RAPD-PCR analysis of *Fusarium* strains – cladistic evaluation of the results

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Cladistic analysis of 42 strains of *Fusarium* is presented based on data obtained with RAPD-PCR analysis using four primers. The six species included in the analysis were *F. avenaceum*, *F. culmorum*, *F. equiseti*, *F. graminearum*, *F. oxysporum* and *F. redolens*. All of the specimens were collected in Finland, primarily from barley, wheat and oats. The results of the cladistic analysis are only partly in accordance with the distinction of the taxa based on morphology. Different strains of *F. avenaceum* were not clearly distinguished from each other except for five strains that consistently formed two separate clades.

Keywords: cladistics, *Fusarium*, phylogeny, RAPD-PCR.

The genus *Fusarium* comprises species of fungi that are saprobes, weak parasites or endophytes under native field conditions. A number of species are commercially important as food contaminants or as parasites that cause vascular wilt diseases which have fostered interest on the taxonomy of the genus (Booth, 1971; Gerlach & Nirenberg, 1982; Bruns & al., 1991; Nelson, 1991; Nelson & al., 1983; Windels, 1991). Because of the limited number and variety of classical morphological characteristics available in the genus, molecular characters are probably more important in *Fusarium* systematics than in other fungi in which more informative morphological characters are available. Studies on strains or species of *Fusarium*, where new molecular methods have been used are exemplified by Kistler & al. (1987), Guadet & al. (1989), Nicholson & al. (1993), Assigbetse & al. (1994), and Yli-Mattila & al. (1996). In addition to molecular methods, vegetative compatibility tests can be used to distinguish different strains of the same *Fusarium* species (Correll, 1991; Gordon & Okamoto, 1992a; 1992b; Bridge & al., 1993; Leslie, 1993; Manicom & Baayen, 1993; Mes

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& al., 1994). Our objectives were: (1) to determine the phylogenetic relationships of a set of strains of *F. avenaceum*, and (2) to determine whether strains of different species of the genus can be consistently distinguished as monophyletic groups based on the results of RAPD-PCR analysis.

Material and methods

RAPD-PCR analysis with four primers (91,299, 91,300, X and Y; Yli-Mattila & al., 1996) of 49 *Fusarium* strains collected in Finland (Tab. 1) provided a data matrix of 125 characters, of which 103 were informative (i.e. excluding constant and autapomorphic characters) for the cladistic analysis (Tab. 2). Characterization of the strains, plus methods of DNA extraction, amplification and electrophoresis are described by Yli-Mattila & al. (1996). Amplification was repeated 2–3 times for each sample, each amplification product was run at least twice on agarose gel and only reproducible results were accepted for the final data matrix. Bands were detected visually from photographs of the gels and recorded as present or absent (1/0) for the data matrix.

The six species included in the analysis were *F. avenaceum, F. culmorum, F. equiseti, F. graminearum, F. oxysporum,* and *F. redolens* (= *F. oxysporum* var. *redolens*). All strains of *F. avenaceum* used in the analysis were collected from barley, wheat or oats (Tab. 1). The other species of *Fusarium* were also collected from cereals, except for one strain of *F. oxysporum*, which was recovered from tomato (Tab. 1). The identification of the strains based on morphology according to Booth (1971) and Gerlach & Nirenberg (1982) was provided by Dr. A. Hannukkala of the Agricultural Research Centre of Finland.

Cladistic analyses of the matrices were made with the programs Hennig86 (Farris, 1988) and PAUP 3.1.1 (Swofford, 1993). The credibility of the results was examined using the program RNA by Farris (1994).

Tab. 1. – List of *Fusarium* strains. Isolate numbers are the stock numbers of strains in the collection of the Agricultural Research Centre of Finland.

Species	Code nr.	Isolate nr.	Host (plant part)	Geographic origin (map number)	Year of isolation
F. avenaceum (Fr.) Sacc.					
	a15	93015	barley (stem base)	Apukka	1992
	a28	93014	barley (stem base)	Apukka	1992
	a51	92003	barley (root)	Honkajoki	1986
	a40	92004	barley (root)	Honkajoki	1986
	a14	92016	barley (root)	Kihniö	1986
	a25	92006	barley (root)	Nousiainen	1986
	a17	92013	barley (stem base)	Kankaanpää	1986
	a19	92020	barley (root)	Rautalampi	1986
	a20	92024	barley (root)	Harjavalta	1986

Tab. 1. – continuation

Species	Code nr.	Isolate nr.	Host (plant part)	Geographic origin (map number)	Year of isolation
	a47	92026	barley (root)	Leppävirta	1986
	a50	92009	barley (root)	Parkano	1986
	a37	92014	wheat (root)	Janakkala	1986
	a38	92015	wheat (root)	Nummi	1986
	a39	93084	wheat (stem base)	Pälkäne	1992
	a46	93071	wheat (stem base)	Pälkäne	1992
	a26	93095	wheat (stem base)	Kokemäki	1992
	a27	93093	wheat (stem base)	Kokemäki	1992
	a41	93088	wheat (stem base)	Kokemäki	1992
	a42	93096	wheat (stem base)	Kokemäki	1992
	a45	93094	wheat (stem base)	Kokemäki	1992
	a21	92005	wheat (root)	Vihti	1986
	a23	92007	wheat (root)	Karjaa	1986
	a16	93097	oats (stem base)	Kokemäki	1992
	a43	93101	oats (stem base)	Kokemäki	1992
	a44	93009	oats (stem base)	Pälkäne	1992
	a18	93010	oats (stem base)	Pälkäne	1992
	a22	93256	strawberry (berry)	Jokioinen	-
	a36	93255	strawberry (berry)	Jokioinen	1988
	a31	93258	thuja (shoot)	Elimäki	1989
	a49	93261	apple (fruit)	Salo	-
	a29	93262	rye (seed)	Salo	1988
	a24	93259	cotoneaster (tuber)	Harviala	1986
	a30	93260	hawthorn (shoot)	Karstula	1989
F. graminearum Schwabe					
	g1	92028	barley (stem base)	Jalasjärvi	1986
	g2	92029	barley (root)	Espoo	1986
	g3	92027	wheat (root)	Pori	1986
F. culmorum (W. G. Smith) Sacc.					
	c4	89009	barley (seed)	Jokioinen	1987
	c13	93003	wheat (stem base)	Pälkäne	1992
	c35	93004	oats (stem base)	Pälkäne	1992
F. equiseti (Corda) Sacc.					
	e6	93044	oats (stem base)	Kokemäki	1992
	e8	93001	oats (stem base)	Kokemäki	1992
	e7	92010	barley (root)	Somero	1986
	e9	92011	barley (root)	Kruunupyy	1986
F. redolens Wollenw. (= *F. oxysporum* var. *redolens* Gerlach & Nirenberg)					
	r10	93139	barley (root)	Siuntio	1985
	r11	93152	barley (root)	Strömfors	1986
	r12	93151	barley (root)	Siuntio	1985
F. oxysporum Schlecht.					
	o32	93144	barley (root)	Lieto	1986
	o33	93138	barley (root)	Espoo	1986
	o34	f. sp. *lycopersici*	tomato (-)	Jyväskylä	1993

Tab. 2.– Data matrix of informative characters used in the analyses. Characters 1–27 are from primer 91,300, characters 28–55 from primer Y, characters 56–79 from from primer 91,299 and characters 80–103 from primer X.

	10 20 30 40 50 60 70 80 90 100
G1	0110101111100000000000011011000001010000110000001100000000001010000010110010000101001000001101101100010
G2	011010111110000000000000?0011000001010000110000001100000000001010000010110010000101001000001101101100010
G3	0110101111100000000000000110000010100001100000011000000000010100000101100100001010110000011011011000 10
C4	0111110100110100110100001000000010100010110000011000000000001000000011110010000101110000000101100101010
C13	0111110100110100110100001000000010100010110000001000000000001000000011110010000101110000000101100101010
C35	0111110100110100110100001000000010100010110000011000000000001000000011110010000101110000000101100101010
E6	0000000001100001001000010000000000000111000001000000000010010000100010100 0?000000000000000010000000111
E7	0000000001100001001000010000000000000111?0010100011100001001000010001010001000000000000000000100000000111
E8	00000000001?00001001000010000000000000111100000000000000001001000010001010001000000000000000000100000000111
E9	000000000?10000100100001000000000000011100000000000000000101100001000100100 1?0000000000000000100000000111
R10	100000001001001110101010000000101000010010000100101001110?000100111000110101000010000000010000101001001
R11	?0000000?0010011101010100000000101000010000000100111001110100010011100010010100001001000000100001010000001
R12	10000000?00100111010101000000001010000100100001001110011101000100111000100101000010000000110000101000001
O32	0000010000000011101001110000001010001101111000000001000101010?000001111001001000010000000000001100100000
O33	0000010100001010000001110000000110001000010000001000000100000000101001100100000010100000110000110101000
O34	0000100100001011000000010000101010001000011000001000000100001000001011001000100000100000010000000000000
a14	0000100000000000100000000000010000100110100000000000010000001000010010100000000000000001010000001001010000
a15	0000100000000010100000000000010000100011100000000000010000000010010010000000000000000000100000000010000000
a16	0011100000000010100000000000010000100110100011000100100000000100100101000000000000000001010000110100100000
a17	0000100000000000101000000000010000000000000000000001011000000101100001001010010010000001100000000010000000
a18	000010000000000010100000000000000000001001000000000101100000000100000100100000000000000001010000110110000000
a19	00001000000000001010000001000000001000000001100000011000000101100001001010010010000001100000000010000000
a20	0000100000000000101000000000001000000101000000110001011000000001000001000000000000000000000001000010010000
a21	0100100000000010100000000000010000101010100011000100100000000010100010000000000000000000101000000010010000
a22	0001100000000000101000000000010000101000100011000101100000000100100100000000000000000001000010000100000000
a23	0001100000000000101000000000010000101000100011000101100000000100100100000000000000000001000010000100000000
a24	1101100000000010100000000000010000101011100011000100100001000100000100000001000000000101001001010100000
a25	00001000000000?010100000000000100001000101000110001011000000000101000100000000000000000001010000001010000000
a26	0000100000000010100000000000010000101010100011000100100000000100100100000000000000000001010000110100100000
a27	00001000000000001010000000000010000010?0??1000110001001000000101000000100100000000000000001000010000100100000
a28	0000100000000010100000000100010000101010100011000000100000000010100010100000100100000001010000110100000000
a29	1001100000000000101000000000010000101010100011000101100000000010100010000000000110000001010000110100000000
a30	0101100000000010100000000000010000101011100011000101100000000010100010000000000100000011100100101100000
a31	00001000000000101010000000000010100101000100011000101100000010100000101100000011000000000001000000000000
a36	1010100000000000010000000000000100001011011000100000001000000101001001000000001001000000111001001010000000
a37	1000100000000000101000000100111000100000000110000101100000010110000100101001011000000111001001010000000
a38	0000000000000000101000000000000000000011000001000010010000000001000000101100000000000000000000001000000000000
a39	000110000000000101000000000000010100101010100011000101100000010101000100000000000000000001010000010110100000
a40	00011000000000001000000000100010000101110100011000001100000000100100100000000000100000010100100101100000
a41	000?100000000010100000000010001000010100110001100010110000000001000001010000000000000000000001000000000000
a42	000010000000001010000000?00010000101010100011000101100000000101000101000000000000000001010000111110100000
a43	0010100000000000010000001000000000001100100000000110001000001000000100000001100000000000000000?0000000001
a44	0010100000000010101000000000001000010110010001100000110000000001010001010001010010000000000010000101000000
a45	00001000000000101000000000000001000010111010001100010110000000001000001010001000110000001010000011101000000
a46	00011000000000001000000000000110000101011100011000100100000001010000010000000000110000001110000000101000000
a47	000010000000001010000000010001000010101000001100010110000000101000000101100000000000000010000001101000000
a49	0010000000100000?01000000000010101010010010000001010000000001010010010100000100000001001000000000100000000
a50	10001000000000001000000000100110110000000000110100001100000010110000100001001001000100111001000010000000
a51	0000100000000010100000000000010000101010100011000101100000100100100100000000000000010100101100010000000011

Results

The initial analysis was carried out using two options from Hennig86: mhennig*, which contructs preliminary cladograms by adding the taxa in several different orders and retains the shortest cladogram for each sequence and bb*, branch-breaker, which generates multiple equally parsimonious trees. This analysis with 42 strains including only those strains of *F. avenaceum* collected on barley, wheat and oats gave the most parsimonious tree with the length of 346 steps (consistency index 0.347, retention index 0.707, Farris 1989). The large size of the data set made it impossible to use algorithms that ensured that all of the most parsimonious trees were found, so 30 random trees

were used as starting points for heuristic searches with the program PAUP 3.1.1. In addition to the tree found in the initial search by Hennig 86, another group (island) with two equally parsimonious trees was identified. A semistrict (combinable component, Bremer 1990) consensus tree is presented in Fig. 1. Another search with 50 replicates of random addition sequence of PAUP did not identify any other equally parsimonious trees.

When seven strains of *F. avenaceum* from hosts other than barley, wheat and oats were added to the matrix, the number of equally parsimonious solutions inflated to 156. A semistrict consensus tree is presented in Fig. 2. Basically the structure is the same as in the tree without these taxa, but with less resolution within the large *F. avenaceum* clade.

Discussion

Every cladogram includes several, frequently conflicting statements about the evolution of the characters, so it is advisable to explore the degree of support for individual branches. The first method for this evaluation was to count the number of characters that supported a particular node. However, as discussed by Sanderson (1989) this measure is not straightforward and actually cannot be used as a criterion to measure robustness of the individual clades. The interdependence of all characters of the data matrix can strongly affect the cladistic analysis and credibility of the results as discussed by Davis & al. (1993). Two methods were used to test the reliability of the trees that were generated.

In addition to the most parsimonious trees, it is also advisable to examine trees that are longer. This is done by constructing the strict consensus tree of all trees one step longer than the most parsimonious one(s), then two steps longer, etc. (Bremer, 1988). The number of extra steps, at which the support for the clade disappears, is its Bremer or branch support (Farris & al., 1994) or decay index (Donoghue & al., 1992; Graham & al., 1991). Calculation of the exact indeces larger than 2–3 for even moderate size matrices, however, exceeds the capabilities of most currently available computers and programs. To circumvent this problem an approximate support tree was calculated with the RNA-program by Farris (1994). Although this tree is different from the consensus tree it does include information of the support of the most consistent groups. The support for almost all the clades within *F. avenaceum* is very weak except for the clade including strains a17, a19, a37 and a50 and for the clade including strain 43. On the other hand values for each species, especially those of *F. culmorum, F. equiseti* and *F. redolens* are very high, supporting monophyly of the strains that are thought to belong to separate species on the basis of their morphology. The monophyly of the strains in each

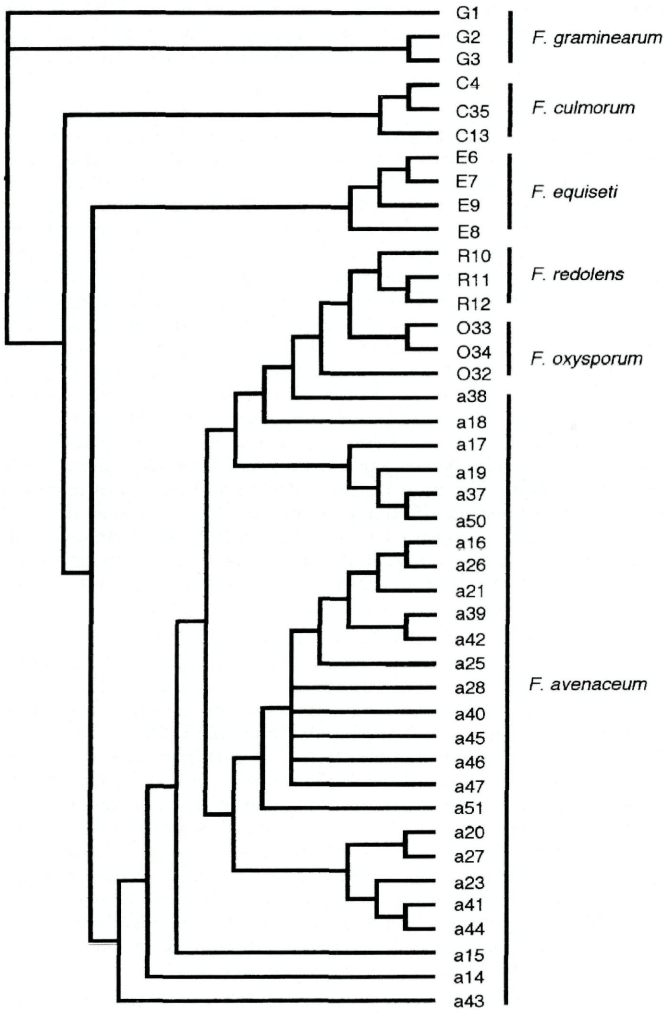

Fig. 1. – The semistrict consensus tree of three equally parsimonious trees for 42 strains of *Fusarium*. Strains marked with a are *F. avenaceum*, with c *F. culmorum*, with e *F. equiseti*, with o *F. oxysporum* and with r *F. redolens*.

species and the clear differences between strains a17, a19, a37, a50 and a43 as compared to other *F. avenaceum* strains are in accordance with the phenetic analysis of the same RAPD-PCR data (Yli-Mattila & al., 1996).

Bootstrapping (Felsenstein, 1985) was originally presented as a means to test the repeatability of the cladistic analyses (Hillis & Bull, 1993). This process works only if the distribution of the characters in the original data set is a good approximation of the distribution of the underlying character universe (Sanderson, 1989). In bootstrapping, a new data matrix is formed by randomly choosing characters from the original matrix with replacement. Accordingly, some of the original characters will not be included at all in the new matrix and some others will be represented by two, three or even more identical replicates. This procedure is repeated a predetermined number of times, and the majority rule (Margush & McMorris, 1981) consensus tree is constructed from the trees resulting from each replication. Support for each clade is given as the percentage of trees in which the particular clade is found. As noted already by Felsenstein (1985) and discussed in detail by Sanderson (1989), bootstrapping in practice identifies only the most unambiguous parts of the tree, as usually there is little statistical support for many of the clades found in the most parsimonious solutions because of character conflict. As can be seen in Fig. 3 on a approximate support tree, the clades with higher bootstrap values usually have also a higher Bremer support. The support values calculated with the program RNA (Farris, 1994) are based on 10,000 samples with replacement from the original data set.

The reliability of the RAPD-PCR results and their suitability for the phylogenetic analysis has been questioned because of the uncertain homology of the bands (Adams & Demeke, 1993; Hillis, 1994). Whether the problem of homology is more severe than with other kind of data has not, however, been proved, and RAPD-PCR results have been used also in phylogenetic studies of closely related species of flowering plants as exemplified by Buren & al. (1994). At least in the present material, the groups that are present in the cladogram are partly congruent with earlier taxonomic conclusions that were based on morphological characters, i.e. strains of the same taxa clustered together. However, it should be noted that the strains of both *F. oxysporum* and *F. redolens* are nested within the large *F. avenaceum* clade. The reliability of the RAPD results would be ideally tested by comparing the congruence (Farris & al., 1994) of different data sets obtained with different methods from the same strains.

In all cladograms presented, the strain a43 was revealed to be distinct from other strains of *F. avenaceum*. This is in accordance with the hybridization analysis of the UP-PCR amplification products (T. Yli-Mattila, N.V. Mironenko, I.A. Alekhina, A. Hannukkala &

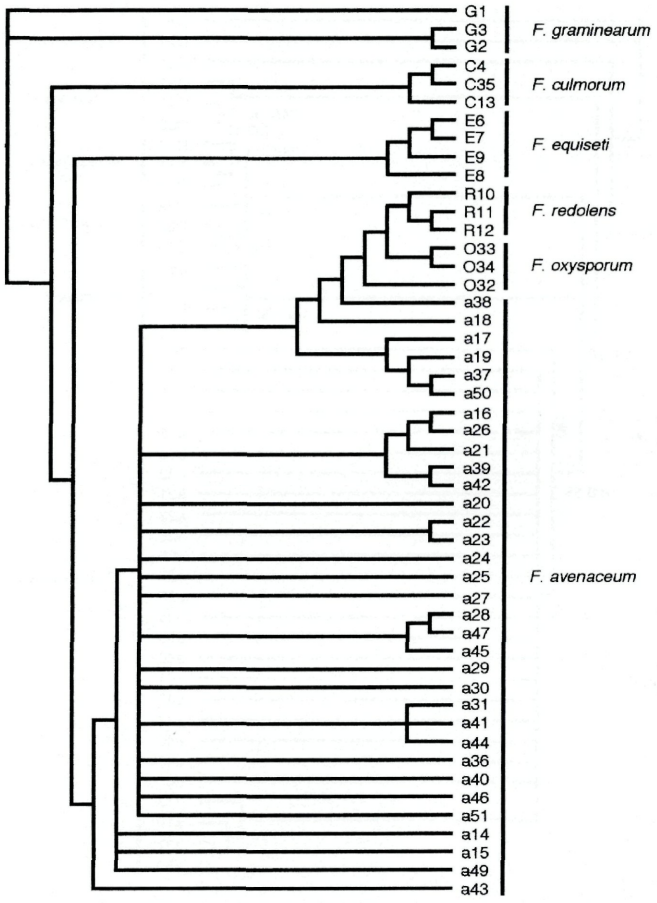

Fig. 2. – The semistrict consensus tree of 156 equally parsimonious trees for 49 strains of *Fusarium*, including 7 strains of *F. avenaceum* collected on other than cereal hosts.

S.A. Bulat; unpublished results, manuscript submitted) and by the preliminary rDNA RFLP study (Yli-Mattila & Paavanen-Huhtala, 1996).

In their morphological studies Nelson & al. (1983) and Baayen & Gams (1988) regarded *F. redolens* conspecific with *F. oxysporum*,

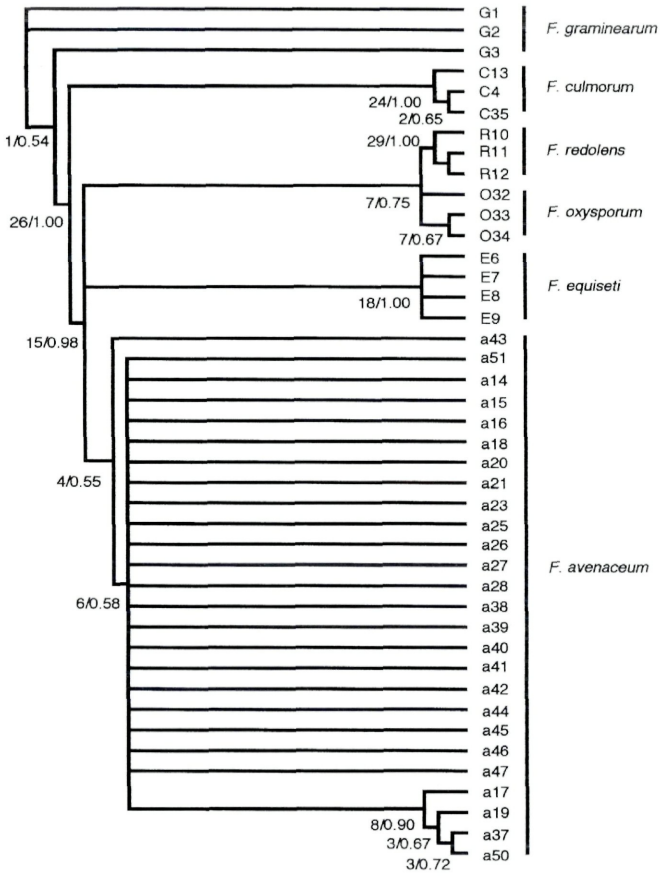

Fig. 3. – Approximate support tree calculated with the program RNA (Farris, 1994) showing branch support (the first number) and bootstrap (the second number, ≥ 0.50) indeces for the clades.

while Gerlach & Nirenberg (1982) considered them as distinct species. The latter view is supported by the rDNA RFLP study of Waalwijk & Baayen (1995). In the approximate support tree of the present study, the strains of all species, except the strain o32 of *F. oxysporum*, for-

med monophyletic groups with high branch support and bootstrap values. Isozyme and RAPD-PCR analyses of more *F. oxysporum* strains from Finland revealed that the strain o32 was only distantly related to other strains of *F. oxysporum* (Paavanen-Huhtala, 1995). This was also supported by the preliminary rDNA RFLP study (Yli-Mattila & Paavanen-Huhtala, 1996) which showed the strain o32 to be more closely related to *F. redolens* than other strains of *F. oxysporum*. Accordingly, the specific status of this strain remains doubtful.

The suitability of cladistic methods for studies below certain taxonomic level has been challenged (e.g. Wheeler & Nixon, 1990). However, as pointed out by Vrana & Wheeler (1992) there is no reason for *a priori* assumptions to be made about the level at which the use of cladistic methods is appropriate. Conclusions about the appropriateness of the results can only be drawn after the analysis is complete. For example, it is not surprising that the large *F. avenaceum* clade is fairly poorly resolved, since it might simply be that there is no hierarchic signal, no differentiated lineages, to be found in the strains of the same taxon collected from a fairly small area. Whether this hypothesis is true in this case can be confirmed only after more thorough analysis including other methods of data retrieval, e.g. direct sequencing.

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