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Isozyme Variation and Phylogenetic Relationships in *Vicia* subgenus *Cracca* (Fabaceae)

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• *Background and Aims* The phylogenetic relationships among 27 vetch species belonging to the subgenus *Cracca* of the genus *Vicia* were studied in comparison with three species of *Lathyrus* section *Lathyrus* on the basis of isozyme variation.
• *Methods* Isozymes encoded by 15 putative loci of ten enzymes were resolved by polyacrylamide gel electrophoresis and isozyme variation was analysed by using parsimony and neighbour-joining methods.
• *Key Results* The analyses revealed 63 parsimony-informative and 36 species-specific orthozymes. Of the latter, 23 are monomophic and are suitable for identification of *V. benghalensis*, *V. palaestina*, *V. dumetorum*, *V. pisiformis*, *V. sylvatica*, *V. onobrychioides*, *V. cappadocica*, *V. cretica*, *V. articulata*, *V. tetrasperma*, *V. ervilia*, *V. hirsuta* and *V. loiseleurii*. Polymorphism with heterozygous and homozygous isozyme genotypes was found for *V. cracca*, *V. tenuifolia*, *V. ochroleuca*, *V. villosa*, *V. sylvatica*, *V. cassubica*, *V. sparsiflora*, *V. megalotropis*, *V. altissima*, *V. onobrychioides*, *V. cassia*, *V. cretica* and *L. heterophyllus*, reflecting outcrossing in these species. By contrast, *V. benghalensis*, *V. palaestina*, *V. disperma*, *V. dumetorum*, *V. pisiformis*, *V. orobus*, *V. pauciflora*, *V. tetrasperma* and *V. loiseleurii* had only homozygous isozyme genotypes at polymorphic loci. Isozyme-based phylogenetic trees are presented.
• *Conclusions* Sections *Cracca*, *Ervum*, *Pedunculatae* and *Lenticula* of traditional taxonomy are monophyletic groups, whereas sections *Oroboideae* (= *Vicilla*) and *Panduratae* appear polyphyletic and section *Cassubicae* is split into two species-couples linked at a low level of support. Treatment of ervoid species in a separate subgenus *Ervum* is not supported because of its polyphyly.

Key words: *Vicia*, subgenus *Cracca*, genetic diversity, isozymes, phylogenetic relationships, monophyletic groups, systematics.

INTRODUCTION

The legume genus *Vicia* L. comprises annual or perennial herbaceous species distributed throughout the temperate regions of Europe, Asia, and North and South America. The genus belongs to the tribe *Vicieae* Adans., together with the genera *Lathyrus* L., *Lens* L. and *Pisum* L. The number of species recognized in the genus varies significantly, estimated from about 150 by Kupicha (1976) to about 210 by Hanelt and Mettin (1989), indicating problems with species circumscription and ranking. The infrageneric taxonomy also poses numerous continuously disputed problems, with several competing taxonomic treatments exisiting, e.g. by Fedtschenko (1948), Ball (1968), Davis and Plitmann (1970), Radzhi (1970), Kupicha (1976) and Tzvelev (1980, 1989). The taxonomic history of the genus is well described by Maxted (1993). Taxonomic treatments of the genus have been based on the traditional morphotypological taxonomy, with subgenera and sections delimited variously by differently selected diagnostic characters. In most treatments (l.c.), *Vicia* species have been grouped into three or four major clusters, *Cracca*, *Ervum*, *Vicia* and sometimes *Faba*, recognized either as subgenera or even as separate genera. In the last monographic treatment of the genus by Kupicha (1976) the genus is divided into only two subgenera *Vicia* and *Vicilla* (Schur) Rouy, with subgenus *Ervum* (L.) S. F. Gray grouped in the second subgenus. The subgenus name *Cracca* (Dum.) Gams was rejected by Kupicha (1976) because it was published later than the subgenera proposed by Rouy in 1899: *Vicilla*, *Ervoidea* and *Pseudoervoidea*. However, Tzvelev (1989) has applied *Cracca* Peterm. as a correct name of the subgenus (published in 1847: *Deutchl. Fl.*, 152) and treated subgenus *Ervum* (L.) S. F. Gray with three sections separately from it.

The type subgenus *Vicia* has been most thoroughly studied with different approaches, e.g. by phenetic analysis of morphology (Maxted, 1993), isozymes (Jaaska, 1997), RAPD and chloroplast restriction fragments (Potokina *et al*., 1999). The isozyme data (Jaaska 1997) regarding monophyletic groups and their relationships are in close agreement with those based on the study of RAPDs and cpDNA RFLP by Potokina *et al*. (1999). A recent publication (Leht and Jaaska, 2002) was a first attempt to apply cladistic and phenetic analysis of morphological and isozyme characters separately and together to evaluate phylogenetic relations between species and sections in the type subgenus. Species groups revealed by isozymes were in a general agreement with traditionally recognized sections, contributing to the debated sectional placement of some species. For example, *V. narbonensis* and its close relatives are frequently treated together with *V. faba* and *V. bithynica* in the same section *Faba* (Miller) Ledeb., e.g. by Ball

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(1968), Kupicha (1976) and Tzvelev (1980). However, cladistic and phenetic analysis of the isozyme data revealed them in separate clades corresponding to sections *Narbonensis* (Radzhi) Maxted, *Faba*, and *Bithynicae* (B. Fedtch. ex Radzhi) Maxted, as treated by Maxted (1993).

There are only few studies describing isozymes in vetches of the subgenus *Cracca*. Yamamoto and Plitmann (1980) studied isozymes of amylase, esterase, aspartate aminotransferse and indophenol oxidase in eight species of the subgenus *Cracca* in comparison with 16 species of the type subgenus without making any phylogenetic inferences. Zhang and Mosjidis (1998) analysed polymorphism of seven enzymes by isoelectric focusing in six species of the subgenus *Cracca* in comparison with five species of the type subgenus to infer their mating system. Black-Samuelsson and Lascoux (1999) studied isozymes in two species of the subgenus and found that *V. dumetorum* was monomorphic for all 14 isozyme loci in all 22 Nordic and central European populations investigated, whereas *V. pisiformis* showed low polymorphism. Leht and Jaaska (2002) described variation of 13 isozymes in *V. dumetorum*, *V. pisiformis* and *V. sylvatica* of the subgenus *Cracca* in comparison with 20 species of the type subgenus.

Previous work by Leht and Jaaska (2002) has shown that isozymes are good taxonomic characters for grouping vetch species into monophyletic sections and also provide additional diagnostic characters for distinguishing between species. The present paper describes variation of isozymes in a set of 27 Eurasian species belonging to nine traditional sections of the subgenus *Cracca* that were attributed to the synonymous subgenus *Vicilla* by Kupicha, in comparison with three species of the sister genus *Lathyrus* L. as an outgroup. Phylogenetic relationships in *Lathyrus* were assessed by Asmussen and Liston (1998) by parsimony analysis of cpDNA RFLP.

The main aim of the present work was a comparative analysis of the variability pattern of isozyme characters based on cladistic and phenetic approaches in order to evaluate species groupings and relationships in the subgenus *Cracca.* Specific goals were: (1) to assess the monophyly of traditional, morphology-based sections and subgenera versus genera in comparison with the isozyme-based groups; and (2) to evaluate the usefulness of isozymes as diagnostic characters to discriminate vetch species and sections.

MATERIALS AND METHODS

Plant material

The list of species and accessions analysed for isoenzymes is given in Table 1. The accessions received from botanical gardens and colleagues were largely collected in the wild from known localities. Taxonomic nomenclature is combined from Kupicha (1976) and Tzvelev (1980, 1989). Taxonomic destinations of accessions received from botanical gardens were verified by the morphology of plants grown from seeds according to species descriptions in Fedtschenko (1948), Ball (1968), Davis and Plitmann (1970) and Tzvelev (1989). Identifications were also checked by the seed characters according to Gunn (1970), Voronchikhin (1981), Perrino *et al*. (1984), and personal observations. Vouchers of species for checking identifications were grown from seed accessions in a common garden of the institute and are preserved in the herbarium of the Estonian Agricultural University (TAA).

Isoenzyme analysis and designation

Enzyme extracts were largely made from scarified seeds imbibed overnight on wet sheets of filter paper in a growth chamber at 28 °C, or germinated for a further 6 d under normal daylight. Individual cotyledons or shoots were crushed in 0·3-mL aliquots of 50 mM Tris – 10 mM EDTA buffer with 5 mM thioglycerol or cysteine as sulphydryl protectors. After adding 0·3 mL of 50 % glycerol and about 50 mg of Sephadex G-200 to increase viscosity, the extracts were stored frozen at −18 °C until electrophoresis in vertical polyacrylamide gel slabs.

Preliminary isozyme screenings included two individuals per accession and the number of individuals analysed was then doubled or tripled for isozymes that revealed polymorphism. A minimum of six individuals were analysed for species represented by 1–3 accessions.

The following ten enzymes were assayed for isozymes: aspartate aminotransferase (AAT, EC 2.6.1.1), diaphorase (DIA, EC 1.6.99.2), formate dehydrogenase (FDH, EC 1.2.1.2), glutamate dehydrogenase (GDH, EC 1.4.1.2), isocitrate dehydrogenase (IDH, EC 1.1.1.42), malate dehydrogenase (MDH, EC 1.1.1.37), 6-phosphogluconate dehydrogenase (PGD, EC 1.1.1.44), phosphoglucoisomerase (PGI, EC 5.3.1.9), phosphoglucomutase (PGM, EC 2.7.5.1), and superoxide dismutase (SOD, EC 1.15.1.1).

The following six gel–buffer systems and three catholytes were combined for different enzymes to achieve better band resolution:

Gel 1: 10 % acrylamide, 0·2 % *N*,*N*′-bisacrylamide (Bis), 0·2 M Tris and 0·1 M HCl; applied for SOD with the glycine catholyte and for PGD with the 2-alanine catholyte.

Gel 2: 10 % acrylamide, 0·2 % Bis, 0·125 M Tris and 0·1 M HCl; applied for MDH and PGM with the glycine catholyte.

Gel 3: 7·5 % acrylamide, 0·2 % Bis, 0·2 M Tris and 0·1 M HCl; applied for FDH with the 2-alanine catholyte.

Gel 4: 7·5 % acrylamide, 0·2 % Bis, 0·125 M Tris and 0·1 M HCl; applied for AAT and MDH with the glycine catholyte and for GDH with the 2-alanine catholyte.

Gel 5: 10 % acrylamide, 0·2 % Bis, 0·1 % triethanolamine hydrochloride and 5 mM Trilon B (disodium EDTA); applied for IDH and PGI with the glycine catholyte.

Gel 6: 7·5 % acrylamide, 0·2 % Bis, 0·05 M histidine, 0·05 M histidine hydrochloride, 5 mM Trilon B; applied for AAT, DIA, IDH and FDH with the HEPES catholyte.

N,*N*,*N*′,*N*′-tetramethylethylenediamine (0·05 mL %), riboflavine (0·5 mg%) and ammonium persulfate (1 mg %) were added to the gel mixtures to initiate and to catalyse their

TABLE 1. *List of the taxa and accessions investigated. The accession numbers with key letters of seed sources are given in parentheses*

Taxon name	Geographical origin and accession numbers with key letters (in parentheses)
I. Genus *Vicia* L. subgenus *Cracca* Peterm. 1847, = subgen. *Vicilla* (Schur) Rouy 1899	
Section *Cracca* S. F. Gray	
1. *V. cracca* L.	Estonia (ML217/87, VJ17/89, VJ20/89, VJ138/96, VJ143/96), France (BCA123/88, BD28/96), Germany (BOU8/88, BBD169/88, BBD 170/88, BHU223/88, BHU225/88, BH13/96, BHA88/99), Italy (BGE34/92), Russia: Siberia (BCS206/87) and Krasnodar (VJ20/92)
2. *V. tenuifolia* Roth	Armenia (G75/91), Czech Republic (G22/93, G29/93), France (BD36/93, BD5/97), Germany (BJ144/90, BJ12/91, BJ13/91, BJ17/93, G32/91), Sweden (G21/79)
3. *V. ochroleuca* Ten.	Bosnia-Herzegovina (BSA186/87, G73/92), Italy (BS163/00)
4. *V. benghalensis* L.	France (IG60798), Italy (IG60790, IG60792, IG60795, IG60819, BS45/97, BS169/00), Palestine (IG60789), Portugal (BCO25/91, BLS66/97, BLS34/99)
5. *V. disperma* DC.	France (BLY47/93), Italy (BGE35/92, BS89/02), Portugal (BCO7/01, BCO69/02, G792/75), Spain (BRK6585)
6. *V. monantha* Retz.	Egypt (G654/77, G655/77), Spain (G778/78, G782/74)
7. *V. palaestina* Boiss.	Jordan (IG62676, IG62678), Lebanon (IG62653), Syria (IG62616, IG62617, IG62638, IG62889), Turkey (SH877019)
8. *V. villosa* Roth	France (MHN13/89, MHN55/02, BCA145/96, BCA34/02), Germany (BBD180/88, BBD181/88, BBD120/96, BBD64/99), Hungary (IG60578, IG62705), Iran (PI268321), Syria (SH867641), Turkey (IG61000, SH877230, SH877287)
Section *Oroboidea* Stankevich 1970, = section *Vicilla* (Schur) A. et G. ex Kupicha 1976	
9. *V. pisiformis* L.	Czech Republic (BPO39/97, BDR19/99, BPO22/99), Germany (BJ16/93, BHU27/93, BHU28/93), Turkey (PI358868)
10. *V. dumetorum* L.	Czech Republic (BPO20/99), Germany (BBD171/88, BH11/96, BH12/96, BBD50/04, BBD51/04), Sweden (BU141/87).
11. *V. sylvatica* L.	Estonia (ML229/87, ML25/89, VJ122/90), France (MHN73/87, MHN53/02), Germany (BBD246/88, BHU116/02), Russia: Siberia (BCS25/87), Slovakia (BBR93/88), Sweden (BU142/87)
Section *Cassubicae* Radzhi 1971	
12. *V. cassubica* L.	Czech Republic (BPA21/99), Estonia (ML24/89), France (BLY15/94), Germany (BBD167/88, BBD27/90, BDR18/99), Italy (BGE33/92), Poland (BW197/87), Slovakia (BBR89/88), Sweden (BU140/87)
13. *V. orobus* DC.	Germany (BLE117/99, BJL147/00), Norway (VIC67/83)
14. *V. sparsiflora* Ten.	Hungary (BBU202/87), Italy (BF58/93)
15. *V. megalotropis* Ledeb.	Russia: Siberia (BCS82/83, G68/83)
Section *Pedunculatae* Rouy	
16. *V. onobrychioides* L.	France (G66/93), Czech Republic (BPO25/99), Spain (G62/81)
17. *V. altissima* Desf.	Italy (BS70/96, BS44/97, BS170/00, BS83/02)
Section *Panduratae* Kupicha	
18. *V. cappadocica* Boiss. et Bal.	Armenia (EN3117, EN3136)
19. *V. cassia* Boiss.	Turkey (IG64015, IG64022, IG64054, IG64056)
20. *V. cretica* Boiss. et Heldr.	Greece (BRK55343)
Section *Ervum* (L.) Taub.	
21. *V. tetrasperma* (L.) Moench, =*Ervum tetraspermum* L.	France (MHN12/89, MHN22/93, MHN23/93, BNA25/93, BCA43/93, BNA60/97), Germany (BH184/87, BOL12/88, BBD179/88, BHU202/88, BHU248/88, BHU29/93, BH20/96), Italy (BS80/96), Turkey (SH877132)
22. *V. pubescens* (DC.) Link	Portugal (BCO127/96, G646/79)
23. *V. parviflora* Cav. =*V. tenuissima* (Bieb.) Schinz & Thell. =*V. laxiflora* Brot., nom. illeg.	Greece (BRK11282), Italy (BF59/93, BS82/97, BP144/02, BS168/00, BS43/03), Portugal (BCO27/91, BCO6/01), Turkey (IG63934, IG63970, IG63974, IG64058)
Section *Lenticula* (Endl.) Aschers. & Graebner	
24. *V. hirsuta* (L.) Gray = *Ervum hirsutum* L.	Estonia (ML12/98), France (BGE36/92, BD2/97, BBE29/01, MHN44/02), Germany (BOL9/88, BH16/96, BHU119/96, BHU92/99), Italy (BGE36/92), Portugal (BCO5/01)
25. *V. loiseleurii* (Bieb.)Litv. =*Ervum loiseleurii* Bieb., =*V. meyeri* Boiss.	Ukraine: Crimea (BNU112/88), Italy (BGE47/94, BS38/03)
Section *Ervoides* (Godr.) Kupicha, = *Cracca* sect. *Ervoides* Godr, = *Vicia* subgenus *Ervoides* Rouy, = *Ervum* sect. *Ervoides* (Godr.) Stankev.	
26. *V. articulata* Hornem. =*Ervum monanthos* L.	Italy (G298/75, G299/75, G300/75), Spain (G869/79, G1008/84, G1009/84), Syria (IG64107), Turkey (IG63654, TR57630)
Section *Ervilia* (Link) Koch, = *Ervilia* Link, = *Vicia* subgenus *Ervilia* (Link) Rouy	
27. *V. ervilia* (L.) Willd., =*Ervum ervilia* L	Armenia (EN3425, EN3426), France (BCA42/93, BCA144/96, BBE146/00), Turkey (SH867981, SH877265, T9/93, G117/79)

TABLE 1. *Continued*

Taxon name	Geographical origin and accession numbers with key letters (in parentheses)
IV. Genus *Lathyrus* L. section *Lathyrus*	
28. *L. sylvestris* L.	Estonia (ML15/98, ML49/01, ML50/01), France (MHN18/97, BNA62/97), Germany (BH5/96, BHU67/97, BHU99/99), Italy (BP150/02)
29. *L. latifolius* L.	France (BNA63/97), Germany (BHU186/88, BJ142/90, BH2/96, BHU118/02), Italy (BS158/00), Portugal (BLS62/97)
30. *L. heterophyllus* L.	France (BBE10/93, MHN14/97), Switzerland (BDR17/99)

B: received from various botanical gardens (BG) coded by key letters following B: BBD, BG of Berlin-Dahlem (Germany); BBE, BG of the Besançon University (France); BBO, BG of Bordeaux (France); BBR, BG of the Bratislava University (Slovakia); BBU, BG of the Budapest University (Hungary); BCA, BG of Caen, France; BCO, BG of Coimbra (Portugal); BCS, Central Siberian BG in Novosibirsk (Russia); BD, BG of Dijon, France; BDR, BG of the Technical University of Dresden (Germany); BF, BG of Firenze (Italy); BGE, BG of Genoa (Italy); BH, BG of Hamburg (Germany); BHF, BG of the Helsinki University (Finland); BHU, BG of the Halle University (Germany); BJ, BG of Jena (Germany); BJL, BG of the Justus-Liebig University in Giessen (Germany); BLE, BG of the Leipzig University (Germany); BLS, BG of the Lisboa University (Portugal); BLY, BG of the Lyon University (France); BNA, BG of Nantes (France); BNU, BG of Nikita (Ukraine: Crimea); BOU, BG of the Oldenburg University (Germany); BP, BG of Palermo (Italy); BPO, BG of Palacky University in Olomouc (Czech Republic); BRK, Royal BG of Kew (England); BS, BG of the University of Siena (Italy); BSA, BG of Sarajevo (Bosnia); BU, BG of the Uppsala University (Sweden); BW, BG of the Wroclaw University (Poland). EN, collected by Dr Estella Nazarova of the Armenian Institute of Botany in Yerevan (Armenia). G (=VIC), the collection of the Institute of Plant Genetics and Crop Plant Research (Gatersleben, Germany). IG, the collection of the International Center for Agricultural Research in the Dry Areas (ICARDA, Aleppo, Syria). MHN, Muséum d'Historie Naturelle in Paris (France). ML, collected by Dr Malle Leht of the Estonian Agricultural University (Tartu, Estonia). PI, the collection of the USDA Regional Plant Introduction Station at the Washington State University (Pullman, WA, USA). SH, previous collection of Southampton University (England). T, received from Dr A. Sahin of Firat University in Elazig (Turkey). TR, the collection of the Aegean Agricultural Research Institute (Izmir, Turkey). VJ, collected by the author. Original numbers are applied for the accessions received from seed banks and Dr Nazarova, whereas accession from botanical gardens and other persons are labelled by numbers in the author's seed collection, the number after a slash indicating the year of receipt. The accessions received from botanical gardens and persons were collected in the wild from known localities.

photopolymerization between two daylight fluorescent bulbs over a period of 1 h.

The three catholytes used consisted of 80 mM glycine, 2-alanine or HEPES with 10 mM Tris. The use of 2-alanine instead of glycine yielded faster band mobilities and better resolution for FDH, GDH and PGD.

The lower anode buffer for gel systems 1–4 was 0·1 M Tris with 0·02 M acetic acid, and it was used repeatedly while the pH remained over 7. The anolyte for gel system 5 consisted of 0·1 M triethanolamine with 0·02 M acetic acid, and that for gel 6 consisted of 0·02 M histidine hydrochloride and 0·08 M histidine.

Electrophoresis in the anodal direction was carried out in an ice-refrigerated Plexiglass apparatus for 120 × 800 × 2-mm vertical gel slabs by applying a pulsed current at 15 mA and 20–30 V cm until the marker dye, bromophenol blue, reached the gel end (about 2·5–3 h). For some isozymes, an overflow of the marker dye for 40–60 min was applied as specified in the Results below in order to obtain better resolution of bands having close mobilities in a set of species.

Isozymes of SOD were also analysed with the isoelectric focusing (IEF) method in a similar vertical polyacrylamide gel as described by Jaaska and Jaaska (1988), in addition to PAGE.

Following electrophoresis, the gels were stained for isozymes by applying standard histochemical methods (Wendel and Weeden, 1989). Isozyme phenotypes were interpreted on the basis of existing knowledge of isoenzyme structure and genetic control (Wendel and Weeden, 1989), as described by Jaaska (1997) for *Vicia* species. Various types of isozymes are specified following the nomenclature described by Jaaska (2001). Thus, isozymes encoded by separate, heterologous loci of a diploid genome are named, in short, as 'heterozymes' (=isozymes *sensu stricto auct.*), and their electrophoretic variants (electromorphs) putatively encoded by different alleles of the same locus as 'allozymes'. Isozymes encoded by duplicated paralogous loci of a diploid genome are named 'parazymes'. Isozymes encoded by orthologous loci in different species are named 'orthozymes' (=allozymes *sensu lato auct.*). Heterozymes and parazymes are designated by upper-case letters followed by numerals to indicate the electrophoretic mobility of their allozymic and orthozymic variants on a scale of 0–100. The mobility values of allozymes and orthozymes are unified for each electrophoretic system, using extracts of selected reference species on the same gel slab in different combinations. In those cases when one allele controls 2–3 modificational isoforms, the mobility value is recorded by the major isoform. The use of an alternative gel-buffer system (gel 6) for FDH, IDH and AAT revealed additional orthozymes that remained cryptic in a standard system. These are labelled using lower-case letters that are added to mobility values in the standard system.

Data analysis

Allozymes and orthozymes were coded for cladistic and phenetic analyses as unordered binary (presence/absence) characters. Cladistic analyses of phylogenetic relationships were conducted using Fitch–Wagner parsimony by applying heuristic search with tree-bisection reconnection (TBS) branch-swapping, multiple parsimony (MULPARS), simple stepwise taxon application of 200 replications, using the program PAUP* 4.0b10 (Swofford, 2002). Phenetic analyses were also performed with the PAUP* program using the neighbour-joining clustering method, with a mean character difference as a distance measure. Reweighting of characters by maximum values of rescaled consistency indexes was applied in order to reduce the misleading

effect of homoplasious characters (Farris, 1969). Branch supports were estimated by bootstrapping with simple stepwise addition of 500 replications and TBS branch-swapping, as implemented in PAUP*.

RESULTS

Description of isozyme characters and diversity

Data on the isozyme variation among the subgenus *Cracca* species in comparison with three *Lathyrus* section *Lathyrus* species are compiled in Table 2. The zymograms for *Vicia* species are similar to those published in previous papers for the Asian *Vigna* subgenus *Ceratotoropis* and American *Phaseolus* species (Jaaska and Jaaska, 1988, 1990; Jaaska, 1996). Therefore, only the zymogram variation pattern of enzymes will be described.

Formate dehydrogenase. FDH zymograms of imbibed cotyledons showed, as a rule, one major band together with 1–2 fainter, evenly spaced bands of higher mobility. The latter are considered to be modified isoforms of the basic isozyme designated FDH-A because changes in the mobility of all bands between species occurred concomitantly in the same direction and to the same degree. FDH-A displayed significant variation, with six shared and six species-specific variants, recorded by the mobility of the slowest major band in gel system 3. The use of an alternative gel system 6 revealed an additional variant that remained cryptic with gel system 3. Thus, *V. tetrasperma* and *V. parviflora 1* of sect. *Ervum* showed A71 in common with species of sect. *Cassubicae* and *Pedunculatae* with the standard system 3, but revealed a different mobility with system 6 (labelled 71s in Table 2). Polymorphism with frequent heterozygous triplet phenotypes with homozygous single bands A60 and A72 was observed in accessions of *V. cracca*, *V. tenuifolia*, *V. ochroleuca* and *V. villosa*, suggesting extensive outcrossing in these species and the dimeric nature of FDH. No intraspecific variation of FDH-A was detected in the remaining species.

Glutamate dehydrogenase. GDH zymograms of cotyledons revealed a series of up to seven evenly spaced, successive bands, indicating a hexameric structure of the plant GDH with two different subunits. The fastest and slowest bands of the seven-banded GDH phenotype reflect homohexameric isoenzymes and the five bands of intermediate mobility represent hybrid heterohexamers. Different interspecific variation patterns of the outer homomeric isoenzymes suggest that they are encoded by duplicated, paralogous gene loci and thus represent paralogous isoenzymes (parazymes), which are designated GDH-A (fast) and GDH-B (slow). The same has been described for GDH of *Phaseolus* (Jaaska, 1996), *Vigna* (Jaaska, 1999, 2001) and *Vicia* subgenus *Vicia* species (Jaaska, 1997). The relative intensity and the number of GDH bands on the zymograms varied depending on seed germinability, seedling age and tissue, as well as on the species. On the cotyledon zymograms of imbibed seeds, the faster band of the GDH-A homomer largely dominated by intensity. GDH-A and GDH-B showed variation, with four and six morphs, respectively, including two unique morphs for both.

Isocitrate dehydrogenase. IDH zymograms of cotyledon extracts gave inconsistent results with the use of standard Tris–HCl gel buffers 1–3 but revealed homozygous two-banded phenotypes with triethanolamine–HCl and histidine–HCl gel buffers 5 and 6. The mobility values were recorded for the dominant band of the doublet in gel system 5, followed by lower-case letters for additional, cryptic variants that could be revealed in gel system 6. In total, five species-specific and eight shared variants were recorded. Most of the variation was between species belonging to different sections. Polymorphism with one- and three-banded homo- and heterozygous phenotypes was observed within accessions of *V. cracca*, *V. tenuifolia*, *V. ochroleuca*, *V. villosa*, *V. cassubica* and *L. heterophyllus*, indicating frequent outcrossing in these species and the dimeric nature of IDH.

Malate dehydrogenase. MDH zymograms revealed symmetrically spaced band triplets characteristic of fixed heterozygosity for a dimeric enzyme. The flanking bands of the triplets revealed an independent variation pattern among the vetch species, suggesting their genetic control by duplicated paralogous loci. Therefore, they are considered to reflect paralogous isoenzymes (parazymes) MDH-A and MDH-B. An additional band with a different variation pattern was attributed to heterozyme MDH-C.

MDH-A revealed variation with three morphs, A59, A53 and A47, each shared by a different set of species. Most species were monomorphic for one of the three orthozymes. Only *V. cracca*, *V. tenuifolia*, *V. sylvatica*, *V. altissima* and *V. cassubica* showed polymorphism with allozymes A59, A53 or A47, segregating together with respective heterozygous triplets within accessions, consistent with outcrossing in these species.

MDH-B displayed variation, with five variants, of which B33 was unique for *V. articulata* and the other four were shared by different sets of species. Variants indicated outcrossing in these species. The other outcrossing species, however, with close mobilities B40 and B41, could be better distinguished from each other using prolonged electrophoresis with a 50-min overflow of the dye front. Some accessions of *V. cracca*, *V. cassia* and *V. cassubica* displayed polymorphism with a hetereozygous triplet combining allozymes B36, B41 or B47. All three *Lathyrus* species had the same invariant triplet phenotype, MDH-A59/B40.

MDH-C has a band that is generally slightly faster or coinciding with MDH-A59. It could be clearly distinguished only on the zymograms of those species or accessions with A53 or A47 instead of the more common A59. MDH-C is thus unsuitable for taxonomic comparisons among vetches.

6-Phosphogluconate dehydrogenase. PGD revealed extensive allozyme variation with homozygous one-banded and frequent heterozygous triplet phenotypes of dimeric PGD-A for many accessions of *V. cracca*, *V. tenuifolia*, *V. ochroleuca*, *V. villosa*, *V. sylvatica*, *V. cassubica*, *V. sparsiflora*, *V. altissima* and *V. onobrychioides*,

TABLE 2. *Unified electrophoretic mobilities (scale 0–100) of formate (FDH), glutamate (GDH), isocitrate (IDH), malate (MDH), 6-phosphogluconate dehydrogenase (PGD), phosphoglucoisomerase (PGI), phosphoglucomutase (PGM-A), superoxide dismutase (SOD), diaphorase (DIA) and aspartate aminotransferase (AAT) orthozymes in Vicia and Lathyrus species*

Taxon	*n*	FDH-A	GDH-A	GDH-B	IDH-A	MDH-A	MDH-B	PGD-A	PGI-A	PGM-A	SOD-A	SOD-B	SOD-C	DIA-B	AAT-A	AAT-C
Vicia* subgenus *Cracca																
V. cracca	15	60;72	75	42	57a;65a;**50**	59;53	41;47r	72;70	75;64	80;84r	78:68;58	54a	38	53;60r	78;71r	38;31
V. tenuifolia	6	60;72	75	42	57a;65a	53;59r	41	72;70	75;64;80	80	78	54a	38	53;60r	78	38;31
V. ochroleuca	3	60;72	75	42	57a;65a	53	41	72;70r	75;80	80;77r	78	54a	38	53	78;71r	31;38
V. benghalensis	11	72	75	**53**	57b	59	41	72	80;75	80	78;68	54a	38	53;**41**	78;84r	38
V. palaestina	8	72	75	51	65b	53	41	76;72	80;75	**86**	78	54a	38	53	71	45a
V. villosa	15	72;60	75	51	65b	53	41	72;78;76	75;80	80;77	68;58	54a	38	53;45	78;71r	45a;38
V. disperma	7	72	75	42	57b;**44**	59	41	70;76	75;80	80	78	54a	38	53;45r	78;71	**24**;38
V. monantha	4	60	75	51	57b	59	41	70	75	77	78	54a	38	53	71	38
V. dumetorum	7	71	75	45	57b	59	36;40	71	**68**	84;80	78	54a	38	53	78	36
V. pisiformis	7	**82**	75	42	72a;**77**	59	47	72	70	73	78	54a	38	53	68w	45b
V. sylvatica	10	**80**	75;80r	42;34	65a	53;47	40	**75**;76;70	**52**	77;73	68	44	38	53	71;68	40
V. cassubica	11	71	75;80r	42	72a;65a	59;53	36;47	72;76;70	70	77;80	78	54a	38,**34**	45;53	78;71;84	45b
V. orobus	3	71	75	42	72a	59	40	72	64	77	78	54a	38	53	78;71	45b
V. sparsiflora	2	71	75	42	72a	59	40	**67**;72	64	77	78	54a	38	53	78	52
V. megalotropis	2	71	75	42	72a	59	47	76	70	77	78	54a	38	45	78	45b
V. altissima	4	71	75	34	65c	59;53	41	72;**65r**	70	84	78	54b	38	53	71	36
V. onobrychioides	3	71	**68**	34;42	65c	59	41	71;76	70;64	80;84	**86**	54b;a	38	53	78;84r	**28**;36r
V. cappadocica	2	**69**	80	45	57b	47	0	78	**57**	77	68	54a	38	53	**75**	38
V. cassia	4	72	75	51	57b	59	41;36	78	84	84	78;68	54a	38	53	78	45a
V. cretica	1	72	75	42	57b	59	41	76	84;73r	84	68;78	54a	38	53	**63**	45b
Vicia* subgenus *Ervum																
V. tetrasperma	15	71s	**88**	42;**61**	72b	59	47	76	70	**70**;77	78	54a	38	53	78	45a;52
V. pubescens	2	84	75	42	72b	59	47	76	70	77	78	54a	38	48	78	45a
V. parviflora 1	8	71s	75	42	72b	59	41	76	70	80	78	54a	38	48	**62**	38
V. parviflora 2	4	84	75	42;51	72b	59	47	76	70	77	78	54a	38	48	84;78	45a
V. articulata	9	**54**	75	42	**48**	53	**33**	76	**65**	77	68	44	38	53	**64**	40
V. ervilia	8	84	75	51	65a	47	40	72	**59**	77	68	54a	38;**47**	53	84	40
V. hirsuta	11	**58**	75	42	**70**	53	40	76	**62**	77	58	42	38	53	84	40
V. loiseleurii	3	**70**	75	42	65a	53	36	76;72	70	77	58	42	38	53	71	40
Genus *Lathyrus*																
L. sylvestris	9	74	75;80r	42	63	59	40	76	70	77	78	54a	33	53	71;78	45b
L. latifolius	5	74	75	42	72a	59	40	76	70	77	78	54a	33;38	53	71	45b;38r
L heterophyllus	3	74	75	42	63;72a	59	40	76	70;75r	77	78	54a	33;38	53	71;78	45b

n, number of accessions analysed; r, rare allozyme; w, weak band; bold entries are species-specific.

reflecting frequent outcrossing in these species. Allozymic polymorphism with the heterozygous triplet A70/76 was observed in accession RBG6585 of *V. disperma*. Intraspecific variation with alternate homozygous allozymes was observed among some accessions of *V. palaestina*, whereas all other species proved monomorphic for a particular orthozyme. In total, PGD-A displayed variation with five shared and two unique morphs among the vetch species and accessions studied. Orthozymes A72 and A76 had the widest distribution, being shared by many vetch species, the latter also by three *Lathyrus* species.

Phosphoglucoisomerase. PGI revealed extensive allozymic polymorphism with homozygous one-banded and heterozygous triplet phenotypes of dimeric PGI-A in some accessions of *V. cracca*, *V. tenuifolia*, *V. ochroleuca*, *V. villosa*, *V. cassubica*, *V. onobrychioides* and *L. heterophyllus*. As in the case of PGD-A, intraspecific variation with alternate homozygous allozymes was observed between some accessions of *V. palaestina*, whereas most other species proved monomorphic for a particular orthozyme. In total, PGI-A displayed variation with five shared and six species-specific orthozymes among the vetch species and accessions studied. Orthozyme A70 had the widest distribution, being shared not only by many vetch species of different sections but also by three *Lathyrus* species.

Phosphoglucomutase. PGM zymograms showed a variation pattern attributed to two monomeric heterozymes, PGM-A and PGM-B. Intrapopulational polymorphism of monomeric PGM-A with one- and two-banded homo- and heterozygous phenotypes was observed in accessions of *V. cracca*, *V. ochroleuca*, *V. villosa*, *V. sylvatica*, *V. cassubica* and *V. onobrychioides*. Most species, however, proved monomorphic for a particular orthozyme. In total, PGM-A revealed variation between species with two species-specific and four shared orthozymes.

PGM-B bands remained too faint or absent on the cotyledon zymograms of many species to be recorded and useful for the phylogenetic comparison.

Diaphorase. DIA zymograms revealed three zones of independent variation pattern among vetch species, indicating the existence of three heterozymes. However, only the most intensely stained DIA-C could be recorded for all species on the cotyledon zymograms. In total, five variants were recorded. Among them, C53 was shared not only by most vetch species but also by the three *Lathyrus* species studied. Allozymic polymorphism with homozygous C53 and C45 together with the heterozygous triplet C45/53 was found in many accessions of *V. cassubica* and *V. villosa*, whereas heterozygous triplet C53/60 was recorded only for a few individuals of *V. cracca* and *V. tenuifolia*. By contrast, *V. benghalensis* showed differentiation only between accessions with two alternate homozygous allozymes, unique C41 and common C53.

Superoxide dismutase. SOD appears on the leaflet and epicotyl zymograms as bands of chloroplastic SOD-A, cytosolic SOD-B and mitochondrial SOD-C (Jaaska, 1997, 1999, 2001). SOD-A remained faint on the PAGE zymograms of cotyledons but could be recorded on the IEF zymograms of cotyledons and PAGE zymograms of seedlings. Thioglycerol and dithiothreitol, when added as sulphydryl protectors to the extraction buffer at >5 mM, severely or totally inhibited Cu/Zn-dependent isoenzymes SOD-A and SOD-B, leaving the Mn-dependent SOD-C unaffected. Cysteine at 5 mM was not inhibitory and was used in the homogenization buffer for SOD.

SOD-A displayed variation in four morphs, with A58, A68 and A78 having wide distribution and A86 being specific for *V. onobrychioides*. Remarkably, all three species of *Lathyrus* shared A78 in common with many vetch species of different sections. Allozymic polymorphism with homozygous A58, A68 or A78 together with respective heterozygous triplets was found in many accessions of *V. villosa*, reflecting outcrossing in this species and the dimeric nature of SOD-A. Most accessions of *V. disperma* lacked the chloroplastic SOD-A even on the zymograms of green seedlings and leaflets, presumably because of the presence of an inhibitory substance.

SOD-B showed four morphs, among which B54a and B54b could be clearly distinguished by IEF. The latter was characteristic only for the two species of section *Pedunculatae*, *V. altissima* and *V. onobrychioides*. The latter species revealed polymorphism with one- and three-banded IEF-phenotypes, reflecting outcrossing in this species and the dimeric nature of SOD-B. Most vetch species shared B54a with the three *Lathyrus* species studied. *Vicia hirsuta* and *V. loiseleurii* of section *Lenticula* were the only species that shared B42. Unexpectedly, *V. sylvatica* and *V. articulata* belonging to different sections shared B44. Electrophoretic variants B42 and B44 were indistinguishable on the IEF zymograms, reflecting their coincident isoelectric points.

SOD-C revealed three morphs, among which C38 had the widest distribution. *Lathyrus heterophyllus* and *L. latifolius* revealed homologous polymorphism of SOD-C with shared allozymes C38 and C32, segregating in accessions together with the five-banded phenotype C38/32, which is typical for co-dominant expression of a tetrameric enzyme and for outcrossing.

Aspartate aminotransferase. AAT zymograms revealed two or three bands of independent intra- and interspecific variation, attributed to heterozymes AAT-A, AAT-B and AAT-C. Bands of AAT-A appeared on the cotyledon zymograms frequently as broad bands consisting of up to three closely spaced isoforms. They could be better distinguished from each other using gel buffer system 6 instead of the currently used system 4. AAT-A displayed variation with seven morphs, including three species-specific orthozymes. A71 and A78 had the widest distribution among the vetch species of different sections and were also shared by *Lathyrus* species. Many accessions of *V. cassubica* revealed polymorphism, with three allozymes in homozygous and three-banded heterozygous phenotypes, reflecting frequent

outcrossing in this species and the dimeric nature of AAT-A.

AAT-B appeared on the cotyledon zymograms of some species as a faint band that could not be recorded for all species.

AAT-C appeared on the zymograms as a band doublet, with the slower band largely dominating in intensity. Variants within and between species differed by equal mobility shifts of both bands, indicating that they represent isoforms of a single allozyme or orthozyme. Differences in the mobilities of AAT-C36, C38 and C40 were small with the currently used gel buffer system 4 with the glycine catholyte. They could be better resolved and checked using a 50-min overflow of the bromophenol blue front, by which time the bands of AAT-A have mostly moved out of the gel. C45 was shared by species of different sections and was also characteristic of the *Lathyrus* species studied. The use of gel system 6 enabled separation of C45 into two variants, labelled 45a and 45b, that were undetected with gel system 4. Several accessions of *V. cracca*, *V. ochroleuca*, *V. tenuifolia* and *V. villosa* revealed polymorphism with homozygous C31, C38 or C45 together with frequent three-banded heterozygous phenotypes C31/38 and C38/45, respectively, reflecting outcrossing in these species and the dimeric nature of AAT-C. Heterozygous AAT-C phenotypes were also recorded for some polymorphic populations of *V. sylvatica* and *V. onobrychioides*. By contrast, *V. disperma* and *V. tetrasperma* revealed only differentiation between accessions for alternate homozygous allozymes.

In total, the analyses revealed 63 parsimony-informative orthozymes that are shared by two or more species, and 36 species-specific orthozymes of 15 isozymes among the 27 vetch species studied. This reflects remarkable genetic diversity and differentiation among vetch species. Of the 36 species-specific orthozymes, 23 were monomophic and can thus be used as diagnostic characters for species identification based on isozyme analyses of seeds. The following 13 vetch species of 27 studied have diagnostic orthozymes suitable for their identification: *V. benghalensis*, *V. palaestina*, *V. dumetorum*, *V. pisiformis*, *V. sylvatica*, *V. onobrychioides*, *V. cappadocica*, *V. cretica*, *V. articulata*, *V. tetrasperma*, *V. ervilia*, *V. hirsuta* and *V. loiseleurii.* Among them, *V. articulata* is the most divergent with five specific orthozymes, followed by *V. cappadocica* and *V. hirsuta* with three. In addition, species-specific allozymes AAT-C24 and PGD-A67 were the most frequent among the accessions of *V. disperma* and *V. sparsiflora*, respectively, aiding in their identification.

Accessions of *V. cracca*, *V. tenuifolia*, *V. ochroleuca*, *V. villosa*, *V. sylvatica*, *V. cassubica*, *V. sparsiflora*, *V. megalotropis*, *V. altissima*, *V. onobrychioides*, *V. cassia*, *V. cretica* and *L. heterophyllus* revealed extensive or moderate allozymic variation with homo- and heterozygous phenotypes of polymorphic heterozymes, reflecting frequent outcrossing in these species. By contrast, *V. benghalensis*, *V. palaestina*, *V. disperma*, *V. dumetorum*, *V. pisiformis*, *V. orobus*, *V. pauciflora*, *V. tetrasperma* and *V. loiseleurii* revealed differentiation between some accessions with alternate homozygous allozymes of polymorphic heterozymes. Accessions of *V. monantha*, *V. cappadocica*, *V. articulata*, *V. ervilia*, *V. pubescens* and *V. hirsuta* studied lacked allozymic variation.

Remarkably, the three species of *Lathyrus* studied had most orthozymes in common with the vetch species, revealing differentiation from the *Vicia* subgenus *Cracca* species by only FDH-A74 and IDH-A63. The latter two were, however, previously detected in *V. peregrina* plus *V. aintabensis* and *V. lathyroides*, respectively (Leht and Jaaska, 2002).

Cladistic and phenetic analysis of relationships between species

The isozyme data matrix for cladistic and phenetic analyses was compiled from Table 2. Rare variants detected in only some individuals of some accessions were not included. Cladistic parsimony analysis of the binary (presence/absence) data matrix of 63 parsimony-informative, shared orthozymes of 28 vetch species compared with three species of *Lathyrus* section *Lathyrus* as an outgroup recovered 16 most-parsimonious trees 213 steps in length. The strict consensus tree (not shown) showed the vetch species in a single clade with six subclades and *V. pisiformis* unresolved. The tree, however, has a low rescaled consistency index, RC = 0·264, reflecting a high level of homoplasy in the isozyme data. This suggests a need for a reweighting of characters in order to minimize the noise resulting from homoplasy. Reweighting of characters based on the maximum RC value gave a single stable tree 71·5 steps in length, with RC = 0·658 (Fig. 1). The tree shows *Vicia* species in a monophyletic core clade of five major hierarchical subclades. The species of section *Cassubicae* form a basal subclade with respect to the remaining vetch species. The *Cassubicae* clade consists of two well-supported subgroups linking *V. cassubica* with *V. megalotropis* and *V. orobus* with *V. sparsiflora* as sister-species couples linked at low bootstrap support. Unexpectedly, *V. pisiformis* of section *Oroboidea* appears basally linked to them, albeit with low bootstrap support. The species of section *Ervum*, *V. tetrasperma*, *V. pubescens* and *V. parviflora*, form a well-supported subclade. The species of sections *Ervilia*, *Ervoides* and *Lenticula*, *V. ervilia*, *V. articulata*, and *V. hirsuta* and *V. loiseleurii*, respectively, form another well-supported monophyletic subclade. Surprisingly, *V. sylvatica* of section *Oroboidea* is nested within it as a sister to *V. articulata* of section *Ervilia*. Remarkably, the type species of Kupicha's section *Panduratae*, *V. cappadocica*, appears basally linked to this subclade with significant bootstrap support. The other two species of Kupicha's section *Panduratae*, *V. cassia* and *V. cretica*, form a highly supported couple that is sister to the *Pedunculatae* species *V. altissima* and *V. onobrychioides*. *Vicia dumetorum* of section *Oroboidea* appears linked to the *Pedunculatae* couple. Thus, the three species of the traditional section *Oroboidea* (=*Vicilla*), *V. dumetorum*, *V. pisiformis* and *V. sylvatica*, appear dispersed in the isozyme cladogram, being linked to species of other sections. The upper subclade comprises all species of the type section *Cracca*.

Phenetic neighbour-joining analysis of the same isozyme matrix yielded a tree 218 steps in length (RC = 0·250, not

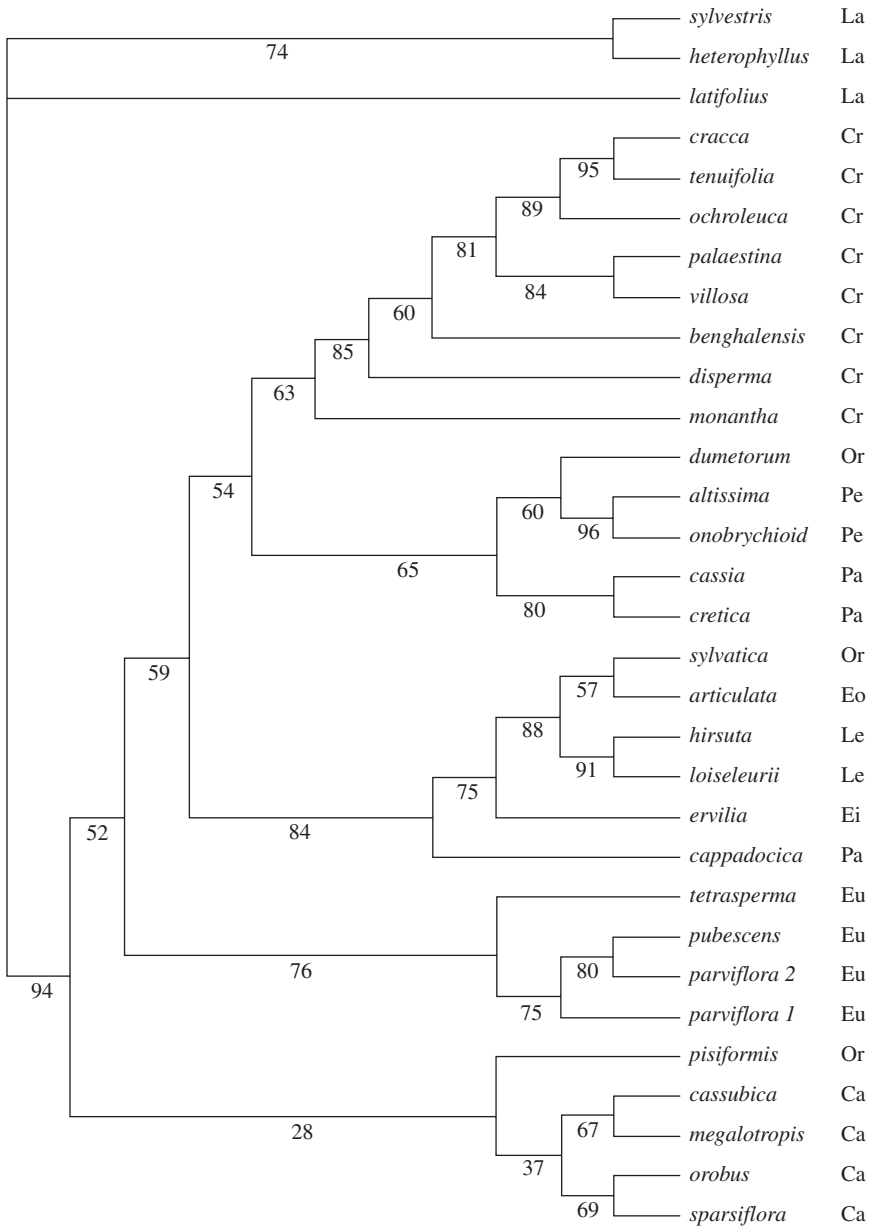

FIG. 1. A single most-parsimonious tree produced by the cladistic analysis of 63 parsimony-informative orthozymes after reweighting once based on maximum RC value: length = 71·5 steps, CI = 0·631, RC = 0·658. Bootstrap supports are given at branches. Sections: Ca, *Cassubicae*; Cr, *Cracca*; Ei, *Ervilia*; Eo, *Ervoides*; Eu, *Ervum*; La, *Lathyrus*; Le, *Lenticula*; Or, *Oroboidea*; Pa, *Panduratae*; Pe, *Pedunculatae*.

shown) that revealed the same species clusters as the most-parsimonious tree. Reweighting of characters once based on maximum RC values gave a tree of 67·6 steps (RC = 0·669, Fig. 2). The reweighted neighbour-joining tree shows essentially the same topology as the reweighted most-parsimonious tree and with same species clusters (Fig. 1), differing from the latter mainly in the position of the *Cassubicae* and *Panduratae* pairs relative to *V. pisiformis.*

DISCUSSION

Several monophyletic groups in the isozyme phylogenetic trees (Figs 1 and 2) correspond to sections delimited on the basis of morphological characters by Radzhi (1970), Kupicha (1976), Tzvelev (1980, 1989) and others. Thus, all species attributed to the section *Cracca* appear in a well-supported monophyletic group on the isozyme trees. The three perennials of the section, *V. cracca*, *V. tenuifolia* and *V. ochroleuca*, form a subgroup of closely related species that revealed extensive homologous polymorphism with shared allozymes without any differentiation by species-specific orthozymes. The annual *V. villosa* is most closely related to them, being differentiated only by the presence of some additional allozymes, AAT-C45, PGM-A77 and DIA-C45. The annual *V. palaestina* is sister to *V. villosa*, being differentiated by species-specific

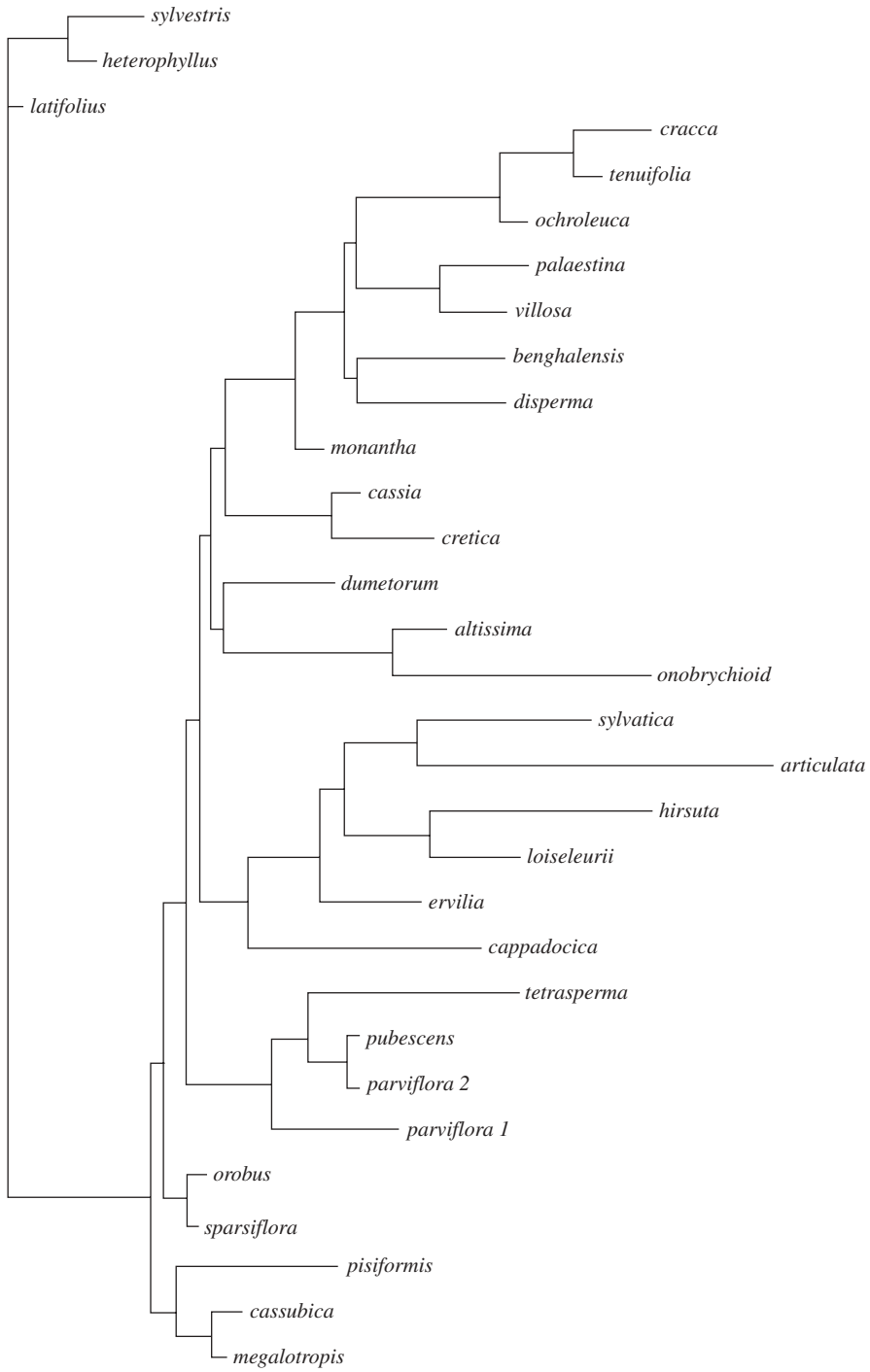

FIG. 2. Neighbour-joining tree produced by the analysis of 63 parsimony-informative and 33 species-specific orthozymes after reweighting once based on maximum RC value: length = 67·6 steps, CI = 0·636, RC = 0·669.

PGM-A86. *Vicia monantha* appears basally sister to the remaining species of the section on both phylogenetic trees.

Species of section *Ervum* form another monophyletic clade in both trees. In this section, *V. parviflora* shows differentiation into two multilocus isozyme lineages, provisionally designated as *V. parviflora 1* and *2*, differing by alternate variants of FDH-A, MDH-B, AAT-A and AAT-C. Such significant difference at four isozyme loci indicates that *V. parviflora* may actually hide two cryptic species. On both phylogenetic trees, *V. parviflora 1* is linked as a sister to *V. pubescens*, whereas *V. parviflora 2* is basally linked to them, preliminarily supporting specific status of all three species. The question of their status, however, requires a further study to identify possible minor morphological differences between the two isozyme lineages of *V. parviflora* and their correspondence to the synonymic species. *Vicia*

pubescens has been treated as a subspecies or variety of *V. tetrasperma* by some taxonomists. The two taxa, however, are placed separately in the *Ervum* clade and are fixed for alternate variants of FDH-A, GDH-A and DIA-C. This evidence supports their recognition as separate species (e.g. Davis and Plitmann, 1970; Zohary, 1972).

The species of sections *Ervilia*, *Ervoides* and *Lenticula*, *V. ervilia*, *V. articulata*, and *V. hirsuta* and *V. loiseleurii*, respectively, are united in a well-supported monophyletic subclade. This result favours the treatment of sections *Ervoides* and *Lenticula* as subsections of section *Ervilia s.l.* The two species of section *Lenticula*, *V. hirsuta* and *V. loiseleurii*, are linked as sister species of the *Ervilia* subclade that is apart from the subclade of section *Cracca*. This result argues against their inclusion in the section *Cracca* by Kupicha (1976) and is consistent with other taxonomic treatments, e.g. by Radzhi (1970), Tzvelev (1980, 1989) and Fedoronchuk (1996). Radzhi (1970) has recognized *Lenticula* as a subsection of section *Ervum*, but our result favours it as a subsection of section *Ervilia s.l.* Despite the fact that *V. loiseleurii* is morphologically quite similar to *V. hirsuta* and has even been treated as a variety of the latter, *V. hirsuta* var. *terronii* Burn., they differ distinctly from each other by alternate orthozymes of FDH-A, IDH-A, MDH-B, PGI-A and AAT-A, which may thus be used for their identification. Morphological features that distinguish the two species are described in detail by Roti-Michelozzi *et al*. (1989).

Sections *Ervum*, *Ervilia*, *Ervoides* and *Lenticula* are treated in many taxonomies, e.g. by Radzhi (1970), Tzvelev (1980, 1989) and Fedoronchuk (1996), in a separate subgenus *Ervum* (L.) S. F. Gray. By contrast, Kupicha (1976) joined them under the enlarged subgenus *Vicilla* (Schur) Rouy 1899 that is a later synonym of subgenus *Cracca* Peterm. 1847 (see Tzvelev, 1980). Sections *Ervum* and *Ervilia s.l.* appear on the isozyme phylogenetic trees as separate monophyletic groups apart from each other. This result argues against the recognition of subgenus *Ervum* because of its paraphyly and supports its inclusion within the enlarged subgenus *Cracca*. Our results disagree with the placement of *V. articulata* and *V. cappadocica* in section *Cracca* by Davis and Plitmann (1970) and favour their position in the *Ervilia s.l.* group.

Two sections, *Oroboidea* (=*Vicilla sensu* Kupicha) and *Panduratae*, appear polyphyletic in the phylogenetic trees. The three species of *Oroboidea*, *V. dumetorum*, *V. pisiformis* and *V. sylvatica*, differ significantly from each other by many isozymes (Table 2) and appear linked to species of sections *Pedunculatae*, *Cassubicae* and *Ervoides*, respectively, in phylogenetic trees. Morphological difference are reflected in their placement in different subsections *Dentatae* Radzhi, *Pisiformes* Radzhi and *Sylvaticae* Tzvel. of section *Oroboidea* by Tzvelev (1980) and Fedoronchuk (1996).

Radzhi (1970) has treated *V. pisiformis* in section *Cassubicae* based on shared dorsally compressed style, placing it in subsection *Pisiformes*. The exact phylogenetic position of *V. pisiformis*, however, remains obscure and needs further study because its link to the *Cassubicae* clade in the isozyme cladogram has low bootstrap support.

Vicia dumetorum is linked basally to the section *Pedunculatae* pair, *V. altissima* and *V. onobrychioides*, in Figs 1 and 2. This linkage is also supported morphologically based on shared dorsally compressed, abaxially tufted styles. Kupicha (1976) still decided to place *V. dumetorum* within section *Vicilla* despite its tufted style, on the grounds of several other morphological characters.

The moderately supported linkage (bootstrap support 57 %) of *V. sylvatica* to *V. articulata* of section *Ervoides* is an unexpected result because the two species differ from each other considerably by many isozymes (FDH-A, IDH-A, MDH-B, PGI-A and AAT-A) as well by morphology. However, they share synapomorphic SOD-A44, which is unique to them. Our results disagree with the inclusion of *V. articulata* into the enlarged section *Cracca* by Davis and Plitmann (1970) and Zohary (1972).

The type species of Kupicha's section *Panduratae*, *V. cappadocica*, appears basally linked to the *Ervoides s.l.* subclade with significant bootstrap support. The other two species of this section, *V. cassia* and *V. cretica*, form a pair of sister species that are basally linked to *V. dumetorum* and section *Pedunculatae* on the cladogram or are basally sister to section *Cracca* in the neighbour-joining tree. This result reflects their intermediate position between sections *Cracca* and *Pedunculatae*. Their linkage to section *Pedunculatae* and *V. dumetorum* is supported by sharing dorsally compressed style, whereas all species of section *Cracca* have laterally compressed style (Kupicha, 1976). Despite only moderate bootstrap support (57 %), the isozyme-based cladogram suggests possible expanded delimitation of section *Pedunculatae* by adding *V. dumetorum*, *V. cassia* and *V. cretica.*

The isozyme phylogenies of the present study reveal some important discrepancies with the recent morphology-based cladistic and phenetic analysis of relationships in *Vicia* subgenus *Cracca* (Leht, 2005). Thus, the morphology-based phylogenetic trees showed all ervoid species in the same monophyletic group, whereas the isozyme trees separated them into two paraphyletic groups. In contrast to result here, parsimony and neighbour-joining analyses of morphology showed most species of section *Oroboidea* (=*Vicilla auct*.) as a monophyletic group when *V. amoena* is removed from it to section *Cassubicae*. Morphological phylogeny shows monophyly of section *Cassubicae* with *V. cassubica*, *V. orobus* and *V. sparsiflora*, whereas the inclusion of *V. megalotropis* by Nikiforova (1985) is not supported. The isozyme phylogeny splits section *Cassubicae* into two well-supported species pairs, *V. cassubica* – *V. megalotropis* and *V. orobus* – *V. sparsiflora*, that are linked only at low bootstrap support. Isozyme trees support the monophyly of section *Pedunculatae* with *V. altissima* and *V. onobrychioides* as close sister species but the morphology-based trees do not.

It should be emphasized that both isozyme- and morphology-based cladistic and neighbour-joining trees provide only preliminary phylogenetic hypotheses that should be tested with the use of DNA markers in future studies. Methodological aspects and limitations of isozymes for phylogenetic purposes due to electrophoretic homoplasy are discussed in Leht and Jaaska (2002). The results of

this and Leht and Jaaska (2002) indicate that isozymes are well suited for inferring phylogenetic relationships between closely related species and for revealing monophyletic groups of species that frequently correspond to traditional sections within genera.

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