Phylogenetic relationships among *Fagopyrum* species revealed by the nucleotide sequences of the ITS region of the nuclear rRNA gene

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The DNA sequences of the internal transcribed spacer (ITS) region of the rRNA gene were determined in 20 taxa of 12 species of *Fagopyrum*. By comparing the sequence data and constructing phylogenetic trees, the phylogenetic relationships among the *Fagopyrum* species were established. The sequences of ITS (ITS1 and ITS2) were about 3–7 times more variable than those of the 5.8S subunit of the rRNA gene. The classification of *Fagopyrum* species based on the DNA sequences of the rRNA gene almost coincides with the classification based on morphology, allozyme variability, and nucleotide sequences of the *rbcL-accD* region of the chloroplast DNA. A close relationship of *F. lineare* with the *F. statice-F. leptopodum* clade was suggested and this was strongly supported by a 68-bp gap in ITS1 found in these three species. A tetraploid *F. cymosum* from Kathi, India, has two distinct rRNA genes probably on homoeologous chromosomes, and was distinguished from the clade of other diploid/tetraploid *F. cymosum* with a high bootstrap value. This strengthened the hypothesis of the polyphyletic origin of the tetraploid *F. cymosum*.

INTRODUCTION

Recently several new species and subspecies were discovered in the genus *Fagopyrum* and their distribution in China and in the Himalayan hills has been clarified (Ohnishi, 1991, 1995, 1998; Ohnishi and Yasui, 1998). Phylogenetic relationships among new and already known *Fagopyrum* species have been investigated from the view points of morphology, allozyme variability, restriction fragment length polymorphisms (RFLP) of chloroplast DNA (cp DNA) (Ohnishi and Matsuoka, 1996), and the nucleotide sequence of the *rbcL-accD* region of cp DNA (Yasui and Ohnishi, 1998). These studies suggested that *Fagopyrum* comprises two major groups, the cymosum group and the urophyllum group. The cymosum group consists of two cultivated species and two wild species, each characterized by large lusterless achenes partially covered with persistent perianths. The urophyllum group contains eight wild species that have small lustrous achenes completely covered with persistent perianths. In the cymosum group, allozyme variability, RFLP of cp DNA, and the nucleotide sequence diversity of cp DNA unexpectedly indicated that *F. cymosum* is more closely related to *F. tataricum* ssp. *tataricum* than to *F. esculentum* ssp. *esculentum* (Ohnishi and Matsuoka, 1996; Yasui and

Ohnishi, 1996, 1998). This was first reported by Kishima et al. (1995) by their RFLP analysis of cp DNA. In the urophyllum group, the *F. statice-F. leptopodum* clade, the *F. gracilipes-F. capillatum* clade, and the *F. callianthum-F. pleioramosum* clade were detected by the nucleotide sequence variability of cp DNA (Yasui and Ohnishi, 1998).

The internal transcribed spacer (ITS) region in the nuclear ribosomal RNA (rRNA) gene is a suitable target to investigate the plant phylogenetic relationships among closely related genera and species because of rapid evolution of the ITS region (e.g., Baldwin, 1992; Suh et al., 1993; Sang et al., 1994; Baldwin et al., 1995) and homogenization among repeat units through concerted evolution (Brown et al., 1972; Arnheim, 1983).

The purpose of this study was to re-examine the classification of *Fagopyrum* proposed by Ohnishi and Matsuoka (1996) and Yasui and Ohnishi (1998) by comparing DNA sequences of the ITS region in the nuclear rRNA gene.

MATERIALS AND METHODS

Plant materials. All the known *Fagopyrum* species were examined in this study (Table 1). These materials were collected by the junior author during his expeditions to China and India in 1986–1995 (Ohnishi 1989, 1991, 1995, and 1998). Species identification follows the criteria of Ohnishi and Matsuoka (1996). *F. cymosum* has diploid

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Table 1. *Fagopyrum* species examined

Species	Accession number and source	Mating system ^a	Growth habit ^b	Chromosome number (2n)	Abbreviation
cymosum group					
1. <i>F. esculentum</i> ssp. <i>esculentum</i> Moench	C9106, Yongsheng, China	O	A	16	ESC
2. <i>F. esculentum</i> ssp. <i>ancestralis</i> Ohnishi	C9136, Yongsheng, China	O	A	16	ANC
3. <i>F. homotropicum</i> Ohnishi	C9139, Yongsheng, China	S	A	16	HOM
4. <i>F. cymosum</i> Meisn.	YC9401, Kangding, China	O	P	16	CYMKD
5. <i>F. cymosum</i>	YC9301, Leshan, China	O	P	16	CYMLS
6. <i>F. cymosum</i>	YC9302, Xiaguan, China	O	P	16	CYMXG
7. <i>F. cymosum</i>	C9051, Dali, China	O	P	32	CYMDL
8. <i>F. cymosum</i>	I8642, Kathi, India	O	P	32	CYMKT
9. <i>F. tataricum</i> ssp. <i>tataricum</i> Gaertn.	C9031, Maerkang, China	S	A	16	TAT
10. <i>F. tataricum</i> ssp. <i>potanini</i> Batalin	C9029, Maerkang, China	S	A	16	POT
urophyllum group					
11. <i>F. urophyllum</i> (Bur. et Franch.) Gross	C8844, Kunming, China	O	P	16	UROKM
12. <i>F. urophyllum</i>	C8842, Dali, China	O	P	16	URODL
13. <i>F. callianthum</i> Ohnishi	C9152, Wenchuan, China	S	A	16	CAL
14. <i>F. lineare</i> Sam.	C9256, Binchuan, China	O	A	16	LIN
15. <i>F. leptopodum</i> Diels	C9253, Yongsheng, China	O	A	16	LEP
16. <i>F. statice</i> (Lévl.) Gross	C9470, Yuanmou, China	O	P	16	STAYM
17. <i>F. statice</i>	C9469, Chengjiang, China	O	P	16	STACJ
18. <i>F. pleioramosum</i> Ohnishi	C9146, Wenchuan, China	S	A	16	PLE
19. <i>F. capillatum</i> Ohnishi	C9149, Yongsheng, China	O	A	16	CAP
20. <i>F. gracillipes</i> Hemsl.	C8938, Kunming, China	S	A	32	GRA

^a O, outcrossing; S, selfing.^b A, annual; P, perennial.

and tetraploid forms and both types were included in this study. Since *F. urophyllum*, *F. statice*, and *F. cymosum* show remarkable morphological variability, two or more accessions from different localities were examined (see Table 1).

DNA sequencing. Total DNA was extracted from fresh leaf tissue by the CTAB method (Murray and Thompson, 1980). The 0.7-kbp fragment containing the complete ITS 1, ITS 2, and the 5.8S subunit of the rRNA gene (the ITS region) was amplified by the polymerase chain reaction (PCR) from these total DNA extracts under standard conditions (Saiki et al., 1988). The amplification primers (Table 2) were chosen after White et al. (1990). Following amplification, the desired products were ligated to pUC18 vector. Nucleotide sequencing reactions were carried out using the Taq dye deoxy terminator sequencing kit (Applied Biosystems). The sequencing primers are also listed in Table 2. After the cycle sequencing reaction and phenol/chloroform purification, electrophoresis was carried out with a 373A DNA sequencer (Applied Biosystems).

Data analyses. Multiple alignments of the nucleotide sequences were obtained using the MALIGN program in the ODEN package (Ina, 1994), with manual modifications to minimize the number of gaps. The phylogenetic relationships were inferred by two methods. The first was parsimony analysis with the PAUP ver.3.1.1 (Swofford, 1993). A heuristic search was carried out 100 times with

Table 2. The amplification and sequencing primers for the ITS region (after White et al., 1990)

Primer	Sequence
ITS1 (a, s) ^a	5'-TCCGTAGGTGAACCTGCGG-3'
ITS2 (s)	5'-GCTGCGTTCTTCATCGATGC-3'
ITS3 (s)	5'-GCATCGATGAAGAACGCAGC-3'
ITS4 (a, s)	5'-TCCTCCGCTTATTGATATGC-3'

^a a, amplification primer; s, sequencing primer.

random-addition-sequence (RANDOM) and tree bisection-reconnection (TBR) branch swapping. The options MULPARS, COLLAPSE, and ACTRAN optimization were selected. The PAUP was also used for performing bootstrap analyses to estimate the relative support for clades (Felsenstein, 1985). The bootstrap values were calculated from 100 replicates using a heuristic search with simple-addition-sequence (SIMPLE) and TBR branch swapping.

The second method was neighbor-joining analysis (Saitou and Nei, 1987) using the PHYLIP ver 3.57 package (Felsenstein, 1993). The number of nucleotide substitutions per site was estimated using Kimura's (1980) two-parameter method with the DNADIST program. Neighbor-joining trees were constructed based on the number of nucleotide substitutions per site by the NEIGHBOR program. The bootstrap analyses were carried out with 100 re-sampling data sets generated by the SEQBOOT program and the bootstrap values were calculated with the CONSENSE program.

RESULTS

Sequence analyses. The PCR amplification produced double bands for tetraploid *F. cymosum* from Kathi, India; all others resulted in a single DNA band corresponding to about 700 bp length (Fig. 1). All these PCR products were cloned, including two types of tetraploid *F. cymosum* from Kathi, and their nucleotide sequences were determined. Aligned DNA sequences of ITS1, ITS2, and the 5.8S subunit for 20 taxa of 12 *Fagopyrum* species are shown in Figure 2. Full accounts of the nucleotide sequences were deposited in the DDBJ/EMBL/GenBank databases under the accession numbers AB000322–AB000342. The 5.8S subunit of 20 taxa of *Fagopyrum* was found to consist of 163 or 164 bp nucleotides, while the length of ITS1 and ITS2 varied from 199 to 265 bp and from 216 to 228 bp, respectively.

The numbers of nucleotide substitutions per site were estimated for each pair of taxa for ITS1, ITS2 and the 5.8S subunit separately, and are summarized in Table 3. In this estimation, the characters positioned from 14, 37–192, 490–514, 608, 635, and 650–710 (Fig. 2) were excluded according to the rules of Morton (1995), because the alignments between the cymosum and the urophyllum groups were ambiguous. In total, 165 nucleotide characters were taken into account in the 5.8S subunit, 139 in ITS 1, and 165 in ITS 2. The numbers of nucleotide substitutions per site between species were greatly varied among the subunits of the ITS region. Within the cymosum group, ITS 1 and ITS 2 have evolved 7.4 and 3.7 times faster than the 5.8S, respectively, and within the urophyllum group, ITS 1 and ITS 2 have evolved 2.8 and 4.0 times faster than the

5.8S subunit, respectively (Table 3). The intraspecific nucleotide diversity of *F. urophyllum* (within species of *F. urophyllum* in Table 3) was higher than the average of interspecific estimates within the urophyllum group (within the urophyllum group in Table 3), while the intraspecific nucleotide diversity of *F. cymosum* and *F. statice* was relatively low (Table 3).

Phylogenetic analyses. Because of high alignment ambiguity between the cymosum and the urophyllum groups (Fig. 2), which probably implies so much differentiation between the two groups, phylogenetic analyses were first carried out for the cymosum group and the urophyllum group, separately, then for all the *Fagopyrum* species. Figure 3 shows one of the two most parsimonious trees (142 steps) for the cymosum group. Three clades were clearly detected, the *F. esculentum*-*F. homotropicum* clade, the *F. tataricum* clade, and the *F. cymosum* clade. In the *F. cymosum* clade, the short and the long types of clones from tetraploid *F. cymosum* from Kathi were not included in the cluster consisting of the other four *F. cymosum* accessions with a bootstrap value of 95%. Topology of the neighbor-joining tree for the cymosum group (figure not shown) perfectly coincided with the parsimonious tree of Figure 3.

The parsimony analysis of the urophyllum group resulted in two most parsimonious trees with 130 steps. One of them is shown in Figure 4. The *F. gracilipes*-*F. capillatum* clade, the *F. callianthum*-*F. pleioramosum* clade, and the *F. statice*-*F. leptopodum* clade were supported with high bootstrap values of 100%, 76%, and 95% respectively. *F. lineare* was united with the *F. statice*-*F. leptopodum* clade, although the bootstrap value was below 50%. Topology of

Fig. 1. Profile of the PCR products of the ITS region in *Fagopyrum*. The DNA markers (1-kb ladder) are on both sides of the figure.

Table 3. Average number of nucleotide substitutions per site in the ITS regions at different taxonomic level

	Region		
	ITS1	ITS2	5.8S
Within species			
<i>F. cymosum</i> (2x)	0.025	0.013	0.004
<i>F. cymosum</i> (4x)	0.025	0.021	0.000
<i>F. urophyllum</i>	0.037	0.054	0.000
<i>F. statice</i>	0.000	0.013	0.006
Between subspecies			
<i>F. esculentum</i> ssp. <i>esculentum</i> vs. <i>F. esculentum</i> ssp. <i>ancestralis</i>	0.023	0.000	0.006
<i>F. tataricum</i> ssp. <i>tataricum</i> vs. <i>F. tataricum</i> ssp. <i>potanini</i>	0.000	0.006	0.006
Between species			
Within the cymosum group	0.067	0.034	0.009
Within the urophyllum group	0.024	0.036	0.009
Between two groups	0.119	0.126	0.036

the neighbor-joining tree (figure not shown) almost coincided with the parsimonious trees and the clades which showed high bootstrap values in parsimony analyses also appeared frequently in the bootstrapped neighbor-joining analyses (100% for the *F. gracilipes*-*F. capillatum* clade and 88% for the *F. statice*-*F. leptopodum* clade).

Phylogenetic analyses were also carried out for all

Fagopyrum species primarily for root placement in two major groups. The characters in 14, 37–192, 490–514, 608, 635, and 650–710 in Figure 2 were excluded because of high alignment ambiguity between the cymosum and the urophyllum groups. The parsimony analysis for all *Fagopyrum* species resulted in nine most parsimonious tree with 118 steps (Fig. 5). Since the nucleotides taken into account in this analysis were different from those used for Figures 3 and 4, the topology in Figure 5 is slightly different from those in Figures 3 and 4. In the cymosum group, the phylogenetic root is placed on the branch between the *F. esculentum*-*F. homotropicum* clade and the *F. tataricum*-*F. cymosum* clade. The neighbor joining method also placed the root on the same branch. It is apparent that *F. cymosum* is more closely related to *F. tataricum* ssp. *potanini* than to *F. esculentum* ssp. *ancestralis*. In the urophyllum group, the *F. gracilipes*-*F. capillatum* clade, the *F. callianthum*-*F. pleioramosum* clade, and the *F. statice*-*F. leptopodum* clade were detected by both parsimony and neighbor-joining methods (see Fig. 5). However, these clades and other species in the urophyllum group were separated with low bootstrap values. Thus, the phylogenetic relationships among them remain to be more strictly clarified and the root placement in the urophyllum group also remains inconclusive.

Gaps were defined by their breakpoints and were ex-

Fig. 3. One of the two most parsimonious trees for 10 taxa of the cymosum group (142 step length). The root was assumed at the midpoint of the tree. Numbers above nodes indicate the bootstrap values for 100 replicate analyses. The bootstrap values below 50% are not shown. The positions of insertion/deletions listed in Table 4 are plotted in this figure: insertions by solid bars, deletions by open bars.

Fig. 4. One of the two most parsimonious trees for 10 taxa of the urophyllum group (130 step length). The root was assumed at the midpoint of the tree. Numbers above nodes indicate the bootstrap values for 100 replicate analyses. The bootstrap values below 50% are not shown. The positions of insertion/deletions listed in Table 4 are plotted in this figure: insertions by solid bars, deletions by open bars.

Fig. 5. The most parsimonious tree for 20 taxa of *Fagopyrum* (118 step length). The root was assumed at the midpoint of the tree. Numbers above nodes indicate the bootstrap value for 100 replicate analyses. The bootstrap values below 50% are not shown in the figure.

Table 4. Summary of detected gaps

Gap	Position ^a	Length ^b
A	52–120	69
B	53–120	68
C	90–101	12
D	117–118	2
E	123–190	68
F	203	1
G	429	1
H	486–506	21
I	640	1

Note: Gaps were critically defined by their breakpoints. Thus, those gaps for which breakpoints could not be solely determined were excluded.

^a Nucleotide positions are corresponding to those in Fig. 2.

^b Length of the gaps is based on the alignment in Fig. 2.

cluded from further analyses when the positions could not be rigidly determined. The gaps detected are summarized in Table 4 and parsimoniously plotted on Figures 3 and 4. None of these gaps showed homoplasy. A large deletion (Gap E in ITS1) supports the *F. lineare*-*F. statice*-*F. leptopodium* clade. The 69-bp gap A characterizes the long and short type clones of tetraploid *F. cymosum* from Kathi. Furthermore, the gap G distinguishes *F. cymosum* Kathi from the clade of other diploid-tetraploid *F. cymosum*. Many gaps (D, F, H, and I) were observed between the cymosum and urophyllum groups.

DISCUSSION

Nucleotide diversity. Intraspecific homogeneity and interspecific heterogeneity of repeat units of the rRNA gene are now known under the term of concerted evolution (Brown et al., 1972; Arnheim, 1983) which is probably caused through gene conversion and unequal crossing-over. Jorgensen and Cluster (1988) pointed out that the sequences of 5.8S subunit are conserved at the same level as other coding regions, while the sequences of ITS are much more variable. In the present study, higher variability in ITS than in the 5.8S subunit was clearly demonstrated (Table 3). Two *F. urophyllum* accessions, one from Kunming and the other from Dali, have been revealed to be highly differentiated (Table 3). This was also pointed out from the nucleotide sequences of the *rbcL-accD* region of cp DNA (Yasui and Ohnishi, 1998). Thus, the observed high nucleotide diversity in *F. urophyllum* is probably due to local differentiation between two populations and this has been acquired through a long history of evolution of this species. This supports the primitiveness of *F. urophyllum*.

Slight differences in the sequences between cultivated species and their wild ancestral subspecies are also clear from Table 3. Nucleotide diversity between the cultivated species and their wild ancestors was less than the corresponding values between two wild populations of the same

species (within species in Table 3). This is a reasonable result, since the start of cultivation of buckwheat is rather recent compared with the long history of the evolution of the *Fagopyrum* species. Close relationships of the cultivated species, *F. esculentum* ssp. *esculentum* and *F. tataricum* ssp. *tataricum*, and their wild subspecies, *F. esculentum* ssp. *ancestralis* and *F. tataricum* ssp. *potanini*, respectively, are now obvious and this supports the idea that these wild subspecies are the direct wild ancestor of cultivated buckwheat.

Phylogeny within the cymosum group. Kishima et al. (1995) first pointed out that *F. cymosum* is more closely related to *F. tataricum* ssp. *tataricum* than to *F. esculentum* ssp. *esculentum* by RFLP analyses of cp DNA. This relationship was confirmed by comparing the nucleotide sequences of the *rbcL-accD* region of cp DNA (Yasui and Ohnishi, 1998), and was also supported by nuclear DNA sequences obtained in the present study. The present study on nuclear DNA sequences also supported this. Since *F. cymosum*, the unique outbreeding perennial species in the cymosum group, was recognized to be a primitive type of this group (Ohnishi and Matsuoka, 1996), we now postulate that *F. esculentum* ssp. *ancestralis* differentiated from *F. cymosum* much earlier than *F. tataricum* ssp. *potanini* did.

The polyphyletic origin of tetraploid *F. cymosum* was first suggested from a study of cp DNA sequences by Yasui and Ohnishi (1998). In the tetraploid *F. cymosum* from Kathi, the two types of the ITS region, distinguished by a gap A, were amplified (Fig. 1). The clade of the long and short types of Kathi was supported with a high bootstrap value of 95% for parsimony and 85% for neighbor-joining analyses and it was separated from the clade of other diploid/tetraploid *F. cymosum* (Figs. 3 and 5). Furthermore, a synapomorphic deletion in the 5.8S subunit (gap G in Fig. 3) was found in the lineage of other diploid/tetraploid *F. cymosum*. Thus, we concluded that tetraploid *F. cymosum* from Kathi was originated independently of the other tetraploid *F. cymosum*. That is to say, tetraploid *F. cymosum* arose more than once.

Phylogeny within the urophyllum group. Phylogenetic analyses suggested three major clades in the urophyllum group; the *F. gracilipes*-*F. capillatum* clade, the *F. callianthum*-*F. pleioramosum* clade, and the *F. statice*-*F. leptopodium* clade. On the contrary, the clade of *F. callianthum*-*F. pleioramosum* was not ascertained in the study of *rbcL-accD* region of cp DNA (Yasui and Ohnishi, 1998). This contradictory result came from our mistake in sampling plants of *F. gracilipes* instead of *F. pleioramosum* in Yasui and Ohnishi (1998). By sequencing the *rbcL-accD* region of true *F. pleioramosum* and subsequent phylogenetic analyses, we confirmed the three major clades detected in the present study (new sequence alignment

data and revised phylogenetic trees are available from the authors). The monophyly of *F. gracilipes* and *F. capillatum*, which is strengthened by the overlapping distribution of these two species in Yunnan province (Ohnishi 1995), may indicate that *F. gracilipes*, a tetraploid species, arose by tetraploidization of *F. capillatum*.

The *F. callianthum*-*F. pleioramosum* clade was not detected by comparing morphological traits (Ohnishi and Matsuoka, 1996). Now, the present data show that *F. callianthum* and *F. pleioramosum* are closely related to each other and these two species are also related to *F. urophyllum*. *F. callianthum* and *F. pleioramosum* are both distributed in the upper Min river valley of Sichuan province (Ohnishi, 1995) and these two species have a common unique mating system, distylous selfing (Table 1). Thus, we may infer that these two species may be annual forms differentiated from perennial species *F. urophyllum*.

The parsimony analysis suggested that *F. lineare* could be united with the *F. statice*-*F. leptopodum* clade (Fig. 4) and this notion was strongly supported by a synapomorphic 68-bp deletion (Gap F in Fig. 4), although it was not detected by the comparison of cp DNA sequences (Yasui and Ohnishi, 1998). Morphological classifications also supported the *F. lineare*-*F. statice*-*F. leptopodum* clade (Steward, 1930; Ohnishi and Matsuoka, 1996). In this clade, nuclear DNA variability indicated that *F. statice* is a sister to *F. leptopodum*. This was also supported by the comparison of nucleotide sequences of the *rbcL-accD* region of cp DNA (Yasui and Ohnishi, 1998). Since *F. statice* is a unique perennial in the *F. lineare*-*F. statice*-*F. leptopodum* clade, *F. lineare* might be differentiated from *F. statice* much earlier than *F. leptopodum*.

On the whole, the phylogenetic trees of *Fagopyrum* based on the nuclear rRNA gene well coincided with those based on cp DNA sequences (Yasui and Ohnishi, 1998). Furthermore, they are also consistent with the classification of *Fagopyrum* based on morphology, isozyme variability, and RFLP of cp DNA by Ohnishi and Matsuoka (1996).

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