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A phylogenetic analysis of *Pisum* based on morphological characters, and allozyme and RAPD markers

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Abstract Cladistic analyses of 17 wild and cultivated pea taxa were performed using morphological characters, and allozyme and RAPD (random amplified polymorphic DNA) markers. Both branch-and-bound and bootstrap searches produced cladograms that confirmed the close relationships among the wild species and cultivars of *Pisum* proposed by a variety of systematic studies. Intraspecific rankings were supported for northern *P. humile*, southern *P. humile*, *P. elatius* and *P. sativum*, which together comprise a single-species complex. *P. fulvum*, while clearly the most divergent of the pea taxa, could also be assigned to the same species complex without violating the hierarchial logic of the cladogram. Its inclusion or exclusion depends on whether the level of interfertility it displays with other pea taxa or its overall morphological and chromosomal distinction are emphasized. As suggested by previous studies, northern *P. humile* was the most likely sister taxon to cultivated *P. sativum*; although, rigorous phylogenetic evaluation revealed a close genealogical affinity among *P. elatius*, northern *P. humile* and *P. sativum*. Despite their limited number, the 16 morphological characters and allozyme markers used precisely organized the pea taxa into established taxonomic groupings, perhaps in part reflecting the role morphology has played historically in pea classification. The RAPD data also generally supported these same groupings and provided additional information regarding the relationships among the taxa. Given that RAPDs are relatively quick and easy to use, are refractory to many environmental influences, can be generated in large numbers, and can complement traditional characters that may be limited in availability, they provide a valuable new resource for phylogenetic studies.

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Introduction

Recent biosystematic treatments of the genus *Pisum* L. (Ben Ze'ev and Zohary 1973; Waines 1975; Marx 1977; Palmer et al. 1985) have focused on relationships among the cultivated garden pea, *P. sativum* L., and the three wild annual species recognized by Boissier (1872) based on morphological characteristics: (1) *P. fulvum* Sibth. and Sm., (2) *P. humile* Boiss. and Noe [= *P. syriacum* (Berger) Lehm.] and (3) *P. elatius* Bieb. Detailed descriptions of the morphology and ecology of the wild species have been reported both by Ben Ze'ev and Zohary (1973) and Waines (1975). *P. fulvum*, an eastern Mediterranean plant usually found in rocky terrain, is a short climbing species characterized by a slender appearance, short pods, small peduncles, markedly toothed leaflets, small orange-brown flowers and dark velvety seeds. *P. humile*, most often found in the open vegetation of the Near Eastern steppe, is characterized as a medium-sized climbing species with medium-sized pods and peduncles, dentate leaf margins and light-blue flowers. It resembles some cultivars of pea in general habit. *P. elatius*, an omni-Mediterranean plant located principally in humid maquis formations, is characterized by a tall climbing feature, large pods and peduncles, large smooth leaves and large colorful flowers with lilac-blue standards and dark-purple wings.

The wild species can also be found in secondary habitats as weeds, often in direct contact with one another or with the domesticated pea. Intergradations involving the *humile* and *elatius* peas have occurred in transition zones between maquis and more xeric open formations. Spontaneous hybridizations also occur between cultivars of *P. sativum* and weedy forms of both *P. humile* and *P. elatius* at the edges of cultivation. With the exception of a single *humile-fulvum* hybrid, no additional examples of hybridization involving *P. fulvum* have been

identified, suggesting that *P. fulvum* is reproductively isolated in nature.

In order to clarify the genetic affinities among the four taxa of wild and cultivated peas, Ben Ze'ev and Zohary (1973) performed cytogenetic studies based on a series of controlled crosses and karyomorphological analyses. They discovered that "northern" *P. humile* isolates (from northeast Israel and Turkey) possess the standard *P. sativum* karyotype, while both "southern" *P. humile* and *P. elatius* isolates differ from the standard karyotype in the sizes of chromosomes 4 and 6. The *P. fulvum* karyotype, by comparison, is distinctly different from the otherwise homologous chromosomes of *humile, elatius* and *sativum.*

Intraspecific crosses for *P. fulvum* or *P. elatius* yield the seven closed bivalents expected in F_1 hybrids of fully fertile diploid plants with a chromosome number of $2n = 14$. Intraspecific crosses for *P. humile* also yield seven bivalents within the northern and southern isolates, but not between them. The five bivalents and one quadrivalent generated by the intervarietal crosses reveal a translocation involving chromosomes 4 and 6 that results in reduced pollen and reduced seed fertility.

Crosses among the *humile, elatius* and cultivated peas indicate general interfertility; although, some reduction in chromosomal compatibility and fertility is observed, once again due to the presence of translocation heterozygotes. Chromosomal incompatibility is far more prevalent for interspecific crosses involving *P. fulvum,* resulting in substantially reduced pollen fertility and seed set. Additionally, normal hybrid seedlings are obtained only when *P. fulvum* is the pollen parent. This apparent cytoplasmic incompatibility between *P. fulvum* and the other taxa reinforces its morphological distinction.

Based on their analyses of morphology, ecology, cytogenetics and hybrid performance, Ben Ze'ev and Zohary (1973) conclude that *P. fulvum* is a fully divergent species, whereas *P. humile, P. elatius* and *P. sativum* form a single-species complex comprised of two main races (*humile* and *elatius*), weedy forms and cultivated derivatives (*sativum*). They further implicate northern *P. humile* as the progenitor of *P. sativum.* The study thus supports the position taken previously by Berger (1928) and Davis (1970) that *Pisum* contains only two biological species, *P. sativum* L. and *P. fulvum.* Electrophoretic examination of albumin and globulin patterns for these taxa reveals limited protein differentiation that is also consistent with this conclusion (Waines 1975); however, these data are unable to clarify the role of *P. humile* in pea domestication.

Other researchers propose that *Pisum* is monospecific, suggesting that the differences observed for *P. fulvum* relative to the other pea taxa are more a matter of degree than the basis for a distinct species (Wellensiek 1925; Lamprecht 1966; Blixt 1974; Marx 1977). Davies (1974) and Marx (1977) further question the thoroughness of the evidence supporting *P. humile* as the wild progenitor of the cultivated pea. A comprehensive analysis of pea chloroplast DNA mutations (Palmer et al. 1985), however, corroborates the conclusion that most *P. sativum* cultivars are derived primarily from the northern *P. humile* populations.

Phylogenetic trees specify ancestry and descent based on a variety of criteria. Phylogenetic systematics, or cladistics, produces such trees, or cladograms, by constructing monophyletic groups exclusively based on shared derived characters (synapomorphies). Cladistics is an especially rigorous approach to systematics because it employs empirical methods to discover natural taxa and to establish genealogical relationships of evolutionary importance (see Wiley et al. 1991). It strictly eschews alternative approaches that are descriptive or narrative. The traits used to construct cladistic hypotheses may be morphological, anatomical, developmental, behavioral, physiological, biochemical or molecular in nature. In recent years, molecular genetics has provided a number of powerful new methodologies with which to construct phylogenetic associations, while avoiding some of the limitations of classical phylogenetic characters. Of particular note is the RAPD, or random amplified polymorphic DNA, procedure developed by Williams et al. (1990) [see also the related AP-PCR, or arbitrarily primed polymerase chain reaction, procedure developed by Welsh and McClelland (1990)].

RAPDs are new molecular markers for comparative analyses that are quick and easy to use, refractory to many environmental influences, and practically unlimited in number. The successful application of RAPDs to taxonomic and evolutionary studies has been documented in a variety of crop plants, including wheat, barley, rice, peanut, mustard and radish (see the review by Demeke and Adams 1994). Kazan et al. (1993) used RAPDs in a clustering analysis of four *Stylosanthes* species that corroborated morphological, cytological and isozyme findings. Tibayrenc et al. (1993) also documented the parity between RAPDs and multilocus enzyme electrophoresis in genetic and evolutionary studies of parasitic protozoa, confirming the suitability of RAPDs for certain phylogenetic reconstructions. Comparisons of RAPDs and RFLPs in determining genetic similarity among *Brassica oleracea* genotypes revealed that these marker systems provide equivalent levels of resolution for determining genetic relationships (dos Santos et al. 1994) Observed differences between the methods were attributed to sampling error rather than fundamental differences in how each approach reveals polymorphism. Other studies using cruciferous species to compare the resolving power of RFLP and RAPD markers in determining genetic relationships have shown RAPDs to be reliable for intraspecific comparisons and among closely-related species, but less reliable for higher taxonomic associations (Hallden et al. 1994), Thormann et al. 1994). We have further corroborated these findings for a variety of legume species, demonstrating both the reliability of RAPDs among closely-related taxa (such as those examined in the present study) and the limitations of RAPD data for producing

expected associations among more divergent taxa (unpublished results).

The purpose of the present study is to examine the relationships among wild and cultivated species of pea by including RAPD data with morphological characters and allozyme markers in a cladistic analysis of selected pea taxa. Our goal is to extend previous morphological, ecological, cytogenetical and chloroplast-based studies to clarify further the genetic affinities defining these taxa and to evaluate the potential progenitor(s) of the cultivated pea.

Materials and methods

Plant materials

Seeds for 14 wild pea populations were obtained through the courtesy of J. G. Waines (Department of Plant Sciences, University of California, Riverside). The populations are described by Ben-Ze'ev and Zohary (1973) and represent the three conventionally recognized wild taxa of pea: *Pisum fulvum* (isolates 701, 702, 703, 706, 707 and 708), *Pisum humile* (southern isolates 711, 712, 713, 714 and northern isolate 716), and *Pisum elatius* (isolates 721, 722 and 723). *Pisum sativum* L. cultivated inbred line A1078-234 was originally obtained through the courtesy of G. A. Marx and N. F. Weeden (New York State Agricultural Experiment Station, Geneva, N.Y.). *P. sativum* L. cv 'Progress #9' seed was purchased from Ferry-Morse (Mountain View, California). *P. sativum* L. cv 'Alaska' seed was purchased from J. Mollema and Son, Inc. (Grand Rapids, Michigan).

Extensive previous work with the three *P. sativum* cultivars has confirmed the absence of any detectable variability within each of the inbred lines. Preliminary studies of the wild pea populations have revealed minimal levels of intrapopulational variability. A representative survey of 184 plants sampled from all 14 populations for four morphological characters and four allozyme loci produced an average of 4% polymorphism per population (data not shown). RAPD-marker surveys involving six plants chosen randomly from each of the 14 populations revealed only minor variations in overall banding patterns among the replicated plants, and no variability among the replicated plants for RAPD bands selected for analytical purposes (see below).

Because of the uniformity exhibited by both wild and cultivated forms, individual plants were used to represent each inbred line and each population isolate studied, for a total of 17 plants. Thus, *P. fulvum*, southern and northern *P. humile*, *P. elatius* and *P. sativum* were represented by six, four, one, three and three populations, respectively. The computational requirements for the exact searches and bootstrapping replications performed for this study practically precluded the inclusion of additional taxa.

Morphological characters

Plants were scored for nine morphological characters (Table 1). Five of these are monogenic, as described by Blixt (1974), with recessive traits placed in parentheses: *D*, presence (absence) of maculum ring; *Fl*, absence (presence) of flecking; *I*, yellow (green) cotyledons; *Pl*, black (unpigmented) hilum; *R*, smooth (wrinkled) seeds. In each case, the dominant character was scored as (a) and the recessive character was scored as (b). Plants were also examined for variation in the degree of dentation of leaves and stipules, although no specific genetic basis was inferred. Leaf dentations (LD) were either markedly serrated (a), mildly serrated (b), or smooth (c). Stipules (SD) either had serrations completely covering them (a) or on the bottom and side only (b). Several plants displayed red coloration (RC) on the leaves and stipules as well, markings distinct from those associated with the maculum ring. Red markings were scored as either present (a) or absent (b). Ten different colors (a–j) of seed coat (or testa, TC) were also scored.

Table 1 Morphological, allozyme and RAPD data for 17 selected taxa of pea. See Materials and methods for character descriptions

Accession		Morphological characters	Allozymes	RAPDs															
		D Fl I Pl R LD SD RC TC	Aat-p Aat-m Skdh Acp-1 Idh Pgd-p Pgd-c	106	108	109	115	116	204	205	215	216	217	218	219	221	223	224	226
P. fulvum	701	b a a b a b b a b	1 2 3 3 1 1 1	+ + 0	− − +	−	− + 0	− −	− + +	− +	− + − − − −	−	+ − −	+ +	−	+ −	0	+ + − +	−
	702	b b a b a b b a c	1 1 4 3 1 1 1	− 0 +	− − +	−	+ + −	− −	− + +	− +	− + − − − +	−	+ + −	+ +	−	+ −	−	− + 0 +	−
	703	b a a b a b b a d	1 1 2 3 1 1 1	0 0 +	− − +	−	+ + −	− −	− + +	− +	− + − − + −	−	− + −	+ +	−	+ −	−	+ + 0 −	−
	706	b a a b a c b a b	1 1 4 3 1 1 1	− 0 0	− − +	−	+ − −	− −	− + +	− +	0 0 0 0 0 0	−	+ + −	+ +	−	+ 0	−	0 0 − +	−
	707	b a a b a b b a b	1 1 2 3 1 1 1	+ − +	− − +	−	+ 0 −	− 0	− + +	− 0	− + − − + −	−	+ + −	+ +	−	+ −	+	+ + 0 +	−
	708	b a a b a b b b b	1 1 1 3 1 1 1	− − −	− − +	−	+ 0 −	+ −	− + +	− +	− + − − − +	−	+ − −	+ +	−	+ −	+	+ − − −	−
Southern *P. humile*	711	b b a a a a a a e	1 1 6 1 1 1 1	− 0 −	0 + −	−	+ 0 −	+ +	− + +	+ −	− − 0 + − −	−	− + −	+ −	−	− +	−	+ − − 0	−
	712	b b a a a a a a e	2 1 6 1 1 1 1	− − −	0 − −	−	− 0 −	− +	0 + +	+ −	+ − 0 + − −	−	+ 0 −	+ 0	−	− +	−	+ − − 0	−
	713	b b a a b a a a e	1 1 6 1 1 1 1	+ − +	+ + −	−	+ + 0	+ +	+ + +	− −	+ − 0 + − −	−	+ + −	+ −	−	− +	−	+ − − 0	−
	714	b a a a a a a a a	1 1 5 1 1 1 1	+ − −	+ + −	−	+ − +	+ +	+ + +	− −	+ − 0 − − −	−	− + 0	+ +	−	− −	−	+ − − +	−
Northern *P. humile*	716	b b a a a a b b f	1 1 3 2 1 1 1	+ 0 +	+ + −	+	+ + +	0 0	+ − −	− −	+ − + − − −	−	+ 0 +	− −	+	− −	−	− − + +	−
P. elatius	721	a a a a a c b b g	1 1 5 2 1 1 1	+ 0 −	+ + −	+	+ + 0	+ +	0 − −	− −	− − − − − −	−	+ + +	− −	+	0 −	−	− − + +	−
	722	a a a a a b b b h	1 1 6 2 2 1 1	0 + 0	+ + −	+	+ − −	+ 0	− − −	0 −	− − − − − −	−	0 + +	− −	+	0 −	−	− − + +	−
	723	a a a a a b b b g	2 1 5 2 2 1 1	0 + −	0 + −	+	+ + +	+ −	− − −	0 −	+ − 0 + − −	−	+ + +	− −	+	+ −	−	− − + +	+
P. sativum cv	A1078-234	a b b b b c 0 b j	2 1 3 3 3 2 2	0 + +	− + −	0	+ + −	+ 0	+ − −	− −	− + + + − −	+	+ + +	− −	+	+ −	−	− − + +	+
	Alaska	b a b b a c b b i	2 2 5 2 2 2 2	+ + +	+ + −	+	+ + +	+ 0	+ − −	− −	− + − − − −	−	+ + +	0 −	+	+ −	−	− − + +	+
	Progress #9	b b b b b c b b i	2 1 5 2 2 1 1	− + +	− + −	0	0 + +	+ 0	0 − −	− −	− + − − − −	+	+ + +	− −	+	+ −	−	− − + +	+

Allozymes

Seven allozyme markers were characterized by horizontal starch-gel electrophoresis generally as described by Weeden and Marx (1984, 1987) and as performed by our laboratory in previous studies (Polans et al. 1985, 1986, 1991; Polans and Folta 1992; Folta and Polans 1992): aspartate amino transferase (*Aat-p* and *Aat-c*), acid phosphatase (*Acp-1*), isocitrate dehydrogenase (*Idh*), 6-phosphogluconate dehydrogenase (*Pgd-p* and *Pgd-c*) and shikimic dehydrogenase (*Skdh*). Bands for each assay are scored 1–6 in Table 1 beginning with the most anodal marker.

RAPDs

DNA for templates was extracted from the leaves of individual plants using a rapid, small-scale CTAB procedure (Saghai-Maroof et al. 1984). Amplification reactions were performed in 25-µl volumes in 0.5-ml microcentrifuge tubes using Perkin Elmer (Cetus) DNA Thermal Cyclers generally as described by Williams et al. (1990). Reaction mixtures contained 100 ng of DNA template, reaction buffer (20 mM Tris-HCl pH 8.55, 16 mM ammonium sulfate, 2.5 mM magnesium chloride and 150 µg/ml bovine serum albumin), 50 µM each of dATP, dCTP, dGTP and dTTP, 0.2 µM of primer (a 10-base oligonucleotide, Table 2), and 1.0 unit of *Taq* DNA polymerase. A drop of mineral oil was added to each tube to seal the reaction volume and to prevent evaporation. A second drop of mineral oil was added to each thermal-cycler well to provide proper contact and temperature exchange between the tube and the thermal-cycler chamber. Amplifications required 45 complete cycles: 1 min at 94 °C (denaturing step), 1 min at 35 °C (annealing step) and 2 min at 72 °C (elongation step) per cycle.

Amplification products were separated electrophoretically in a 1.4% agarose gel using the Tris-Borate-EDTA (TBE) buffer system for approximately 1 h at 125–150 V (approximately 9–11 V/cm). Fractionated bands were detected by ethidium-bromide fluorescence under UV light and were photographed using a MP-4 Polaroid Land camera and Royal Pan film (see Fig. 1). Bands were scored (Table 1) as present (+) or absent (−), and band lengths were determined by comparison with a 1-kb ladder (BRL) included in each gel as a size standard. Missing data were represented as (0).

RAPD patterns can include weak or minor bands that are especially vulnerable to variations in protocol. It is widely accepted that procedure optimization and consistent reaction conditions are important elements in reducing these fluctuations and ensuring reproducible results (e.g., see Rafalski and Tingey 1993). Penner et al. (1993) tested the reproducibility of RAPDs by comparing the amplification products of the same primer/template combinations produced in different laboratories. They demonstrated the general reliability of the method, observing that some laboratories generated different size ranges of DNA fragments, variations attributed principally to differences in thermocyclers.

The selection of polymorphic bands for inclusion in the current data set was based on both band characteristics and reliability, but was otherwise random. Band characteristics included clarity, signal strength, and resolution from nearby bands. Reliability was directly confirmed in some cases by replicated runs. It was also tested in several RAPD-marker surveys in which six plants were chosen randomly from each of the 14 wild pea populations and compared for variations in banding patterns after amplification in different thermocyclers. In these tests, samples of each individual plant were run in replicative fashion using both a Perkin Elmer DNA Thermal Cycler and an older Perkin Elmer Cetus DNA Thermal Cycler located in our laboratory and two different PTC-100 (MJ Research, Inc.) units located in another laboratory. While some very minor band variation was detected, overall banding patterns were uniform from machine to machine (as well as among samples from the same population). There was no variability detected among replicates for RAPD bands that had been chosen as characters for parsimony analysis. When similar bands selected for genetic anaysis were examined, they were also generally reproducible, appearing consistently throughout parental, F_1 and F_2 generations in accordance with Mendelian expectations.

Unlike DNA fingerprinting or genetic-distance measures that may include all the component bands of a RAPD profile in a complete analysis, cladistic treatments require only a selection of informative bands to provide the shared derived characters upon which monophyletic groups and phylogenies may be constructed.

Cladistic analysis

Cladistic analyses were performed using the PAUP (version 3.1.1) computer package (Swofford 1993). Data sets were entered as qualitative (binary or multistate) characters that are freely reversible and unordered. Data were also scaled so that each character was equally weighted irrespective of the number of character states. Parsimony analysis was first performed using a heuristic method of searching in combination with tree bisection-reconnection, a branch swapping option that is effective in finding shorter trees. Equally parsimonious trees were then sorted into consensus trees to confer conservative results. Many variations of this standard approach were also implemented to achieve a thorough evaluation of the data, including branch-and-bound searches and bootstrapping.

Results

Cladistic analyses were performed for 17 pea taxa as described in Materials and methods. Each plant was scored for nine morphological characters, seven allozymes, and 38 RAPD bands (see Table 1, Fig. 1). The

Table 2 RAPD primer sequences and corresponding band sizes

Primer	Sequence	%GC	Band size (bp)
106	5′-ACG GGG ACT A-3′	60	1080, 655 555
108	5′-AAG AGG GCA T-3′	50	1040, 615, 480
109	5′-CCA GAG TGA T-3′	50	515
115	5′-CGG CCC CTG T-3′	80	1020, 540, 460
116	5′-TGG TCA CAG A-3′	50	555, 420
204	5′-TTC GGG CCG T-3′	70	370, 320, 220
205	5′-CGG TTT GGA A-3′	50	320, 200
215	5′-TCA CAC GTG C-3′	60	590, 505, 430, 380, 340, 320
216	5′-CAT AGA CTC C-3′	50	215
217	5′-ACA GGT AGA C-3′	50	520, 345, 275
218	5′-CTC AGC CCA G-3′	70	455, 355
219	5′-GTG ACC TCA G-3′	60	500
221	5′-CCC GTC AAT A-3′	50	300, 265
223	5′-GAT CCA TTG C-3′	50	515
224	5′-TCT CCG GTA T-3′	50	630, 540, 460, 320
226	5′-GGG CCT CTA T-3′	60	465

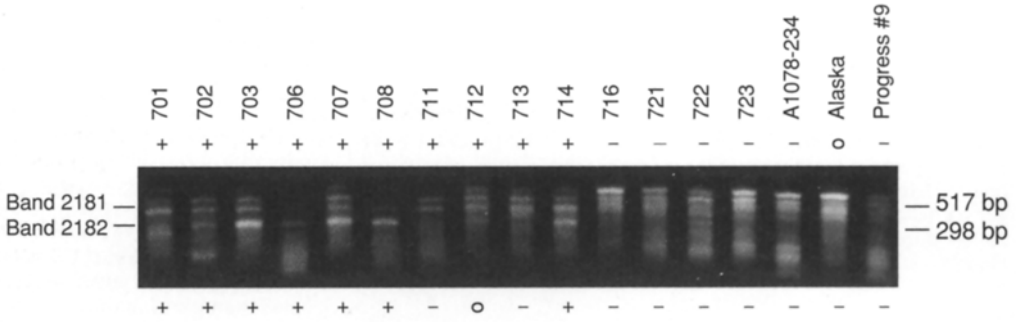


Fig. 1 RAPD band patterns for 17 pea taxa using primer 218. Scores for bands 2181 and 2182 appear on the top and bottom of the photograph, respectively, where +, − and 0 represent band presence and absence, and missing data, respectively. Flanking size standards are designated on the right-hand side of the photograph

multistate data were scaled such that each unordered character was weighted equally and the six *P. fulvum* population isolates were designated as an outgroup in keeping with the established distinction between *P. fulvum* and the other three pea taxa (see Introduction).

When the 16 morphological traits and allozyme markers were analyzed independently of the RAPD data set, a branch-and-bound search generated 117 most-parsimonious trees and the corresponding 50% majority-rule consensus cladogram shown in Fig. 2 a. Despite the relatively small number of characters available for these comparisons, the resulting tree corroborates past hypotheses describing the relationships in the genus *Pisum*. The *P. fulvum* isolates (701, 702, 703, 706, 707, 708) are retained as a monophyletic outgroup to the more closely associated *P. humile* (711, 712, 713, 714, 716), *P. elatius* (721, 722, 723) and *P. sativum* (A1078-234, Alaska, Progress #9) isolates which constitute an ingroup of three equally related and fully differentiated clades. The *P. humile* clade includes northern *humile* 716 as a sister taxon to the southern *humile* group, which consists of both a 711, 712 and 713 multifurcation and sister taxon 714. *P. elatius* isolates 722 and 723 form a sister group to *elatius* 721. A heuristic bootstrap search of these same data involving 1000 replications produced a 50% majority-rule consensus cladogram (Fig. 2 b) that continues to group the southern *humile*, *elatius* and *sativum* as described in the preceding branch-and-bound search. The six *fulvum* isolates, however, are not retained as a monophyletic clade; instead, both *P. fulvum* and northern *humile* 716 comprise a polytomy.

When the 38 RAPD bands were analyzed independently of the morphological traits and allozyme markers, a branch-and-bound search generated 44 most-parsimonious trees and the corresponding 50% majority-rule consensus cladogram shown in Fig. 3 a. As with the findings for Fig. 2 a, this tree generally supports the established relationships among the species of *Pisum*. Once again, the *fulvum* isolates aggregate as a monophyletic outgroup. The remaining 11 taxa constitute a monophyletic ingroup consisting of a southern *humile* clade and a second clade including the northern *humile* isolate, the *elatius* isolates and the *sativum* cultivars. *P. elatius* 721 and 722 are unresolved, whereas *elatius* 723 serves as a sister taxon to a monophyletic group comprising *sativum* and northern *humile* 716. A heuristic bootstrap search of these same data involving 1000 replications produced a 50% majority-rule

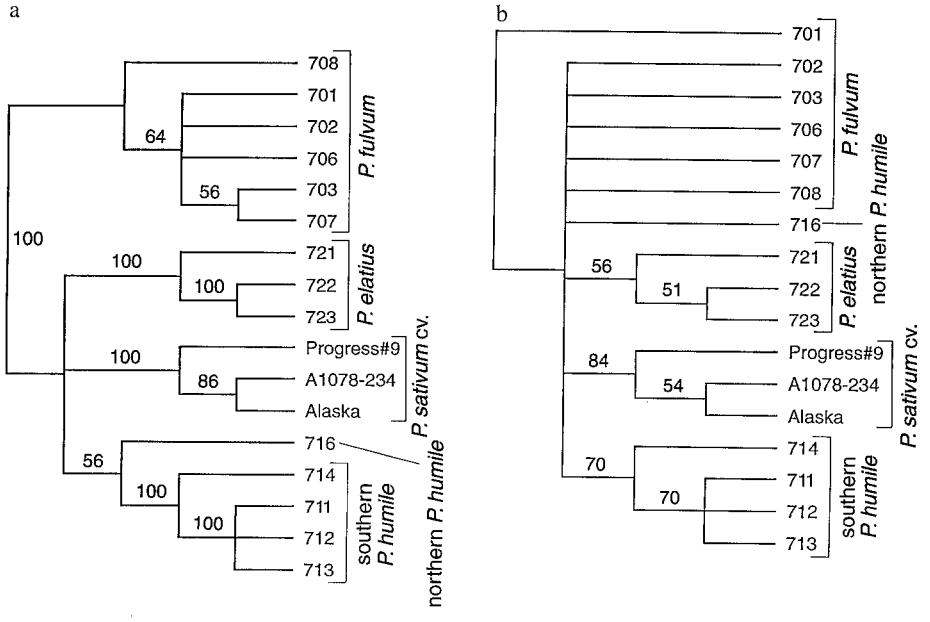


Fig. 2a,b The 50% majority-rule consensus cladograms of 17 pea taxa based on nine morphological characters and seven allozyme marker loci. **(a)** A branch-and-bound search (117 minimal trees, scaled trees length = 2664, CI = 0.54, RI = 0.72). **(b)** A heuristic bootstrap search (1000 replications).

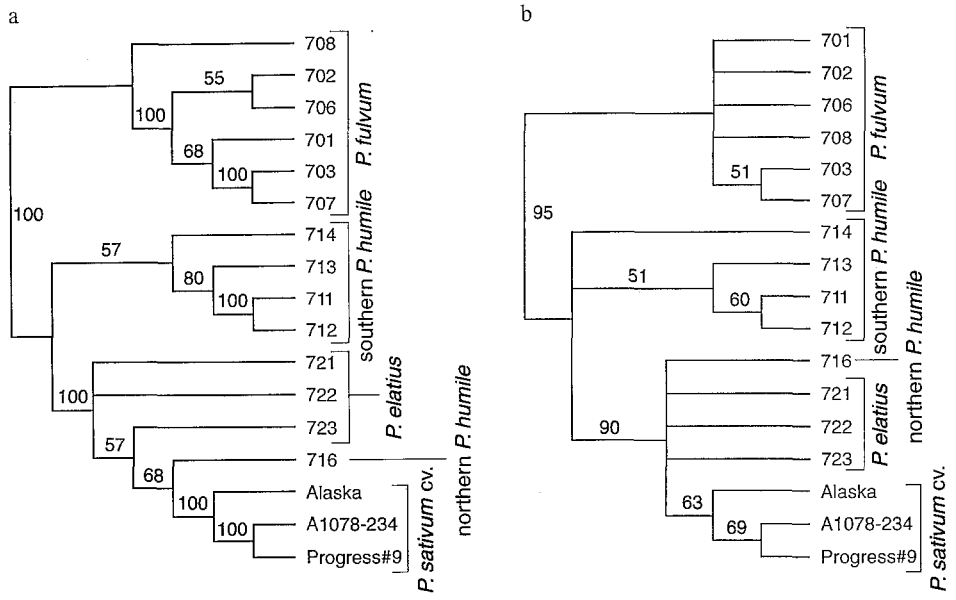
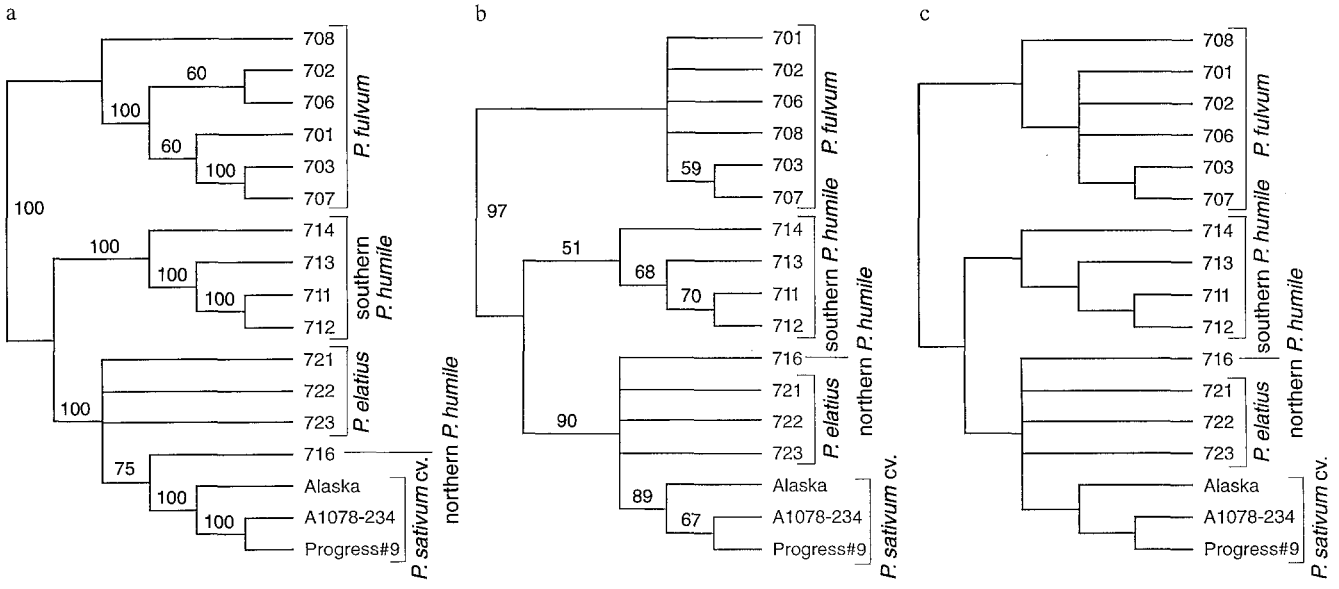


Fig. 3a,b The 50% majority-rule consensus cladograms of 17 pea taxa based on 38 RAPD markers. **(a)** A branch-and-bound search (44 minimal trees, scaled tree length = 6480, CI = 0.53, RI = 0.76). **(b)** A heuristic bootstrap search (1000 replications).

consensus cladogram (Fig. 3 b) in which southern *humile* 714 and both northern *humile* 716 and the *elatius* isolates are unresolved with respect to the associations produced by the preceding branch-and-bound search.

When all 54 morphological, allozyme and RAPD scores were combined, a branch-and-bound search generated 20 most-parsimonious trees and the corresponding 50% majority-rule consensus cladogram shown in Fig. 4a. The resulting composite cladogram is nearly identical topologically to Fig. 3a, the cladogram generated exclusively with RAPD data. The only exception is *elatius* 723, which joins the remaining two *elatius* isolates to form an unresolved group in Fig. 4a. A heuristic bootstrap search of the same data involving 1000 replications produced a 50% majority-rule consensus cladogram (Fig. 4b) in which northern *humile* 716 joins the unresolved *elatius* group. While Fig. 4a and b clearly reflect the influence of the larger RAPD data set on the composite analysis, the contribution of the morphological and allozyme data becomes more evident in the corresponding strict consensus cladogram shown in Fig. 4c in which the *fulvum* outgroup forms a hierarchial arrangement identical to that found in Fig. 2a.

Fig. 4a–c The 50% majority-rule and strict consensus cladograms of 17 pea taxa based on 54 combined morphological, allozyme and RAPD scores. **(a)** The 50% majority-rule consensus cladogram of a branch-and-bound search (20 minimal trees, scaled tree length = 9369, CI = 0.52, RI = 0.74). **(b)** The 50% majority-rule consensus cladogram of a heuristic bootstrap search (1000 replications). **(c)** Strict consensus cladogram of the branch-and-bound search described in (a)



Discussion

A cladistic analysis of *Pisum*

The extensive genetic variability commonly associated with pea as the classical Mendelian organism is the basis for its multiplicity of forms. Systematists have often interpreted this variation by collectively recognizing many distinct pea species in a variety of different relationships (see Marx 1977). Genetical, cytogenetical, hybridization and biochemical studies have inspired several systematic interpretations as well (see Waines 1975 for a review). Most investigators currently recognize only one or two legitimate species, assigning the wealth of diversity to taxa of subspecific rank (Marx 1977). Berger (1928) and Davis (1970) accept only two pea species, *P. fulvum* and *P. sativum*, a view supported by the comprehensive study of Ben Ze'ev and Zohary (1973). Wellensiek (1925), Lamprecht (1966), Blixt (1974) and Marx (1977) regard *Pisum* as monospecific, emphasizing the overall genetic compatibility among taxa. Although the origin and ancestry of the cultivated pea remains unresolved, a wild counterpart never having been located, Ben Ze'ev and Zohary (1973) and Palmer et al. (1985) present additional evidence that supports (the northern isolates of) *P. humile* as the probable progenitor. Davies (1974) and Marx (1977) question this conclusion.

The cladistic analyses performed in the present investigation using 54 morphological, allozyme and RAPD characters (see Fig. 4a–c) support the close relationships among the wild species and cultivars of pea proposed by most recent systematic studies. In each case, *P. fulvum* is clearly the most distinct of the pea taxa, while *P. humile*, *P. elatius* and *P. sativum* form a monophyletic group. Within the *humile-elatius-sativum* ingroup, the southern *P. humile* isolates form a clade that is distinct from the remaining taxa, including northern *P. humile*. With respect to the northern *humile-elatius-sativum* clade, the cultivars form a monophyletic group, while the *elatius* isolates comprise a polytomy. The precise placement of northern *P. humile* is also less clear. The 50% majority-rule consensus cladogram derived from the branch-and-bound search (Fig. 4a) reveals that 75% of the equally minimal trees assign northern *humile* 716 as the sister taxon to the cultivars, supporting the position taken by Ben Ze'ev and Zohary (1973) and Palmer et al. (1985). The 50% majority-rule consensus cladogram derived from the bootstrap search (Fig. 4b) places northern *humile* 716 with the unresolved *elatius* isolates. The contrasting information provided by the minimal tree and resampling procedures regarding the location of 716 necessitates following the more conservative assignment for northern *P. humile* shown in the strict consensus cladogram (Fig. 4c) until a more thorough resolution becomes possible (see below). Because strict consensus requires that 100% of the contributing trees support assignment to a clade, northern *humile* 716 is currently included with the unresolved *elatius* isolates (consistent with the results of the bootstrap search).

The results summarized in Fig. 4c underscore a strong affinity among the selected cultivars which are less closely related to southern *P. humile* than they are to the unresolved *elatius* and northern *humile* taxa. Evidence that southern *P. humile* and *P. elatius* share a chromosome 4/6 translocation, while northern *P. humile* possesses the standard *P. sativum* karyotype, has suggested that southern *P. humile* and *P. elatius* are closely related and that northern *P. humile* is the probable progenitor of the cultivated pea (Ben Ze'ev and Zohary 1973). In accordance with the findings of the current study, however, the recent common ancestor of the three cultivars is shared with both northern *P. humile* and *P. elatius*. In this regard, it is important to note that cultivars do not derive directly from extant wild taxa, but from their antecedents; wild and cultivated forms both having evolved since the time of cultivation. Thus, the cladograms reveal genetic affinities between *P. sativum* and its nearest wild relative(s) based on shared derived characters, and they include hypothetical common ancestors to establish closely shared history and germplasm. It must also be emphasized that the cultivars selected for this study do not represent a comprehensive sampling of domesticated peas. Additional cultivars must be introduced to these analyses to assess in a rigorous fashion whether all domesticated peas derive from a single wild source or whether they are derived from several wild taxa and/or are the products of multiple introgression events.

The cladogram shown in Fig. 4c (and discussed above) is generally consistent with a number of more descriptive systematic treatments. Unlike these, however, the cladogram is based on an estimate of genealogy rather than similarity, and it allows classification based on evolutionary principles that can be interpreted using a variety of hierarchial schemes, including the classical system of Linnaeus (see Wiley et al. 1991). The obvious and undisputed affinity between the northern and southern *P. humile* varieties, despite their placement in separate clades, strongly supports their continued assignment to the same species. In keeping with the hierarchial logic of the cladogram, their conspecificity requires that members of the southern *P. humile* monophyletic group and the northern *P. humile-P. elatius-P. sativum* monophyletic group belong to the same species as well. A case may be made, however, for intraspecific rankings distinguishing *P. sativum*, southern *P. humile* and both northern *P. humile* and *P. elatius* (based, in particular, on Fig. 4a and the other minimal tree analyses). Whether the degree of divergence displayed by *P. fulvum* warrants separate species status cannot be determined by the present analysis alone. Because the entire ingroup is monospecific, the *P. fulvum* monophyletic sister group could be included with it as part of a single-species complex in a logically consistent manner. The relative degree of interfertility it exhibits with the other pea taxa

supports this inclusion, while its overall distinction, particularly in nature, militates against its inclusion.

The use of RAPDs as cladistic characters

Systematic analyses are sometimes limited by the number of characters available for comparative purposes, especially among closely-related organisms with few readily discernible differences. Molecular markers, such as the RAPDs used in the present study, provide valuable supplements to important, but often small, data sets comprised of more traditional characters. The 16 morphological traits and allozyme markers available for the current analysis of *Pisum* clearly organize the 17 taxa of interest into the four established taxonomic groupings depicted in Fig. 2a. The six *P. fulvum* isolates form a completely undifferentiated monophyletic sister group, while the ingroup is equally divided into *P. humile*, *P. elatius* and *P. sativum*. The precision of the intraspecific groupings given the relatively small number of characters is impressive, but perhaps not entirely unexpected given the role morphology has played in most previous (descriptive) evaluations of pea systematics. Aside from the unambiguous assignment of the *P. fulvum* isolates as a monophyletic sister group, the interspecific relationships among these taxa are comparatively uninformative.

Given the relative size of the RAPD data set, it is also not entirely surprising that the 38 RAPD markers generate cladograms (Fig. 3a and b) that bear striking similarities to the composite cladograms shown in Fig. 4 a and b, respectively. It is interesting to speculate whether additional RAPD data might ultimately place all the southern *P. humile* isolates within a single independent clade in a more convincing fashion than depicted in Fig. 3a and b, or whether more RAPDs might be a key to resolving the relationship between northern *P. humile* and *P. elatius* with respect to the origin of cultivated peas. The successful application of RAPDs to these and other issues rests with their relative value as cladistic characters. Do morphological traits, which may be shaped to a greater degree by selective pressures, provide different or more valuable systematic information than RAPDs and similar types of molecular data; or, conversely, is it possible that morphological traits and allozyme markers actually characterize taxa in a biased fashion and do not reflect evolutionary relationships as accurately? Should either supposition be correct, combined character sets would need to be weighted in accordance with their composition. Because all synapomorphies are considered to be equally informative in parsimony analysis, it is a principal tenet of cladistics to avoid weighting or pre-judging characters. Given that all traits are roughly equivalent for cladistic purposes, then, in theory, character types that are available in greater numbers, such as RAPDs, should be able to augment characters available only in limited number.

It is arguable that the most reasonable initial approach to cladistic analyses is to include as many character types as possible in order to sample the genome in a representational fashion and to reduce biases in the data. Because RAPDs are abundant and reflect largely unselected genetic alterations, they provide a useful complement to most traditional systematic characters. In future cladistic studies of pea, we anticipate increasing the number of RAPD characters included in the analyses to evaluate any quantitative effect additional RAPD data points may have on informativeness. By increasing the number of RAPD characters and by also increasing the number of pea cultivars in future studies, we anticipate resolving more completely the close affinity among northern *P. humile*, *P. elatius* and *P. sativum*, as well as the origin(s) of domesticated peas.

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References

Ben-Ze'ev N, Zohary D (1973) Species relationships in the genus *Pisum*. Israel J Bot 22:73–91

Berger A (1928) Peas. In: Hedrick UP, Hall FH, Hawthorn LR, Berger A (eds) Vegetables of New York. Report of the NY State Agricultural Experiment Station, pp 1–132

Blixt S (1974) The pea. In: King RC (ed) Handbook of genetics, vol 2. Plenum Press, New York, pp 181–221

Boisser E (1872) Flora Orientalis, vol 2, pp 622–624

Davies DR (1974) Peas. In: Simmonds NW (ed) Evolution of crop plants. Longman, New York, pp 172–174

Davis PH (1970) Flora of Turkey, vol 3. Edinburgh University Press, pp 370–373

Demeke T, Adams RP (1994) The use of PCR-RAPD analysis in plant taxonomy and evolution. In: Griffin HG, Griffin AM (eds) PCR technology: current innovations. CRC Press, Boca Raton, Florida, pp 179–191

Folta KM, Polans NO (1992) Evidence supporting the revision and integration of the pea chromosome-7 linkage map. *Pisum* Genet 24:44–47

Hallden C, Nilsson N-O, Rading IM, Sall T (1994) Evolution of RFLP and RAPD markers in a comparison of *Brassica napus* breeding lines. Theor Appl Genet 88:123–128

Kazan K, Manners JM, Cameron DF (1993) Genetic variation in agronomically important species of *Stylosanthes* determined using random amplified polymorphic DNA markers. Theor Appl Genet 85:882–888

Lamprecht H (1966) Die Entstehung der Arten und höheren Kategorien. Springer Verlag, Vienna

Marx GA (1977) Classification, genetics and breeding. In: Sutcliffe JF, Pate JS (eds) Physiology of the garden pea. Academic Press, New York, pp 21–43

Palmer JD, Jorgensen RA, Thompson WF (1985) Chloroplast DNA variation and evolution in *Pisum:* patterns of change and phylogenetic analysis. Genetics 109:195–213

Penner GA, Bush A, Wise R, Kim W, Domier L, Kasha K, Laroche A, Scoles G, Molnar SJ, Fedak G (1993) Reproducibility of random amplified polymorphic DNA (RAPD) analysis among laboratories. PCR Methods Appl 2:341–345

Polans NO, Folta KM (1992) The mapping of *Pea2* supports the assignment of *Vc-5* and *rb* to a specific region of chromosome 3. *Pisum* Genet 24:79–81

Polans NO, Weeden NF, Thompson WF (1985) Inheritance, organization, and mapping of *rbcS* and *cab* multigene families in pea. Proc Natl Acad Sci USA 82:5083–5087

Polans NO, Weeden NF, Thompson WF (1986) Distribution, inheritance and linkage relationships of ribosomal DNA spacer length variants in pea. Theor Appl Genet 72:289–295

Polans NO, Folta KM, Elliott RC, Thompson WF (1991) The inheritance and linkage mapping of *Ferredoxin-1* in pea. J Hered 82:259–261

Rafalski JA, Tingey SV (1993) Genetic diagnostics in plant breeding: RAPDs, microsatellites and machines. Trends Genet 9:275–280

Saghai-Maroof MA, Soliman KM, Jorgensen RA, Allard RW (1984) Ribosomal DNA spacer length polymorphism in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc Natl Acad Sci USA 81:8014–8018

Santos JB dos, Nienhuis J, Skroch P, Tivang J, Slocum MK (1994) Comparison of RAPD and RFLP genetic markers in determining genetic similarity among *Brassica oleracea* L. genotypes. Theor Appl Genet 87:909–915

Swofford DL (1993) PAUP: Phylogenetic Analysis Using Parsimony, Version 3.1. Computer program distributed by the Illinois Natural History Survey, Champaign, Illinois

Thormann CE, Ferreira ME, Camargo LEA, Tivang JG, Osborn TC (1994) Comparison of RFLP and RAPD markers to estimating genetic relationships within and among cruciferous species. Theor Appl Genet 88:973–980

Tibayrenc M, Neubauer K, Barnabe C, Guerrini F, Skarecky D, Ayala FJ (1993) Genetic characterization of six parasitic protozoa: parity between random-primer DNA typing and multilocus enzyme electrophoresis. Proc Natl Acad Sci USA 90: 1335–1339

Waines JG (1975) The biosystematics and domestication of peas (*Pisum* L.). Bull Torrey Bot Club 102:385–395

Weeden NF, Marx GA (1984) Chromosomal locations of twelve isozyme loci in *Pisum sativum*. J Hered 75:365–370

Weeden NF, Marx GA (1987) Further genetic analysis and linkage relationships of isozyme loci in the pea. J Hered 73:153–159

Wellensiek SJ (1925) Genetic monograph on *Pisum*. Bibliogr Genet 2:343–476

Welsh J, McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 18:7213–7218

Wiley EO, Siegel-Causey D, Brooks DR, Funk VA (1991) The Compleat Cladist. The University of Kansas Museum of Natural History Special Publication, Lawrence, Kansas

Williams JGK, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 18:6531–6535