# ITS sequence variation in selected taxa of *Pisum*

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Most investigators currently recognize only one or two legitimate species of *Pisum* (5). These usually include *P. fulvum* and a *P. sativum* complex comprised of two main races (*humile* and *elatius*), weedy forms and cultivated varieties (2). Two distinct isolates of *humile* have also been described, a "northern" form that possesses the standard *sativum* karyotype and a "southern" form that exhibits the same chromosomal translocation as *elatius*. Despite these distinctions, there is an unmistakably close genealogical affinity among all the wild and cultivated taxa of pea (4,5). One approach to characterizing the nature and degree of these genetic affinities among the various pea taxa is by comparing the nucleotide sequences of their ribosomal DNA.

Nuclear ribosomal DNA (nrDNA) is organized as individual chromosomal units that are repeated thousands of times in most higher plant genomes. Each of these units contains the three genes that encode the 18S, 5.8S and 26S ribosomal RNA subunits, as well as several different spacer DNA regions. The nucleotide sequence variation found in both of the internal transcribed spacer regions (ITS-1 and ITS-2, Fig. 1) is used extensively for the systematic analysis of closely related taxa, at least in part due to the speedy rate of evolutionary change characterizing these DNA regions (1). In this preliminary study, ITS-1 and ITS-2 DNA sequence variation is assessed for five pairs of wild and cultivated pea taxa selected to approximate the range of *Pisum*. The goal of the exercise is to examine the similarity of the sequences within paired accessions, the overall level of genetic variation found across the entire genus, and the topological relationships established among the five selected groups of taxa.

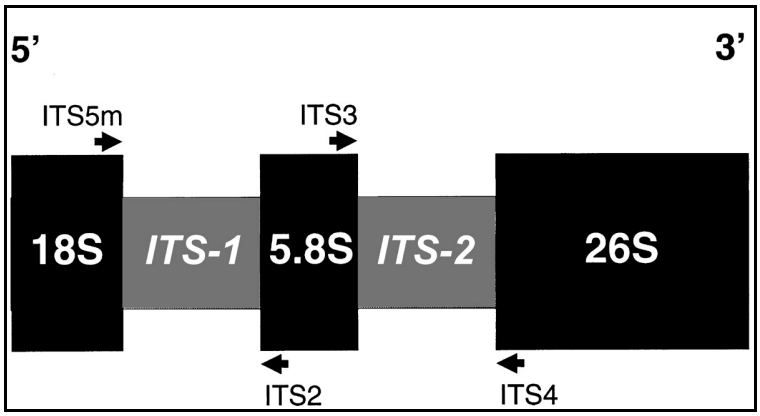


*Fig. 1. The three coding and two internal transcribed spacer regions of the nuclear ribosomal DNA repeat unit of a typical angiosperm (not drawn to scale). Arrows indicate approximate locations of the four primers used for PCR amplification.*

**Materials and Methods**

DNA for the ITS procedure is extracted from the leaves of the individual pea plants listed in Table 1 using a CTAB protocol (7). Primers ITS2, ITS3 and ITS4 are described elsewhere (10), as are the PCR amplification cycle and the modified ITS5m primer (8). Gel purification (3) precedes DNA sequencing with an Applied Biosystems model 373 DNA sequencer. PCR is performed with Perkin Elmer (Cetus) DNA thermal cyclers. Forward and reverse DNA sequences are compared to resolve ambiguities using PC Gene software and the resulting sequences aligned with the Clustal X computer program. Sequence data are analyzed using the PAUP computer package (9).

**Results and Discussion**

The pea ITS-1 and ITS-2 regions examined in this study contain 298 and 349 alignable base pairs (bp), respectively, totaling 647 bp for each of the plants analyzed. Four ambiguous pyrimidine sites are denoted by the IUPAC/IUB symbol "Y." Of the 647 ITS bp sequenced for each individual plant, 629 (>97%) of these

**Table 1. Variable ITS sites for 10 wild and cultivated taxa of pea.**

| | | Nucleotide Position* | |
|---|---|---|---|
| | | ITS-1 | ITS-2 |
| | | 1111111112222 | 11223 |
| | | 0112333490346 | 34080 |
| Taxon | Accession | 3584259508407 | 78818 |
| *P. fulvum* Sibth.&Sm. | 701 | GTTGGGACCGATG | TTTAG |
| | 702 | GTTGGGACCGATG | TTTAG |
| *P. sativum* L. var. *humile* | 712 | ATCAGAGCTACCA | CCAAC |
| Boiss.&Noe–(southern) | 713 | ATCAAAGCTACCA | YCAAC |
| *P. sativum* L. var. *humile* | 716 | GTCGGGGCTACCA | CCATC |
| Boiss.&Noe–(northern) | JI1794 | GTCGGGGCTACCA | CCATC |
| *P. sativum* L. var. *elatius* | 721 | GCCGTAGYTACCA | CCATC |
| Bieb. | 722 | GCCGTAGYTACCA | CCATC |
| *P. sativum* L. cv. 'Alaska' | | ACCGAAGYTACCA | CCATC |
| 'Austrian Winter' | JI711 | ACCGAAGCTACCA | CCATC |

***In the 5'->3' direction (see Fig. 1), beginning with those bases nearest primer ITS5m (for ITS-1) or primer ITS3 (for ITS-2). Complete sequences are available through GenBank for ITS-1 and ITS-2, respectively, as follows: 701(AF305582, AF305920), 702(AF305583,AF305921), 712(AF305584,AF305922), 713(AF305585,AF305923), 716(AF305586,AF305924), JI1794(AF305587,AF305925), 721(AF305588,AF305926), 722 (AF305589,AF305927), Alaska(AF305202,AF305928), JI711(AF305590,AF305929).**

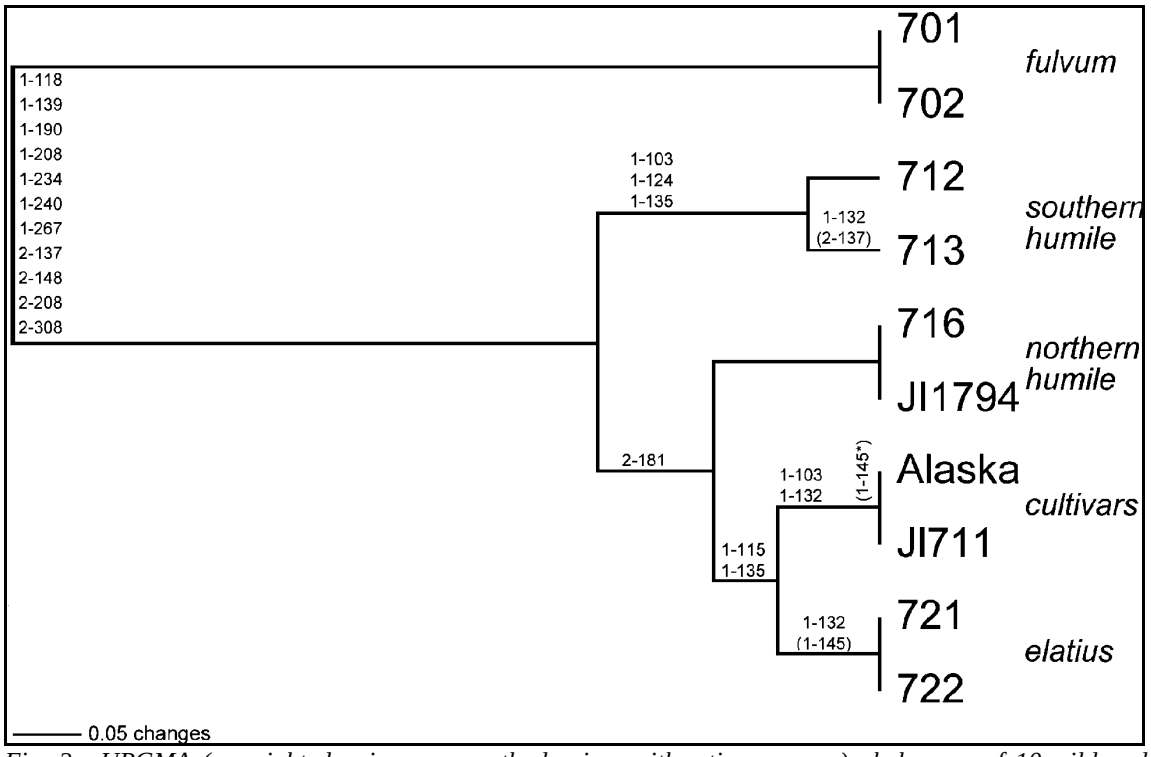


*Fig. 2. UPGMA (unweighted pair group method using arithmetic averages) phylogram of 10 wild and cultivated pea taxa based on 18 variable ITS sites. Nucleotide substitutions (as shown in Table 1) are located on the appropriate branches, the first number of the designation denoting whether the variant is derived from the ITS-1 or ITS-2 region and the other three numbers denoting the assigned nucleotide position within either spacer region. Parentheses indicate an ambiguous substitution, and the asterisk indicates an ambiguous substitution within the cv. Alaska terminus. Branch length distances are drawn with reference to the 0.05 length standard.*

sites are constant among the 10 pea taxa. Only 18 of the sites are polymorphic (and only 17 are parsimony informative). Despite its smaller size, ITS-1 contains 13 of the polymorphic sites, as compared with the five found for ITS-2 (Table 1). These numbers attest persuasively to both the very close evolutionary relationships that must exist within the genus and the limited ITS information available with which to differentiate pea taxa. By contrast, when *Vicia montbrettii* (GenBank AF228075), a single taxon representing a sister genus to *Pisum*, is included in the data set for comparative purposes, more than three times as many polymorphic sites become available.

A standard UPGMA distance analysis of the data is presented in Fig. 2. *P. fulvum*, phylogenetically the most diverged from the cultivars, is assigned as the outgroup. Actual nucleotide substitutions (24 in all) are placed on the phylogram branches, with approximately one-half of these base changes supporting the differentiation of *fulvum* from the larger *sativum* ingroup. Within *sativum*, all eight accessions pair according to their traditional taxonomic designations. The selected pairs of northern *humile* and *elatius* each displays completely identical nucleotide sequences (at 647 sites), as does the pair of *fulvum* lines comprising the outgroup. The cultivars differ at only one ambiguous site, and the southern *humile* differ at only one ambiguous and one unambiguous site. It should be noted here that accession JI1794, listed by the John Innes Institute simply as *P. humile*, seems to possess the morphological features of a northern *humile*. It is thus identified in this study in accordance with its perfect ITS sequence identity with northern *humile* 716.

According to the UPGMA analysis depicted in Fig. 2, *elatius* is the closest taxon to the cultivated *sativum*, followed by northern *humile.* Southern *humile* is the taxon within the ingroup most distinct from the cultivars. The close clustering of the northern and southern forms of *humile* would seem intuitive, while their resolution in the phylogram supports their established distinctiveness as well. Parsimony analyses of this same small data set do not resolve these relationships as thoroughly as the distance model, although they produce many of the same branches and much of the same topology. Only the node joining the *sativum* ingroup with the *fulvum* outgroup receives strong (100%) support using parsimony methods. Neither branch-and-bound nor bootstrap searches generate high clade values among the four ingroup taxa; the single exception being a 77% bootstrap value at the node joining *elatius* and the cultivars.

It has been postulated that northern *humile*, rather than *elatius*, is the closest wild progenitor of the cultivated pea, based in part on a shared chromosomal translocation (2) and detailed chloroplast studies (6). This compelling relationship, however, is inconsistent with the UPGMA findings presented in Fig. 2. Northern *humile* is even further removed from the cultivars in a number of the (fourteen) most parsimonious trees, in these instances reversing its position in Fig. 2 with that presently shown for southern *humile*. Irrespective of the relative phylogenetic positions of northern and southern *humile*, neither this ITS data set, nor other more extensive data sets (not shown), support northern *humile* as the taxon closest to the cultivars.

**Conclusion*s***

ITS sequence variation for the selected taxa of this study suggests: 1) very close genetic affinities throughout *Pisum*, with *P. fulvum* exhibiting the greatest degree of divergence, 2) support for the established taxonomic categories of the genus based upon identical or near identical sequences within group pairs, 3) the assignment of JI1794 as a "northern" *humile*, 4) the validity of northern and southern *humile* as closely-related, but distinct, lines, 5) the apparent independent evolution of a pea chromosomal translocation and 6) a close relationship between *elatius* and the cultivated *sativa.*

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