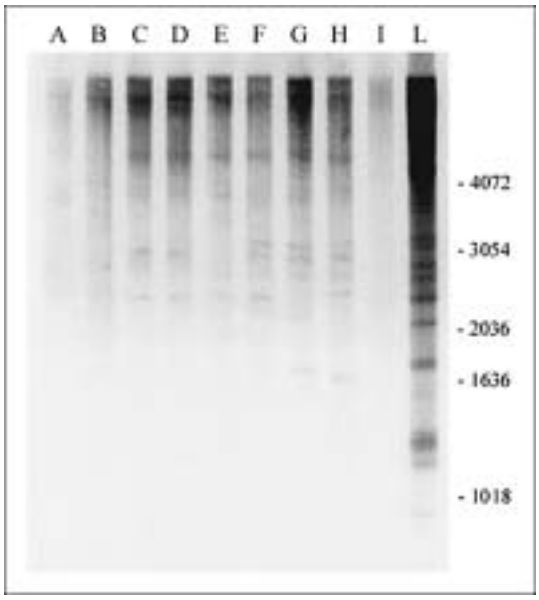

Fig. 3 — Band patterns of genomic DNAs of the analysed species digested with EcoRI restriction endonuclease after Southern blot hybridization with digoxigenin-labelled *Vf*B probe. Numerals indicate molecular sizes in base pairs as determined using a 1-kb DNA ladder as a marker.
A=*V. sativa*; B=*V serratifolia*; C=*V. johannis*; D=*V. hyaeniscyamus;* E = *V. galilaea*; F=*V. kalakhensis*; G=*V. narbonensis*; H=*V. eristalioides*; I=*V. bithynica*; L=*V. faba.*

the species even if to a minimum extent in *V. sativa* and even less in *V. bithynica.* Inside the *Narbonensis* section, the banding pattern clearly differentiate *V. serratifolia* in comparison to the other species, which, on the contrary, shared several bands and a greater similarity is evident between *V. johannis* and *V. hyaeniscyamus*.

The tandem array of *Vf*M consists of 4 repetitions of a sequence which doesn't show any homology with sequences reported in EMBL database, as evidenced from Fasta alignment (Pearson and Lipman 1988). The length is invariably 60 bp, with the exception of the fourth incomplete repeat of 46 bp (Fig. 4). A+T content of *Vf*M repeats is 53.8 % and the nucleotide sequence is highly conserved among the repeats: the homology ranges from 88.33% to 95%.

No typical ladder of multimers of about 60 bp was produced in the blots of *V. faba* DNA after digestion with different restriction endonucleases (BamHI, EcoRI, TaqI, RsaI, Sau3AI, AluI which don't find any cleavage site and HinfI restriction endonuclease, which presents three cleavage sites in the sequence) and hybridization with digoxigenin-labelled *Vf*M (Fig. 5); lack of signal was also observed after hybridization of labelled probe to the metaphase chromosomes of *V. faba,* suggesting a moderate representativeness of the sequence in the genome of field bean.

Lack of signal has been observed after hybridization of digoxigenin-labelled *Vf*M to the genomic DNAs of all the species, digested with HinfI restriction endonuclease (data not shown). With the aim to study the organization of this DNA element in the genome of *V. faba*, we have analysed the genomic library at our disposal with *Vf*M labelled probe and three positive clones containing sequences homologous to *Vf*M repeats were obtained (Fig.6).

Fig. 7 shows the alignment of the repeats (except for the incomplete repeats) of the three clones in addition to *Vf*M and a similarity index of 80,6% was determined. In all the clones, the most conserved region was the initial one and this sequence was used as a single primer in DAMD-PCR experiments.

The DAMD-PCR (Fig. 8) profiles reveal variations among *Vicia* species. There are from one to four distinguishable bands, in addition to several other faint bands amplified in the different species. The number and positions of these bands depend on species and in many cases clearly discriminate among them. In particular *V. faba* presents two evident and eight faint bands, while *V. bithynica* as *V. sativa* evidence only one band, but of different molecular weight. The species of *Narbonensis* section present a different banding pattern not only in comparison with *V. faba, V. bithynica* and *V. sativa* but also if compared one to each other inside the section. Anyway, some species share more bands respect to the other species. This is the case of *V. eristalioides* and *V. kalakhensis* and *V. hyaeniscyamus* and *V. johannis,* respectively.

```
          10         20         30         40         50         60
           |          |          |          |          |          |
a CCTGTGTAGG GTTGCTTCAT GATGAGTTCG TGTAAAACTG CATCTTGGAT TGAGAAGGAA
b CCTGTGTAGG GTTTCCTGAT GATGAGTCCG TGTAAAACTG CATCTGGAGT TGAGAAGGAA
c CCTGTGTAGG GTTGCTTCAT GATGAGTCCG TGTAAAACTG CATCTGGAGT TGAGAAGGAA
d CCTGTGTAGG GTTGCTTCAT GATGAGTCCG TGTAGAACTG CATCTT
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Fig. 4 — Nucleotide sequence of *Vf*M element. The different repeats are indicated with different letters.

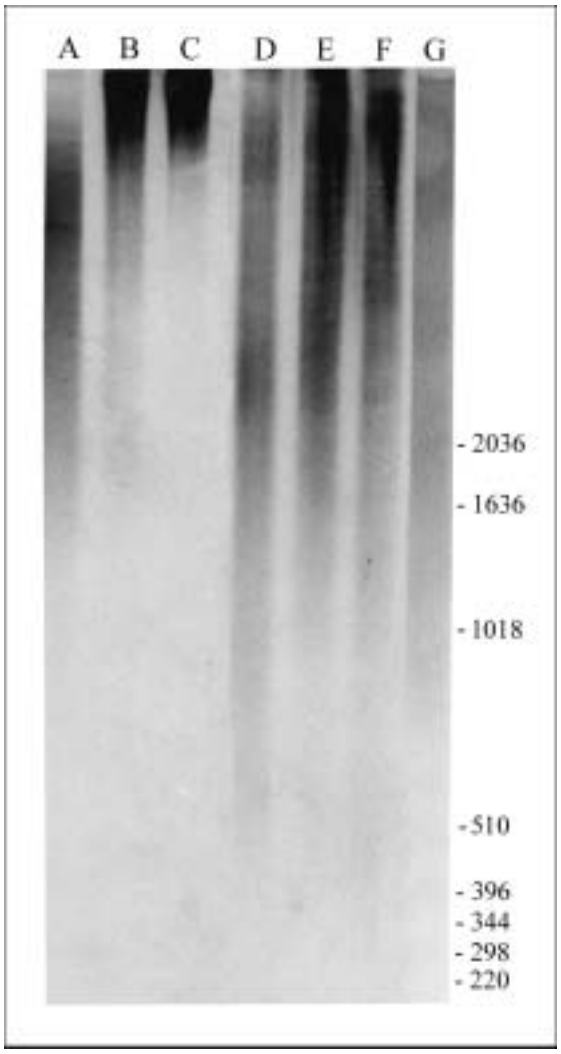

Fig. 5 — Band patterns of genomic DNA of *V. faba* digested with A=RsaI; B=EcoRI; C=BamHI; D=Sau3AI; E=AluI; F=TaqI; G=HinfI restriction endonucleases, after Southern blot hybridization with digoxigenin-labelled *Vf*M probe. Numerals indicate molecular sizes in base pairs as determined using a 1-kb DNA ladder as a marker.

DISCUSSION

MAXTED (1993) proposed a new classification of subgenus *Vicia* where nine sections are present and the old *Faba* section was substituted by three distinct sections: *Bithynicae* and *Faba,* monospecific, and *Narbonensis* which contains the seven species before referred to as the Narbonensis complex. In this connection, is noteworthy that the phylogenetic data generated from the analysis of the sequences of Internal Transcribed Spacers (ITS) of rDNA and from karyomorphological data (VENORA *et al*. 2000), provided support for this new classification.

In this paper we report further evidence obtained by using four different DNA repeated sequences. The results obtained by studying the FokI elements in the genomes of the species belonging to the sections *Faba*, *Bithynicae* and *Narbonensis,* in addition to *V. sativa,* allow us to conclude that they are almost species-specific to *V. faba.* Indeed FokI elements are detectable only in *V. narbonensis*, even if to a minimum extent. Faint hybridization signal is also present in *V. sativa*, which is included in sect. *Vicia* of the subgenus *Vicia,* according to the Maxted's classification (MAXTED 1993).

Analogous results are obtained by hybridizing p*Vf1* sequences on the analysed species. Indeed, strong hybridization signal is obtained on *V. faba* blots only, even if p*Vf1* sequences are detectable in *V. narbonensis* and *V. sativa*, but in this case the hybridization signal is stronger in *V. sativa* than in *V. narbonensis.*

Lack of hybridization to our Southern blots cannot be equated with absence of homologous sequences, however our findings may be taken as indicating different degrees of homology between sequences and then differing phylogenetic distances between species. However, *V. sativa* which is not included in the three sections we have analysed, shares more genomic similarity with *V. faba* than the other species.

*Vf*B elements are represented in the DNA of the species of sect. *Narbonensis* but to a reduced extent compared with that of *V. faba* and even less in that of *V. sativa* and of *V. bithynica*. It is noteworthy that this molecular marker discriminates also inside the *Narbonensis* section, showing a greater similarity between *V. hyaeniscyamus* and *V. johannis* and clearly differentiating *V. serratifolia* from the remaining species.

The fourth molecular marker, *Vf*M, is a tandemly-arrayed AT-rich sequence, with a monomeric unit of 60 bp. The lack of typical ladder in the hybridization patterns after Southern blotting and the negative results of cytological hybridization, indicate that *Vf*M elements are moderately represented in the genome of *V. faba* and/or they are not organized as a simple tandem arrays in *V. faba* genome, but rather in clusters dispersed throughout the genome. *Vf*M elements are species-specific, indeed when they are used as a probe in experiments of Southern blot hybridization on all the selected species, the signal is present in the genomic DNA of *V. faba* only.

The collected results, in particular the length of the repeats and the organization in the genome prompt us to consider *Vf*M as a minisatellite-like sequence and to use the most conserved region of the sequence as a primer in DAMD-PCR experiments.

***Vf*R3**

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a)CCTGGGTAGG GTTGCTTCAT AGGAGTCCGT TTAAACTACA CTAGAATTTG GAGAAGGAT
b)CCTGGGTAGG GATGCTTTAG GATGGGTTCG TGTGAAACTG CATCTGGAAA GAGAAGGAA
c)CCTGGGTAGG GTTTCTTCAT GATGAGTTCG TGTAAAACTT CATCTGGGAA GAGAAGGAA
d)CCTGGGTAGG GTTGCTTCAT GATAAATCAG TGTAAAACTT CATATGGAAA GAGAC GAA
e)CCTGGGTAGG GTTGCTTCAT GATGAGTCCA TGTAAAACTT CATATGGAAA TGTATGACAA GGAA
f)CCTGGGTAGG GTTACTTCAT
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***Vf*R9**

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a)CCTGGGTAGG GTTTCCTTGA TGATGAGTCT GTGTAAAACT TTCATTTTGG AAAGAAGGAA
b)CCCTGTGTAG GGTTTCTTGA TGATGAGTCC GTGTAAAACT TCATCTTGGA AAGAAGGAA
c)CCTGTGTAGG GTTTCTTTGA TGATGAGTCT GTGTAAAACT GCA
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***Vf*R10**

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a)CCTGTGTAGA GTTGCTTCAT GATGAGTCCG TGTAAAACTG CATCTTGGAA AGAAGGAA
b)CCTGTGTAGG GTTTCTTGAT GATGAGTCCA GTGTAAAACT TCATCTTGGA AAGAAGGAA
c)CCTGTGTAGG GTTTCTTGAT GATGAGT
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Fig. 6 — Nucleotide sequences of DNA fragments homologous to *Vf*M element identified in the clones *Vf*R3, *Vf*R9 and *Vf*R10. In each clone the different repeats are indicated with different letters.

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VfMb    CCTGTGTAGGGTTTCCTGATGATGAGTCC.GTGTAAAACTG.CATCTGGAGTT....GAGAAGGAA. 60
VfMa    CCTGTGTAGGGTTGCTTCATGATGAGTTC.GTGTAAAACTG.CATCTTGGATT....GAGAAGGAA. 60
VfMc    CCTGTGTAGGGTTGCTTCATGATGAGTCC.GTGTAAAACTG.CATCTGGAGTT....GAGAAGGAA. 60
VfR9b   CCTGTGTAGGGTTTCTTGATGATGAGTCC.GTGTAAAACTT.CATCTTGGAA......AGAAGGAA. 58
VfR3a   CCTGGGTAGGGTTGCTTCATGAGGAGTCC.GTGTTAAACTA.CACCTAGAATT.TGAGAGAAGGAT. 63
VfR3b   CCTGGGTAGGGATGCTTTAGGATGGGTTC.GTGTGAAACTG.CATCTGGAAAT....GAGAAGGAA. 60
VfR3c   CCTGGGTAGGGTTTCTTCATGATGAGTTC.GTGTAAAACTT.CATCTGGGAAT....GAGAAGGAA. 60
VfR3d   CCTGGGTAGGGTTGCTTCATGATAAATCA.GTGTAAAACTT.CATATGGAAAA....GAGACG.AA..59
VfR3e   CCTGGGTAGGGTTGCTTCATGATGAGTCC.ATGTAAAACTT.CATATGGAAATGTATGACAAGGAA. 64
VfR9a   CCTGGGTAGGGTTTCTTGATGATGAGTCT.GTGTAAAACTTTCATTTTGGAA......AGAAGGAAC 60
VfR10b CCTGTGTAGGGTTTCTTGATGATGAGTCCAGTGTAAAACTT.CATCTTGGAA......AGAAGGAA. 59
VfR10a CCTGTGTAGAGTTGCTTCATGATGAGTCC.GTGTAAAACTG.CATCTTGGAA......AGAAGGAA. 58

Cons.   CCTG.GTAGgGtT.CtT.AtGAtgagTcc.gTGTaAAACT..Cat.T.G.a...... AgAaGgAa
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Fig. 7 — Comparison of the different repeats of *Vf*M, *Vf*R3, *Vf*R9 and *Vf*R10 (as reported in Figs. 4 and 6) with consensus sequence where in uppercase are reported the identical bases and in lowercase the bases well conserved. Only the complete repeats are reported and hyphens represent gaps introduced to maximize homology.

This method allows us to characterize the different species, since the number and the size of the PCR fragments is generally different in the species. Our results are consistent with Maxted's classification (MAXTED 1993) since the banding patterns clearly differentiate *V. faba*, *V. bithynica* and the species of *Narbonensis* section.

Many reports in the literature confirm our results and Maxted's classification. Indeed ZIMMIAK-PRZYBYLSKA and PRZYBYLSKA (1995) evidenced, by the electrophoretic analysis of seed globulin, that *V. faba* was clearly different from the species of *Narbonensis* section. JAASKA (1997), PRZYBYLSKA *et al.* (1998) and LETH and JAASKA (2000), by isozymes analyses, showed that *V. faba* and *V. bithynica* were most distantly related to one another and to the species of sect. *Narbonensis*. POTOKINA *et al.* (1999), by RAPD analysis of genomic DNA and PCR-RFLP analysis of chloroplast genes, highlighted that *V. faba* was more closely related to *V. bithynica* than to the other spe-

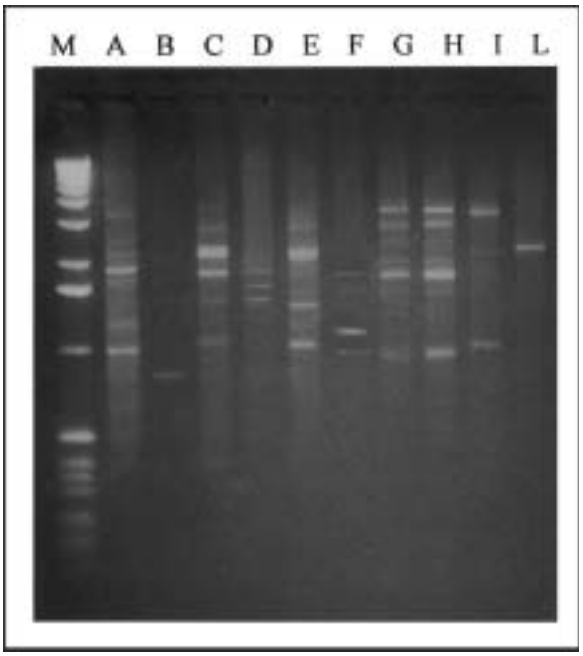

Fig. 8 — DAMD-PCR amplification of DNAs of *Vicia* species. A=*V. faba*; B=*V. bithynica*; C=*V. eristalioides*; D=*V. narbonensis;* E= *V. kalakhensis*; F= *V. galilaea*; G= *V. hyaeniscyamus*; H=*V. johannis*; I=*V. serratifolia*; L= *V. sativa.* M=1-Kb ladder.

cies of sect. *Narbonensis* and suggested that these two species could have the same ancestors as species belonging to the *Peregrinae* section, as suggested by Jaaska (1997), too.

Our results also evidence that the banding patterns obtained by DAMD-PCR experiments discriminate inside this last section, showing a greater similarity between *V. hyaeniscyamus* and *V. johannis* and, at less extent, between *V. eristalioides* and *V. kalakhensis*. Although it is not known if the common fragments amplified were from the same loci in the different species, it is possible to suppose that more bands in common may signify more affinity.

In this connection, it is noteworthy that the present data partially agree with the spatial representation of the karyological indices that highlighted four distinct group inside the *Narbonensis* section, including in the same group *V. eristalioides* and *V. kalakhensis*, while ITS analyses did not enable clarification of the phylogenetic relationships among the species of the section (Venora *et al*. 2000).

Maxted (1993) subdivided sect. *Narbonensis* in two series: *Rhombocarpae,* with *V. eristalioides* only, and *Narbonensis* including the other species. Up today only one report confirms this subdivision of the sect. *Narbonensis*, indeed Przybylska and Zimmiak-Przybylska (1995), by the analysis of seed albumins, evidenced that *V. eristalioides* was a peripheral member of the sect. *Narbonensis*. Other reports, by isozyme analysis, have evidenced specific relationship inside the *Narbonensis* section: two lineages *V. narbonensis-V. serratofolia* and *V. johannis-V. galilaea* (Jaaska 1997), one lineage *V. johannis* and *V. galilaea* (Przybylska *et al.* 1998). Potokina *et al*. (1999) subdivided the sect. *Narbonensis* in two subclusters, the former with *V. narbonensis* and *V. serratifolia* and the latter with V. *galilaea, V. johannis, V. hyaeniscyamus* and *V. kalakhensis*.

Therefore from the four markers we have used it is evident that *V. faba*, *V. bithynica* and the species of sect. *Narbonensis* are three distinct taxonomic groups according to Maxted's classification (Maxted 1993), but *Vf*B and *Vf*M only are useful for a discrimination inside the sect. *Narbonensis*.

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