Multilocus sequence analysis of *Ensifer* and related taxa

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Multilocus sequence analysis (MLSA) was performed on representatives of *Ensifer* (including species previously assigned to the genus *Sinorhizobium*) and related taxa. Neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) phylogenies of *dnaK*, *gltA*, *glnA*, *recA*, *thrC* and 16S rRNA genes were compared. The data confirm that the potential for discrimination of *Ensifer* species is greater using MLSA of housekeeping genes than 16S rRNA genes. In incongruence-length difference tests, the 16S rRNA gene was found to be significantly incongruent with the other genes, indicating that this gene should not be used as a single indicator of relatedness in this group. Significant congruence was detected for *dnaK*, *glnA* and *thrC*. Analyses of concatenated sequences of *dnaK*, *glnA* and *thrC* genes yielded very similar NJ, MP and ML trees, with high bootstrap support. In addition, analysis of a concatenation of all six genes essentially produced the same result, levelling out potentially conflicting phylogenetic signals. This new evidence supports the proposal to unite *Ensifer* and *Sinorhizobium* in a single genus. Support for an alternative solution preserving the two genera is less strong. In view of the opinions expressed by the Judicial Commission, the name of the genus should be *Ensifer*, as proposed by Young [Young, J. M. (2003). *Int J Syst Evol Microbiol* **53**, 2107–2110]. Data obtained previously and these new data indicate that *Ensifer adhaerens* and '*Sinorhizobium morelense*' are not heterotypic synonyms, but represent separate species. However, transfer to the genus *Ensifer* is not possible at present because the species name is the subject of a pending Request for an Opinion, which would affect whether a novel species in the genus *Ensifer* or a new combination based on a basonym would be created.

INTRODUCTION

The rhizobia are a large and diverse group of soil bacteria that are capable of root nodule formation and nitrogen fixation and exist in symbiosis with leguminous plants. In recent years, their classification has undergone major changes and revisions and several novel species have been described (for reviews see Young & Haukka, 1996; Zakhia & de Lajudie, 2001; Broughton, 2003; Sawada *et al.*, 2003). The current classification of rhizobia, as for other bacterial groups, is strongly influenced by polyphasic classification, taking into account phenotypic characteristics as well as genetic characteristics (Vandamme *et al.*, 1996), with 16S rRNA gene phylogeny providing the backbone of the

classification (Willems & Collins, 1993; Young & Haukka, 1996; Yanagi & Yamasato, 1993; Young *et al.*, 2001).

The rhizobia that belong to the *Alphaproteobacteria* include the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* and *Sinorhizobium* (de Lajudie *et al.*, 1994, 1998; Dreyfus *et al.*, 1988; Jordan, 1982; Jarvis *et al.*, 1997). In 16S rRNA gene-based phylogeny, *Agrobacterium* and *Allorhizobium* species are phylogenetically dispersed between *Rhizobium* species (Willems & Collins, 1993; de Lajudie *et al.*, 1998; Terefevork *et al.*, 1998; van Berkum *et al.*, 2003). This observation, and the absence of clear phenotypic distinctions, has led to a proposal to merge these three genera into a single genus, *Rhizobium* (Young *et al.*, 2001). However, this scheme has been challenged and is not generally accepted (Farrand *et al.*, 2003). It rests largely on the 16S rRNA gene phylogeny and the resulting genus *Rhizobium* would have the phylogenetic breadth of a bacterial family when neighbouring groups in the alphaproteobacterial 16S rRNA gene tree are considered (see Fig. 1 of Young *et al.*, 2001). There is no single phylogenetic breadth for ranks above species (Brenner *et al.*,

Abbreviations: AIC, Akaike Information Criterion; ILD, incongruence-length difference; ML, maximum-likelihood; MLSA, multilocus sequence analysis; MP, maximum-parsimony; NJ, neighbour-joining.

The GenBank/EMBL/DDBJ accession numbers of new sequence data for *dnaK*, *glnA*, *gltA*, *recA*, *thrC* and 16S rRNA genes are given in Table 1.

2001); however, as pointed out by Konstantinidis & Tiedje (2005), there is great comparative value in having taxonomic ranks that are comparable among lineages. Although this may not be achievable over the whole of prokaryote diversity, we can endeavour to establish broadly comparable groups within this part of the alphaproteobacterial tree, taking into account several genes and ultimately genomes (Konstantinidis & Tiedje, 2005). We agree with Young *et al.* (2001) that *Agrobacterium rhizogenes* is best regarded as a member of *Rhizobium*, as it is always found to be closely related to the type species *Rhizobium leguminosarum*. However, at present we consider that uniting *Agrobacterium*, *Allorhizobium* and *Rhizobium* in a single genus (Young *et al.*, 2001) is not a better solution. It is hoped that the taxonomy of these organisms will be resolved in the near future on the basis of a comprehensive analysis of additional markers (sequence and non-sequence). In this paper we use the *Agrobacterium* nomenclature, except for *Rhizobium rhizogenes*.

The 16S rRNA gene phylogeny was considered originally to be a good model for the so-called organismal phylogeny (Olsen & Woese, 1993). In recent years, several studies have shown that the rRNA genes of rhizobia may occasionally undergo lateral transfer and genetic recombination, resulting in sequence mosaicism (Eardly *et al.*, 1996, 2005; Sullivan *et al.*, 1996; van Berkum *et al.*, 2003; Vinuesa *et al.*, 2005). These observations imply that the rhizobial rRNA gene phylogeny may not always accurately reflect prokaryotic phylogeny. A further disadvantage of 16S rRNA gene phylogeny is that closely related species cannot always be distinguished because of the high level of sequence conservation. To overcome these limitations, protein-encoding genes, with a higher level of sequence divergence than rRNA genes, but sufficiently conserved to retain phylogenetic signal and to allow primers to be designed, have been suggested as alternative phylogenetic markers (Stackebrandt *et al.*, 2002). Ideally, these would be single copy chromosomal genes that are universally distributed among prokaryotes. Examples of genes that are used frequently as molecular markers in various bacterial groups include *recA* (Eisen, 1995), *gyrB* (Yamamoto & Harayama, 1995), *rpoB* (Mollet *et al.*, 1998), *rpoD* (Yamamoto *et al.*, 2000), *groEL* (Viale *et al.*, 1994) and *atpD* (Ronner *et al.*, 1991). Several housekeeping genes have already been used as alternative phylogenetic markers within rhizobia: for example, *atpD* and *recA* (Gaunt *et al.*, 2001), *glnA*, *glnB* (Turner & Young, 2000; Wernegreen & Riley, 1999), *dnaK* (Stepkowski *et al.*, 2003) and *gltA* (Hernández-Lucas *et al.*, 2004). Their phylogenies are not always in agreement with 16S rRNA gene phylogeny. Indeed, trees based on single genes do not always reflect organismal phylogeny because of possible horizontal gene transfer or recombination, variable mutation rates and simple stochastic variation (Gevers *et al.*, 2005; Coenye *et al.*, 2005).

The ad hoc committee for re-evaluation of the species definition suggested that 'species should be identifiable by readily available methods (phenotypic, genomic)' and

that one promising approach towards this end was the determination of a minimum of five housekeeping genes (Stackebrandt *et al.*, 2002). Zeigler (2003) even suggested that sequence analysis of less than five suitable housekeeping genes might be sufficient for a reliable classification. Multilocus sequence analysis (MLSA), using the sequences of multiple protein-coding genes for genotypic characterization of a group of prokaryotes (Gevers *et al.*, 2005), is used increasingly to study and elucidate taxonomic relationships between species (Wertz *et al.*, 2003; Adékambi & Drancourt, 2004; Christensen *et al.*, 2004; Holmes *et al.*, 2004; Naser *et al.*, 2005; Thompson *et al.*, 2005). MLSA is distinct from multilocus sequence typing (Maiden *et al.*, 1998), a method that is used mostly in epidemiology to characterize strains at an infraspecific level by comparing allelic mismatches in housekeeping genes.

In this study we used *Sinorhizobium* (now renamed *Ensifer*, see below) as a model system to assess the use of MLSA for taxonomic purposes. This genus contains eleven species, most of which have been described in polyphasic studies: *Sinorhizobium fredii*, *Sinorhizobium xinjiangense* (Chen *et al.*, 1988), *Sinorhizobium meliloti*, *Sinorhizobium saheli*, *Sinorhizobium teranga* (de Lajudie *et al.*, 1994), *Sinorhizobium medicae* (Rome *et al.*, 1996), *Sinorhizobium arboris*, *Sinorhizobium kostense* (Nick *et al.*, 1999), *Sinorhizobium kummerowiae* (Wei *et al.*, 2002), '*Sinorhizobium morelense*' (Wang *et al.*, 2002) and *Sinorhizobium americanum* (Toledo *et al.*, 2003). From 16S rRNA gene comparisons, it is apparent that the monotypic genus *Ensifer* is highly related to *Sinorhizobium* (Balkwill, 2005). This organism comprises predatory soil bacteria that were originally described in a separate genus as *Ensifer adhaerens*, based mainly on morphological and phenotypic characteristics (Casida, 1982). Phylogenetic analyses of 16S rRNA and *recA* genes (Balkwill, 2005; Willems *et al.*, 2003) indicate that *Ensifer* forms a well-supported cluster together with *Sinorhizobium*. From a polyphasic study, Willems *et al.* (2003) concluded that the genera *Ensifer* and *Sinorhizobium* can be considered as a single taxon and therefore should be merged. According to Rule 38 of the Bacteriological Code (Lapage *et al.*, 1992), the oldest legitimate name, *Ensifer* Casida 1982, has priority over *Sinorhizobium* Chen *et al.* 1988. However, after discussion in the Subcommittee on the taxonomy of *Rhizobium* and *Agrobacterium* (Lindström & Martínez-Romero, 2002) and in view of several arguments against abandoning the name *Sinorhizobium*, Willems *et al.* (2003) in a Request for an Opinion proposed that an exception might be made to Rule 38, allowing the transfer of *Ensifer adhaerens* to *Sinorhizobium* as *Sinorhizobium adhaerens* comb. nov. In a separate Request for an Opinion, Young (2003) proposed that the combination *Sinorhizobium adhaerens* is illegitimate because it contravenes Rules 23 and 24 of the Code and consequently proposed to transfer all *Sinorhizobium* species to the genus *Ensifer*. Recently, the Judicial Commission of the International Committee on Systematics of Prokaryotes ruled that the case for granting an exception to the Rules is too weak (P. De Vos, personal

communication). We have therefore adopted the *Ensifer* nomenclature (Young, 2003).

In the present study, an MLSA approach was used to investigate the relationships of these bacteria by determining the phylogeny of the housekeeping genes *recA* (recombinase A), *glnA* (glutamine synthetase type I), *gltA* (citrate synthase I), *dnaK* (heat-shock protein Hsp70) and *thrC* (threonine synthase). Their phylogenies were examined and compared to that of the 16S rRNA gene. In addition, several combined analyses were performed. The results and their possible taxonomic implications are discussed below.

METHODS

Strains and culture conditions. A total of 33 *Ensifer* strains was used in this study (Table 1): 26 strains representing all known former *Sinorhizobium* species (except for *S. kummerowiae*, for which we could not obtain a bona fide strain, and *S. americanum*, for which we could not obtain the complete set of genes) and seven strains representing the three genomovars (A, B and C) of *E. adhaerens*. In addition, 12 rhizobial strains were included as reference strains. These represent the different groups of the 16S rRNA gene phylogram of rhizobia and belong to the genera *Bradyrhizobium*, *Rhizobium*, *Mesorhizobium* and *Agrobacterium*. All bacterial strains were grown on yeast extract mannitol agar at 28 °C.

DNA preparation. Bacterial DNA was prepared using the alkaline lysis method as described previously (Baele *et al.*, 2000).

Primers for amplification and sequencing. The following genes were studied: *recA*, *glnA*, 16S rRNA, *gltA*, *dnaK* and *thrC*. To design primers for PCR amplification and sequencing of these genes, we used corresponding sequences derived from the genome sequences of related bacteria: *Agrobacterium tumefaciens* C58 (Wood *et al.*, 2001; Goodner *et al.*, 2001), *S. meliloti* 1021 (Galibert *et al.*, 2001), *Mesorhizobium loti* MAFF 303099 (Kaneko *et al.*, 2000), *Brucella melitensis* 16M^T (DelVecchio *et al.*, 2002) and *Bradyrhizobium japonicum* USDA 110 (Kaneko *et al.*, 2002). The *dnaK*, *glnA*, *gltA*, *recA* and *thrC* sequences were compared using the program BioNumerics (Applied Maths) to identify conserved regions. The primers designed are listed in Table 2. Primers for the amplification of *recA* and *dnaK* were obtained from the literature (Gaunt *et al.*, 2001; Stepkowski *et al.*, 2003).

PCR amplification and sequencing of the genes. PCR amplification was performed by means of a T-Gradient Thermocycler (Biometra) in 50 µl reaction mixtures containing 2.5 µl DNA extract, 10 mM Tris/HCl pH 8.3, 1.5 mM MgCl₂, 50 mM KCl, 0.001 % (w/v) gelatin, 1 U AmpliTaq DNA polymerase (Applied Biosystems), 200 µM each deoxyribonucleotide and 50 ng each primer. The temperature and cycling conditions used are given in Table 2. The PCR products and their concentration were verified by electrophoresis of 3 µl product on a 1 % agarose gel and staining with ethidium bromide. A molecular mass marker (Smartladder-Eurogentec) was included to estimate the length of the amplicons.

The amplified products were purified using a Qiaquick PCR purification kit (Qiagen). The purified DNA was sequenced using the dideoxynucleotide chain-termination method with fluorescent ddNTPs (Applied Biosystems) on an ABI Prism 3100 capillary sequencer, according to the manufacturer's instructions (Applied Biosystems). Consensus sequences were constructed using the Auto-Assembler software (Applied Biosystems).

Phylogenetic data analysis. Nucleotide sequence alignments were made using CLUSTAL_X (Thompson *et al.*, 1997), taking into account the corresponding amino acid alignments for protein-coding genes. To assess the influence of noise due to saturation of the third codon position, we performed incongruence-length difference (ILD) tests (Farris *et al.*, 1994), as implemented in PAUP* version 4.0b10 (Swofford, 2002), using the different codon positions as separate partitions in 1250 replications. As a further test to assess saturation, we used the index of substitution saturation described by Xia *et al.* (2003) as implemented in DAMBE version 4.2.13 (Xia & Xie, 2001). The same set of strains was used for all genes and sequence data for *Caulobacter crescentus* CB15, extracted from the complete genome sequence (Nierman *et al.*, 2001), were used as an outgroup. Neighbour-joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) analyses were performed with PAUP*. NJ analyses were performed using the Kimura-2 correction and 1000 bootstrap replications; MP analyses were performed using the heuristic search option. For the 16S rRNA gene, this resulted in 46 best trees, which were reduced to five by repeating the analysis using the Goloboff-fit criterion (GK=2; Goloboff, 1993). For ML analyses, the optimal models of nucleotide substitution were estimated using the program MODELTEST 3.7 (Posada & Crandall, 1998), using both hierarchical likelihood ratio tests and the Akaike Information Criterion (AIC). Using both options, the same model was obtained in all cases, except for the 16S rRNA gene and *dnaK*, where models obtained with AIC were used (Posada & Buckley, 2004). The MP trees were used as starting trees for the heuristic search procedure. Bootstrap analyses were performed using 1000 replications of heuristic searches for MP and 100 replications for ML. The ILD test implemented in PAUP* and using 1250 replicates was used to assess incongruence between datasets. To assess particular hypotheses, the Shimodaira-Hasegawa test (Shimodaira & Hasegawa, 1999) as implemented in PAUP* was used. The significance of differences in the likelihood scores was assessed by a bootstrap test with 1000 replications.

RESULTS AND DISCUSSION

We analysed comparatively the sequences of six genes (five housekeeping genes and the 16S rRNA gene) for 33 *Ensifer* strains and 12 representatives of related taxa (Table 1). We also included corresponding sequences retrieved from the complete genome sequences of *Agrobacterium tumefaciens* C58, *Brucella melitensis* 16M^T, *Brucella suis* 1330^T, *C. crescentus* CB15, *Rhodopseudomonas palustris* CGA009 and *Bradyrhizobium japonicum* USDA 110, available in GenBank/EMBL/DDBJ. The choice of genes was guided by the following requirements: single copy occurrence, relatively high degree of conservation (as established from the literature) and separate location on the main chromosome (as assessed from the complete genome of *S. meliloti*). Sequence alignments were inspected visually to identify positions with uncertain alignment to be omitted from further analyses. The lengths of the alignments used for further analysis were: 498 bp for *recA*, 975 bp for *glnA*, 639 bp for *gltA*, 285 bp for *dnaK*, 636 bp for *thrC* and 1419 for the 16S rRNA gene. No parts of the sequences were omitted, except in the case of the 16S rRNA gene for the type strain of *Rhizobium leguminosarum* where, in two different positions, a single C residue was omitted because it was absent from all other taxa included in the alignment.

Table 1. Strains used and GenBank/EMBL/DDBJ accession numbers

Accession numbers of new data are in bold.

Name	16S rRNA	recA	glnA	dnaK	thrC	gltA	Source
<i>Ensifer adhaerens</i> gv. A							
LMG 9954 (=BR819)	AM181735	AJ505599	AM182970	AM182112	AM181721	AM181647	<i>Leucaena leucocephala</i> , Brazil
LMG 10007 (=BR8606)	AM181734	AJ505600	AM182969	AM182111	AM181720	AM181646	<i>Pithecellobium dulce</i> , Brazil
R-9451 (=HAMBI 1631)	AJ420774	AJ505598	AM182968	AM182110	AM181719	AM181645	<i>Sesbania grandiflora</i> , Sri Lanka
<i>Ensifer adhaerens</i> gv. B R-7457 (=5D19)	AJ420773	AJ505597	AM182967	AM182109	AM181718	AM181644	<i>Medicago sativa</i> , Spain
<i>Ensifer adhaerens</i> gv. C							
LMG 20216 ^T (=ATCC 33212 ^T)	AM181733	AJ505595	AM182966	AM182108	AM181717	AM181643	Soil, central PA, USA
R-14067 (=ATCC 33499)	AM181731	AM182150	AM182964	AM182106	AM181715	AM181641	Soil, central PA, USA
LMG 20582 (=R-6387)	AJ420775	AJ505596	AM182965	AM182107	AM181716	AM181642	Agricultural soil, Pittem, Belgium
<i>Ensifer arboris</i>							
LMG 14919 ^T (=HAMBI 1552 ^T)	AM181744	AM182130	AM182944	AM182086	AM181695	AM181621	<i>Prosopis chilensis</i> , Kosti, Sudan
LMG 14917 (=HAMBI 1396)	AM181752	AM182131	AM182945	AM182087	AM181696	AM181622	<i>Prosopis chilensis</i> , Kenya
LMG 19223 (=HAMBI 1704)	AM181738	AM182129	AM182943	AM182085	AM181694	AM181620	<i>Acacia senegal</i> , Khartoum, Sudan
<i>Ensifer fredii</i>							
LMG 6217 ^T (=USDA 205 ^T)	X67231	AM182145	AM182959	AM182101	AM181710	AM181636	<i>Glycine max</i> , Honan, China
LMG 8317 (=USDA 191)	X77123	AM182144	AM182958	AM182100	AM181709	AM181635	Soil, Shanghai, China
LMG 6218 (=USDA 206)	AM181740	AM182146	AM182960	AM182102	AM181711	AM181637	<i>Glycine max</i> , Honan, China
LMG 6216 (=LMG 8317)	AM181749	AM182147	AM182961	AM182103	AM181712	AM181638	Soil, Shanghai, China
<i>Ensifer kostiensis</i>							
LMG 19227 ^T (=HAMBI 1489 ^T)	AM181748	AM182142	AM182956	AM182098	AM181707	AM181633	<i>Acacia senegal</i> , Kosti, Sudan
LMG 14911 (=HAMBI 1502)	AM181750	AM182143	AM182957	AM182099	AM181708	AM181634	<i>Acacia senegal</i> , Tendelti, Sudan
LMG 19225 (=HAMBI 1484)	AM181747	AM182141	AM182955	AM182097	AM181706	AM181632	<i>Prosopis chilensis</i> , Kosti, Sudan
<i>Ensifer medicae</i>							
LMG 19920 ^T (=A 321 ^T)	L39882	AM182135	AM182949	AM182091	AM181700	AM181626	<i>Medicago truncatula</i> , Aude, France
LMG 19921 (=R-481)	AM181754	AM182136	AM182950	AM182092	AM181701	AM181627	<i>Medicago truncatula</i>
LMG 18864 (=HAMBI 1809)	AM181746	AM182137	AM182951	AM182093	AM181702	AM181628	<i>Medicago truncatula</i> , Syria
<i>Ensifer meliloti</i>							
LMG 6133 ^T (=NZP 4027 ^T)	X67222	AM182133	AM182947	AM182089	AM181698	AM181624	<i>Medicago sativa</i> , VA, USA
LMG 4289 (=INRA 2011)	AM181751	AM182134	AM182948	AM182090	AM181699	AM181625	<i>Medicago sativa</i>
LMG 6130 (=NZP 4009)	AM181739	AM182132	AM182946	AM182088	AM181697	AM181623	<i>Medicago sativa</i> , Australia
' <i>Sinorhizobium morelense</i> ' LMG 21331 (=CFN E1007)	AM181737	AJ505601	AM182971	AM182113	AM181722	AM181648	<i>Leucaena leucocephala</i> cv. Cunningham in cultivated soils
<i>Ensifer saheli</i>							
LMG 7837 ^T (=ORS 609 ^T)	X68390	AM182138	AM182952	AM182094	AM181703	AM181629	<i>Sesbania cannabina</i> , Dakar, Senegal
LMG 11864 (=ORS 600)	AM181753	AM182140	AM182954	AM182096	AM181705	AM181631	<i>Sesbania pachycarpa</i> , Senegal
LMG 8310 (=ORS 611)	AM181742	AM182139	AM182953	AM182095	AM181704	AM181630	<i>Sesbania grandiflora</i> , Dakar, Senegal
<i>Ensifer teranga</i>							
LMG 7834 ^T (=ORS 1009 ^T)	X68388	AM182153	AM182974	AM182116	AM181725	AM181651	<i>Acacia laeta</i> , Dakar, Senegal
LMG 11859 (=ORS 52)	AM181743	AM182154	AM182975	AM182117	AM181726	AM181652	<i>Sesbania rostrata</i> , Senegal
LMG 6464 (=ORS 51)	AM181741	AM182152	AM182973	AM182115	AM181724	AM181650	<i>Sesbania rostrata</i> , Dakar, Senegal
<i>Ensifer xinjiangensis</i>							
LMG 17930 ^T (=CCBAU 110 ^T)	AM181732	AM182148	AM182962	AM182104	AM181713	AM181639	<i>Glycine max</i> , Xinjiang, China
R-16438 (=CCBAU 83834)	AM181736	AM182149	AM182963	AM182105	AM181714	AM181640	–
<i>Ensifer</i> sp. LMG 20571 (=R-4955)*	AJ420776	AM182151	AM182972	AM182114	AM181723	AM181649	Agricultural soil, Pittem, Belgium
<i>Mesorhizobium loti</i> LMG 6125 ^T (=NZP 2213 ^T)	X67229	AM182156	AM182977	AM182118	AM181728	AM181654	<i>Lotus corniculatus</i> var. tenuifolium, New Zealand
<i>Mesorhizobium mediterraneum</i>	AM181745	AM182157	AM182978	AM182119	AM181729	AM181655	<i>Cicer arietinum</i> L., Spain
LMG 17148 ^T (=UPM-Ca36 ^T)							
<i>Bradyrhizobium elkanii</i> LMG 6134 ^T (=USDA 76 ^T)	AF362942	AM182155	AM182976	AJ431152	AM181727	AM181653	<i>Glycine max</i> , USA
<i>Bradyrhizobium japonicum</i> LMG 6138 ^T (=NZP 5549 ^T)	X66024	AM182158	AM182979	AM182120	AM181730	AM181656	<i>Glycine max</i> , Japan
<i>Rhizobium galegae</i> LMG 6214 ^T (=HAMBI 540 ^T)	X67226	AM182127	AM182941	AM182083	AM181692	AM181618	<i>Galega orientalis</i> , Finland
<i>Rhizobium gallicum</i> R-4387 ^T (=R602sp ^T)	AM181756	AM182124	AM182938	AM182080	AM181689	AM181615	<i>Phaseolus vulgaris</i> , France
<i>Rhizobium giardinii</i> R-4385 ^T (=H152 ^T)	AM181755	AM182123	AM182937	AM182079	AM181688	AM181614	<i>Phaseolus vulgaris</i> , France
<i>Rhizobium huautlense</i> LMG 18254 ^T (=S02 ^T)	AF025852	AM182128	AM182942	AM182084	AM181693	AM181619	<i>Sesbania herbacea</i> , Sierra de Huautla, Mexico
<i>Rhizobium leguminosarum</i> LMG 14904 ^T (=ATCC 10004 ^T)	AM181757	AM182125	AM182939	AM182081	AM181690	AM181616	<i>Pisum sativum</i> , IL, USA

Table 1. cont.

Name	16S rRNA	recA	glnA	dnaK	thrC	gltA	Source
<i>Rhizobium rhizogenes</i> LMG 150 ^T (= ATCC 11325 ^T)	AY945955	AM182126	AM182940	AM182082	AM181691	AM181617	–
<i>Agrobacterium radiobacter</i> LMG 140 ^T (= ATCC 19358 ^T)	AM181758	AM182121	AM182935	AM182077	AM181686	AM181612	–
<i>Agrobacterium rubi</i> LMG 17935 ^T (= ATCC 13335 ^T)	AM181759	AM182122	AM182936	AM182078	AM181687	AM181613	<i>Rubus ursinis</i> var. <i>loganobaccus</i> , USA

*Strain LMG 20571 was previously assigned to ‘*S. morelense*’ (Willems *et al.*, 2003).

Individual gene analyses

For each *Ensifer* species (or genomovar) studied, sequences of the same gene for the various strains included (two to four per species) were highly similar (ranging from 98.0–100 % for *gltA* to 98.7–100 % for *glnA*; values for 16S rRNA genes were 99.6–100 %). Initial NJ trees that included all strains revealed that, in all cases, strains belonging to a single species clustered very closely together. For this reason, the type strains only were selected for further analysis in order to reduce computing times (except for ‘*S. morelense*’ where both strains were included, *E. adhaerens* where the three genomovars were represented and *B. japonicum* where subgroups I and Ia were

represented). Among *Ensifer* species, the sequence similarities of the same housekeeping genes were clearly lower, ranging from 79.5–94.2 % for *thrC* to 87.5–96.9 % for *glnA* (for the 16S rRNA genes values were in the range 97.9–99.8 %). For the calculation of these inter-species sequence similarity ranges, *Ensifer xinjiangensis* (two strains) was excluded because, in all comparisons, it was identical or very similar to *Ensifer fredii* [four strains; 99–100 % sequence similarity for all genes except *gltA* (98.3–100 %)], strongly suggesting that the two species are in fact synonymous. The clear gap between within-species and between-species sequence similarity values (Fig. 1) demonstrates the greater potential for species discrimination using protein-encoding housekeeping genes

Table 2. Oligonucleotide primers used and PCR cycling conditions

Primers given in bold were used for initial amplification.

Primer	Position*	Sequence 5'–3'	PCR cycling
glnA144F	144–164	GTCATGTTTCGACGGYTCYTCG	10 min 95 °C, 2 × (2 min 94 °C, 2 min 62 °C, 1 min 72 °C), 32 × (30 s 94 °C, 1 min 61 °C, 1 min 72 °C), 10 min 72 °C
glnA1142R	1162–1142	TGGAKCTTGTCTTGATGCCG	
glnA572F	572–591	GGACATGCGYTCYGARATGC	
glnA572R	591–572	GCATYTCRGARCGCATGTCC	
gltA428F	428–446	CSGCCCTCTAYCAYGACTC	5 min 95 °C, 3 × (2 min 94 °C, 2 min 53 °C, 1 min 72 °C), 30 × (30 s 94 °C, 1 min 53 °C, 1 min 72 °C), 5 min 72 °C
gltA1111R	1130–1111	GGGAAGCCSAKCGCCTTCAG	
gltA1089R	1106–1089	ATRCGSGARTAGAAGTCG	
gltA667R	683–667	GCRTGCAGGATGAANAT	
thrC1231R	1250–1231	GGRAATTTDCCGGRGTGSGC	5 min 95 °C, 3 × (2 min 94 °C, 2 min 55 °C, 1 min 72 °C), 30 × (30 s 94 °C, 1 min 55 °C, 1 min 72 °C), 5 min 72 °C
thrC577F	577–597	GGCAMPKTTTCGACGAYTGCCAG	
thrC766F	766–782	GGCAATTTTCGCGGAYAT	
thrC766R	782–766	ATRTCGCCGAAATTGCC	
thrC925R	944–925	GASGARAYCTGGATRTCCAT	
recA6F†	6–31	CGKCTSGTAGAGGAYAAATCGGTGGA	5 min 95 °C, 32 × (45 s 94 °C, 1 min 60 °C, 1 min 30 s 74 °C), 5 min 72 °C
recA555R†	555–530	CGRATCTGGTTGATGAAGATCACCAT	
recA63F†	63–85	ATCGAGCGGTCTGTCGGCAAGGG	
recA504R†	504–523	TTGCGCAGCGCTGGCTCAT	
dnaK1466F‡	1466–1488	AAGGARCANCAGATCCGCATCCA	1 min 94 °C, 35 × (1 min 94 °C, 1 min 64 °C, 40 s 72 °C), 5 min 72 °C
dnaK1777R‡	1777–1799	TASATSGCCTSRCCRAGCTTCAT	

*Position relative to the sequences taken from the complete genome of *E. meliloti* 1021 (Galibert *et al.*, 2001).

†From Gaunt *et al.* (2001).

‡From Stepkowski *et al.* (2003).

Fig. 1. Summary of the percentage sequence similarity results for six genes for *Ensifer* strains.

compared with 16S rRNA genes, where there is in fact no gap. With any of the housekeeping genes used here, discrimination between the existing species and genomovars (except *E.*

fredii and *E. xinjiangensis*) is clear and thus, for species identification purposes in *Ensifer*, these genes are superior to the 16S rRNA gene.

According to ILD tests using first, second or third codon positions as separate partitions in pairwise comparisons, potential third codon saturation was found not to cause conflicting phylogenetic signals for most genes. For *thrC*, the ILD test gave a *P* value of 0.868 for the datasets containing only the first and the second positions, respectively, and 0.0376 for the datasets containing the first and third codon positions, respectively. However, based on the *P* value of 0.5816 for a comparison of the partitions containing the second and the third codon positions, respectively, we decided to include the third position in further analyses. Furthermore, the indices of substitution saturation (Xia *et al.*, 2003) did not indicate any significant saturation for any of the genes (data not shown).

Sequence characteristics are provided in Table 3 and demonstrate that the housekeeping genes are more informative than 16S rRNA genes. *glnA* was the largest gene fragment sequenced (975 bp) and contained the greatest number of parsimony-informative characters (388), representing 40 % of sequenced positions. By comparison, *thrC* was one-third shorter in length, contained 60 % informative characters and was the most information-rich of the genes studied. *dnaK* was the shortest fragment but, of its 285 positions, 51 % were still parsimony informative.

For each of the genes, separate phylogenetic trees constructed using NJ, MP or ML methods gave similar groupings, although the precise branching pattern showed some variation (data not shown). For the housekeeping genes, we also prepared NJ trees of the amino acid sequences.

Table 3. Sequence information

IS, Invariable sites; No. nt, number of nucleotides; PI, parsimony-informative sites; VS, variable but parsimony-uninformative sites.

Locus	No. nt	IS	VS	PI (%)	Length MP tree/no. of trees	ML model/invariant sites/gamma shape parameter*	Frequency A/C/G/T	−ln L ML tree†	ILD vs 5 genes: <i>P</i> value‡
16S rRNA	1419	1120	72	227 (16)	696/46	GTR + I + G/0.6382/0.4966	0.2341/0.2418/0.3210/0.2031	5568.26548	0.0008
<i>glnA</i>	975	483	104	388 (40)	1794/4	GTR + I + G/0.3547/0.6280	0.1889/0.4025/0.2805/0.1281	9201.08097	0.3272
<i>gltA</i>	639	304	76	259 (41)	1291/3	GTR + I + G/0.3312/0.5664	0.1740/0.3914/0.3069/0.1277	6459.28809	0.0016
<i>thrC</i>	636	196	58	382 (60)	2025/3	TVM + I + G/0.2286/0.9101	0.1708/0.3385/0.3492/0.1415	4964.14372	0.0696
<i>recA</i>	498	258	29	211 (42)	1003/3	GTR + I + G/0.4569/1.0978	0.1421/0.3888/0.3226/0.1465	9151.14012	0.0320
<i>dnaK</i>	285	103	36	146 (51)	677/1	TrN + G/0/0.4401	0.1901/0.3384/0.3791/0.0924	3316.16551	0.1560
3 genes	1896	782	198	916 (48)	3232/1	GTR + I + G/0.2772/0.7002	0.1707/0.3888/0.3075/0.1330	21949.29759	–
6 genes	4452	2464	375	1613 (36)	6395/2	GTR + I + G/0.4059/0.6047	0.1899/0.3478/0.3109/0.1514	40205.60491	–

*Estimated using the AIC with the program MODELTEST. G, shape parameter of the gamma distribution; +G, indicates rate variation among sites is taken into account; GTR, General time-reversible model; I, proportion of invariable sites; +I + G, indicates invariable sites are included and rate variation among sites is taken into account; TrN, Tamura and Nei model; TVM, transversal model.

†*L*, likelihood.

‡ILD test of this gene versus a partition consisting of a concatenation of the other five genes.

However, these sequences were much less divergent and resulted in trees with relatively less clear separation of species and groups (data not shown). We therefore performed further analyses on the gene sequences. Most variation was observed for *dnaK* where some *Rhizobium* and *Mesorhizobium* species grouped among *Ensifer* species in the MP and ML trees. For *thrC*, the three algorithms produced very similar branching patterns. To illustrate the differences observed between the gene phylogenies, the ML trees are shown in Fig. 2. Significant groupings in the different phylogenies are discussed below; however, it is clear that the different genes yield phylogenies that can differ to varying extents. This may be due to lateral gene transfer events, but also to the size of the dataset (in particular, the *dnaK* sequence is relatively short) and the taxa included. Bootstrap support for the observed groups was not always high in the ML trees, which generally have lower bootstrap values than corresponding NJ and MP trees (see below and Table 4).

It is clear that single housekeeping gene phylogenies should be interpreted as such and cannot be taken automatically to represent organismal phylogeny. To assess the latter, a combination of several genes seems indispensable, although it is unclear what the optimal number of genes should be.

Concatenated sequence analyses

One approach to combined data analysis is to combine only congruent data and to exclude data partitions with a significant level of incongruence, as they can introduce errors that may obscure reliable data and lead to erroneous topologies (Bull *et al.*, 1993; Miyamoto & Fitch, 1995).

The congruence of the different gene phylogenies was assessed using pairwise ILD tests. This revealed that *dnaK*, *thrC* and *glnA* had compatible phylogenetic signals (P values of 0.112–0.0936) and *recA* also showed significant congruence with *glnA* ($P=0.0640$). For *dnaK* this was surprising as visual comparison of gene trees (Fig. 2) shows a rather aberrant grouping. We assume that the tree represents a reduced picture of the information content and in this case highlights particular relationships that do not have significant bootstrap support. This may be related to the relatively short sequence used for this gene so that a strong spurious signal can dominate the information content. In the ILD test, this effect appeared to be levelled out to reveal the underlying signal, which in this case appears congruent with *thrC* and *glnA*.

Surprisingly, none of the housekeeping genes showed significant congruence with the 16S rRNA gene ($P<0.01$). This may be due to intragenic mosaicism, which has been reported previously to occur in these organisms (Eardly *et al.*, 2005; Vinuesa *et al.*, 2005). We divided the alignment into three partitions (1–357, 358–744 and 745–1419) that were reported previously to have different phylogenetic affiliations in *Rhizobium galegae* (Eardly *et al.*, 2005). ILD tests were repeated for each of these partitions against each of the five housekeeping genes. No significant congruence

was observed ($P<0.05$ in all combinations), indicating that mosaicism may be present in these partitions. To establish this with certainty, more detailed analyses of the alignments will be needed.

As a further test to detect aberrant phylogenetic signals, we performed an ILD test for each gene versus a partition comprising the remaining five genes (Table 3, last column). This test gives an indication of how well the phylogenetic signal in one gene is in-line with the phylogenetic signal of the other five genes taken together. Again, *glnA*, *dnaK* and *thrC* gave P values of greater than 0.05, whereas *recA* yielded a P value of 0.0320 and 16S rRNA and *gltA* had P values of less than 0.01. The values for the 16S rRNA gene and *gltA* could not be improved by eliminating one or two taxa potentially responsible for incongruence as deduced by visual inspection of the trees (data not shown). However, in the case of *recA*, exclusion of *Rhizobium huautlense*, *Rhizobium galegae* or both yielded P values of 0.3024, 0.0944 and 0.1064, respectively, indicating that these species contribute strongly to the incongruence of the *recA* dataset. In view of the ILD test results, we concatenated the aligned sequences for *dnaK*, *thrC* and *glnA* only and obtained an alignment of 1896 nucleotides, comprising 782 invariable sites, 198 variable but parsimony-uninformative sites and 916 (48 %) parsimony-informative sites. MODELTEST was used to select the optimal substitution model for ML analysis (Table 3). NJ, MP and ML trees were now very similar and bootstrap support was generally higher (Fig. 3).

An alternative to the above approach of combining only congruent partitions is to calculate trees based on a concatenation of all six genes. Despite the possible presence of incongruent signals, this approach may improve the total resolution of the tree because the more conserved 16S rRNA gene may resolve other parts of the tree than the housekeeping genes. In many of the rhizobial groups, there is little divergence in the 16S rRNA gene between species. Therefore, in a combined tree, the 16S rRNA gene will not contribute much to branching patterns near the branch endings (here representing species level). The housekeeping genes we used, although still conserved, are more variable (Fig. 1) and will contribute more strongly to the branching patterns near the tips, whereas in the deeper parts of the tree their phylogenetic signal may be less clear because of noise from the higher number of substitutions. However, the effect of noise from individual partitions may be reduced in the larger complete dataset (Barrett *et al.*, 1991; Kluge, 1989). The concatenated alignment of the six genes comprised 4452 positions, 2464 and 1613 (36 %) of which were constant and parsimony informative, respectively. MODELTEST was used to select the most suitable substitution model for ML analysis. Again, NJ, MP and ML trees were very similar and bootstrap support was high (Fig. 4). There was excellent agreement with the trees obtained based on the concatenation of *dnaK*, *thrC* and *glnA*. In this case, the combination of six genes with varying levels of congruence

Table 4. Bootstrap support in the various analyses

10, 100 % Bootstrap support; 9, 90–99 % bootstrap support; 8, 80–89 % bootstrap support.

Cluster*	<i>gltA</i>			<i>dnaK</i>			<i>recA</i>			<i>glnA</i>			<i>thrC</i>			16S rRNA			Three genes			Six genes		
	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML	NJ	MP	ML
Arad, Atum	9			9			10	9	10	10	9	9	10	9	9				10	10	10	10	10	10
Arad, Atum, Arub	10	10	10				8		8	9	8	9	9	9	9	10	10	10	10	10	10	10	10	10
Bruc	10	10	10	10	10	9	9	10	10	10	10	10	10	10	10	10	10	9	10	10	10	10	10	10
Ensi							8						9	8	9	9			10	9	10	10	10	10
Smor	9	9	8	9	9	8	9	9	9	9	9	9	9	8	9	10	9	9	10	10	10	10	10	10
EadhABC, Smor							8			9	8	8	10	9	9	9			10	9	10	10	10	9
Ensi except Smor and Eadh							8		8				9	9	8				10	9	9	10	9	10
EadhAB													8											
EadhBC				8						9	9	8							8	9		9	9	9
EadhBC, Smor										9														
EadhABC				9									9	9	9				10	9	10	9	9	10
Earb, Emel	9						8												9			9		
Earb, Emed															8									
Earb, Emel, Emed	8		9	10	9	9	9	8		9	8	8	10	9	9	10	10	9	10	10	10	10	10	10
Efre, Exin	10	10	10	10	10	9	10	10	10	10	10	9	10	10	10				10	10	10	10	10	10
BjapI, BjapIa	10	10	9	9	9	9	10	9	9	9	9	9	10	10	9	9			10	10	10	10	10	10
Belk, BjapI, BjapIa	10	9					10	10	9	10	10	9							10	10	10	10	10	
Belk, Rhps				8		9																		
BjapI, BjapIa, Rhps																10	9	9						8
Belk, BjapI, BjapIa, Rhps	10	10	9	10	9	9	10	10	9	10	9	9	10	10	9	10	10	10	10	10	10	10	10	10
Meso	10	10	10	10	10	10	10	9	10	9	9	9	10	10	10	10	10	9	10	10	9	10	10	10
All except Caul, Rhps, Brad	9	9		9	9					10	10	9	10	10		10	10		10	10	10	10	10	10
All except Caul, Rhps, Brad, Meso	9	8																	9	8		10	9	
All except Caul, Rhps, Brad, Meso, Bruc				8			8						9	8					9	9	9	9	10	10
Rgal, Rhuat				9	9					9						10	10	8	9	9	10	10	10	10
Rgal, Rhuat, Arad, Atum, Arub				8															9		8	9	8	8
Rgal, Rhuat, Rgiar										8	8	8												
Rgal, Rhuat, Rgalli, Rleg, Rrhi																		8						
Rrhi, Rleg																		9	8	8				
Esah, Eter																		8						
Rgalli, Rleg							8						9		8				9	9	9		8	
Rgalli, Rleg, Rrhi																8			9	9	10	10	10	10

*Arad, *Agrobacterium radiobacter*; Arub, *Agrobacterium rubi*; Atum, *Agrobacterium tumefaciens*; Belk, *Bradyrhizobium elkanii*; BjapI, *Bradyrhizobium japonicum* I; BjapIa, *Bradyrhizobium japonicum* Ia; Brad, *Bradyrhizobium*; Bruc, *Brucella*; Caul, *C. crescentus*; EadhABC, *E. adhaerens* gv. A, B and C; Earb, *E. arboris*; Efre, *E. fredii*; Emed, *E. medicae*; Emel, *E. meliloti*; Ensi, *Ensifer*; Esah, *E. saheli*; Eter, *E. terangae*; Exin, *E. xinjiangensis*; Meso, *Mesorhizobium*; Rgal, *Rhizobium galegae*; Rgalli, *Rhizobium gallicum*; Rgiar, *Rhizobium giardinii*; Rhps, *Rhodopseudomonas palustris*; Rhuat, *Rhizobium huaulense*; Rleg, *Rhizobium leguminosarum*; Rrhi, *Rhizobium rhizogenes*; Smor, ‘*S. morelense*’ (2 strains).

appeared to level out aberrant signals that may be present in partly incongruent partitions, to yield trees that are highly similar, both in topology and bootstrap support, to those obtained by concatenating only congruent partitions (Fig. 3).

Significantly grouped taxa

Table 4 presents a simple but effective way of assessing the most strongly supported groups in all 24 NJ, MP and ML analyses. As was expected, the two reference strains of

Fig. 2. Phylogenetic trees based on six genes calculated using the ML method. The complete genome sequence of *C. crescentus* strain CB15 was used as the outgroup. Bootstrap values of 75 % or more (using 100 replicates) are indicated at branch points. Bars, 0.1 expected nucleotide substitutions per site. A., *Agrobacterium*; B., *Bradyrhizobium*; Bru., *Brucella*; R., *Rhizobium*; Rp., *Rhodopseudomonas*.

Fig. 3. Phylogenetic trees based on concatenated sequences of *dnaK*, *glnA* and *thrC*, calculated using NJ, MP and ML. Strains used are the same as in Fig. 2. Bootstrap values of 75 % or more (using 1000 replicates for NJ and MP; 100 for ML) are indicated at branch points. Bars, 0.1 estimated nucleotide substitutions per site (NJ and ML); 10 base changes between nodes (MP). A., *Agrobacterium*; B., *Bradyrhizobium*; Bru., *Brucella*; R., *Rhizobium*; Rp., *Rhodopseudomonas*.

Brucella and those of *Mesorhizobium* each grouped together always. Similarly, *Rhodopseudomonas palustris* always grouped with *Bradyrhizobium* species, peripheral to the *Bradyrhizobium* cluster in the *recA*, *gltA*, *thrC* and *glnA* phylogenies and inside the *Bradyrhizobium* cluster according to 16S rRNA and *dnaK* gene phylogenies. In the case of *dnaK*, *Rhodopseudomonas palustris* grouped closer to *Bradyrhizobium elkanii* whereas, according to the 16S rRNA

gene phylogeny, the same species is closer to *Bradyrhizobium japonicum* groups I and Ia. *Agrobacterium radiobacter*, *Agrobacterium tumefaciens* and *Agrobacterium rubi* were grouped together with high bootstrap support in all analyses except the *dnaK* phylogeny. *Rhizobium galegae* and *Rhizobium huaulense* grouped together with high bootstrap support in the 16S rRNA gene and in the two combined phylogenies, whereas *Rhizobium gallicum*,

Fig. 4. Phylogenetic trees based on concatenated sequences of *dnaK*, *glnA* and *thrC*, *gltA*, *recA* and 16S rRNA genes calculated using NJ, MP and ML. Strains used are the same as in Fig. 2. Bootstrap values of 75 % or more (using 1000 replicates for NJ and MP; 100 for ML) are indicated at branch points. Bars, 0.1 estimated nucleotide substitutions per site (NJ and ML); 100 base changes between nodes (MP). A., *Agrobacterium*; B., *Bradyrhizobium*; Bru., *Brucella*; R., *Rhizobium*; Rp., *Rhodopseudomonas*.

Rhizobium leguminosarum and *Rhizobium rhizogenes* grouped together with significant support only in the combined analyses. In *Ensifer*, several groupings recurred, but not always with strong bootstrap support. A close relationship between *E. meliloti*, *E. arboris* and *E. medicae* was strongly supported. *E. adhaerens* grouped particularly with 'S. morelense'; bootstrap support for this relationship was strong in the *glnA* and *thrC* phylogenies and the

combined analyses. For 'S. morelense' we included the type strain and strain LMG 20571. The latter strain was previously assigned as representing 'S. morelense' on the basis of phenotypic and protein profile similarities (Willems *et al.*, 2003). Our present data suggest that it may actually represent a separate but closely related genospecies. Our data do not support the proposal (Young, 2003) that 'S. morelense' is a later heterotypic synonym of *E. adhaerens*, but

confirm that it represents a separate species, as has been established previously (Willems *et al.*, 2003). The name of the species '*S. morelense*' is considered to not be validly published because, at the time of publication, the type strain was not deposited in two publicly accessible culture collections in different countries (Euzéby & Tindall, 2004). The type strain is now available from the BCCM/LMG (Belgium, <http://bccm.belspo.be/about/lmg.php>) and NBRC (Japan, <http://www.nbrc.nite.go.jp/e/index.html>) culture collections and thus fulfils the criteria for validation. However, because the name is the subject of a pending Request for an Opinion (Euzéby & Tindall, 2004), '*S. morelense*' cannot be transferred to the genus *Ensifer* before a ruling is published. The name of the species will only be established when an opinion from the Judicial Commission is approved and published. Therefore, at present we cannot adopt the *Ensifer* nomenclature for this species and the species name '*S. morelense*' is used. It is evident from most trees that *Ensifer adhaerens* groups with the former *Sinorhizobium* species, although the bootstrap support for the whole group is strong only in the *thrC* phylogeny and in the two combined analyses of three and six genes. Our data thus support the proposal to unite the genera *Ensifer* and *Sinorhizobium*. The topologies of some trees suggest the possibility that *E. adhaerens* and '*S. morelense*' together form a separate group that may be regarded as a sister taxon to the rest of *Ensifer* (the former *Sinorhizobium*). However, bootstrap support is high only in the *thrC* phylogeny and in the two combined analyses. We tested this hypothesis by using the Shimodaira–Hasegawa test (Shimodaira & Hasegawa, 1999): for each gene, new ML trees (using the substitution models selected in MODELTEST) were constructed with the constraint that *E. adhaerens* and '*S. morelense*' should form a group, the remaining *Ensifer* species should form a second group and that the two groups form a single cluster separate from the other taxa included. The likelihood of the constrained tree was compared with the likelihood of the original unconstrained ML tree using the Shimodaira–Hasegawa test. The test showed significant support for the hypothesis in all five housekeeping genes and for the 16S rRNA gene (Table 5). Running the test for the concatenated

dataset of *dnaK*, *glnA* and *thrC* and for the concatenation of the six genes yielded *P* values of 1 and 0.1454, respectively.

Taxonomic conclusions

An important observation from our data is that, according to ILD tests, the phylogenetic signal of the 16S rRNA gene is not congruent with that of the five housekeeping genes used. This is in line with reports of possible recombination events among 16S rRNA genes of rhizobia (Eardly *et al.*, 1996; van Berkum *et al.*, 2003; Vinuesa *et al.*, 2005). Taken together, these observations indicate that, at least in this group, 16S rRNA gene data should be interpreted with caution and should always be compared with data from other genes so as to minimize the impact of possible recombination events when assessing phylogenetic relationships for taxonomic purposes.

The new data presented here confirm the close relationship between *Ensifer* and the former genus *Sinorhizobium* and provide supporting evidence for uniting the two genera, as proposed by Young (2003). An alternative scenario in which *Ensifer* would be restricted to *E. adhaerens* and '*S. morelense*' and the genus *Sinorhizobium* maintained for the other *Ensifer* species had some support as apparent from the Shimodaira–Hasegawa test and analyses of concatenated sequences, but little support from the individual gene analyses. Furthermore, redefining genera in this group would require additional biological data. We conclude that, from the present evidence of six genes, classification as a single genus *Ensifer* is most appropriate.

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Table 5. Shimodaira–Hasegawa test

Gene	Δ -ln L*	P
16S rRNA	6.64264	0.1257
<i>glnA</i>	2.77208	0.3926
<i>gltA</i>	4.36147	0.3855
<i>thrC</i>	0	1
<i>recA</i>	2.05614	0.2005
<i>dnaK</i>	6.46891	0.2865
<i>dnaK</i> + <i>thrC</i> + <i>glnA</i>	0	1
Six genes	15.01006	0.1454

*L, likelihood.

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