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Minireview

Symbiovars in rhizobia reflect bacterial adaptation to legumes

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ABSTRACT

Legume specificity is encoded in rhizobial genetic elements that may be transferred among species and genera. Dissemination (by lateral transfer) of gene assemblies dictating host range accounts for the existence of the same biological variant (biovar) in distinct microbiological species. Different alternative biovars may exist in a single species expanding their adaptation to different niches (legume nodules). A review of all reported biovars is presented. Instead of the term biovar, symbiotic variant (symbiovar) is proposed as a parallel term to pathovar in pathogenic bacteria. Symbiovars should be determined based on the symbiotic capabilities in plant hosts, distinguished by the differences in host range and supported by symbiotic gene sequence information.

Introduction

Nitrogen fixing bacteria in legume nodules collectively designated as rhizobia have been known since 1888, reviewed in [58]. They were the first biofertilizers produced and allow savings of millions of dollars in chemical fertilizers [23] that may contaminate soil and water. Interest in these bacteria is increasing as plants to produce biofuels may profit from bacterial nitrogen fixation to attain a sustainable process. Nodule formation culminating in nitrogen fixation has been well studied and different symbiosis genes such as *nod*, *nif* and *fix* genes are known. Different host specificities may be determined by the symbiotic gene content. Excellent reviews have been published on the molecular basis of nodulation and on Nod factors, the modified lipochitooligosaccharides that induce nodule formation [10,11,16,27,43,56]. Nod factors, type 3 secretion systems and other rhizobial functions are needed to establish symbioses with legumes [43]. The *nod* gene similarity found in some cases in *Rhizobium*, *Agrobacterium* and *Sinorhizobium* species evidences the mobilization of symbiosis genes between these genera, reviewed in [41].

Biovars (biological variants) have been described in diverse bacterial species and reveal the different biochemical and enzymatic characteristics within a species. A biovar represents a group of bacterial strains distinguishable from other strains of the same species on the basis of physiological or biochemical characters. Biovars were formerly known as biotypes. In rhizobia biovars have been used to distinguish symbiotically distinct subgroups within a single rhizobial species. Biovars can be shared by different species due to the lateral transfer of symbiotic information. Biovars were first described in *Rhizobium leguminosarum* in a taxonomical revision of rhizobial species [28]. Since then biovars have been identified in other *Rhizobium* species, in *Ensifer* (*Sinorhizobium*), *Mesorhizobium* and *Bradyrhizobium* (Fig. 1A, Table 1, Supplementary Fig. 1) [2,5,35,45,59,62,66,76,77,79] but not so far in beta-rhizobia. Lateral transfer of *nod* genes from alpha rhizobia to *Burkholderia* and from these bacteria to *Ralstonia* seems to account for the existence of nodulating species in these genera [1,3,8,47].

Biovars in *Rhizobium*

Biovars viciae (nodulating pea), trifolii (nodulating clover) and phaseoli (nodulating *Phaseolus vulgaris* beans) were all ascribed to *R. leguminosarum* [28] considering that there was a single bacterial species (a common chromosomal background) that could alternatively contain symbiotic plasmids with different specificities. In a multilocus enzyme electrophoresis study (though with few metabolic enzymes), there were electrophoretic types common in all biovars supporting their belonging to a single taxon [82]. These results were confirmed by RFLP (restriction fragment length polymorphism) in hybridization assays using chromosomal probes showing the same pattern types in isolates from different host species [30]. Although there was a taxonomy revision of *R. leguminosarum* in 1984, officially the species *R. phaseoli* and *R. trifolii* were never rejected and according to taxonomy rules these species were still valid. When the *R. phaseoli* type strain (ATCC 14482) was characterized by DNA–DNA hybridization and sequencing of 16S rRNA, *recA* and *atpD* genes it was found that it was different from all described species and clearly did not correspond to *R. leguminosarum* bv. phaseoli [60]. The biovar of the *R. phaseoli* type strain (ATCC 14482) is bv. phaseoli as it nodulates *P. vulgaris* bean and has

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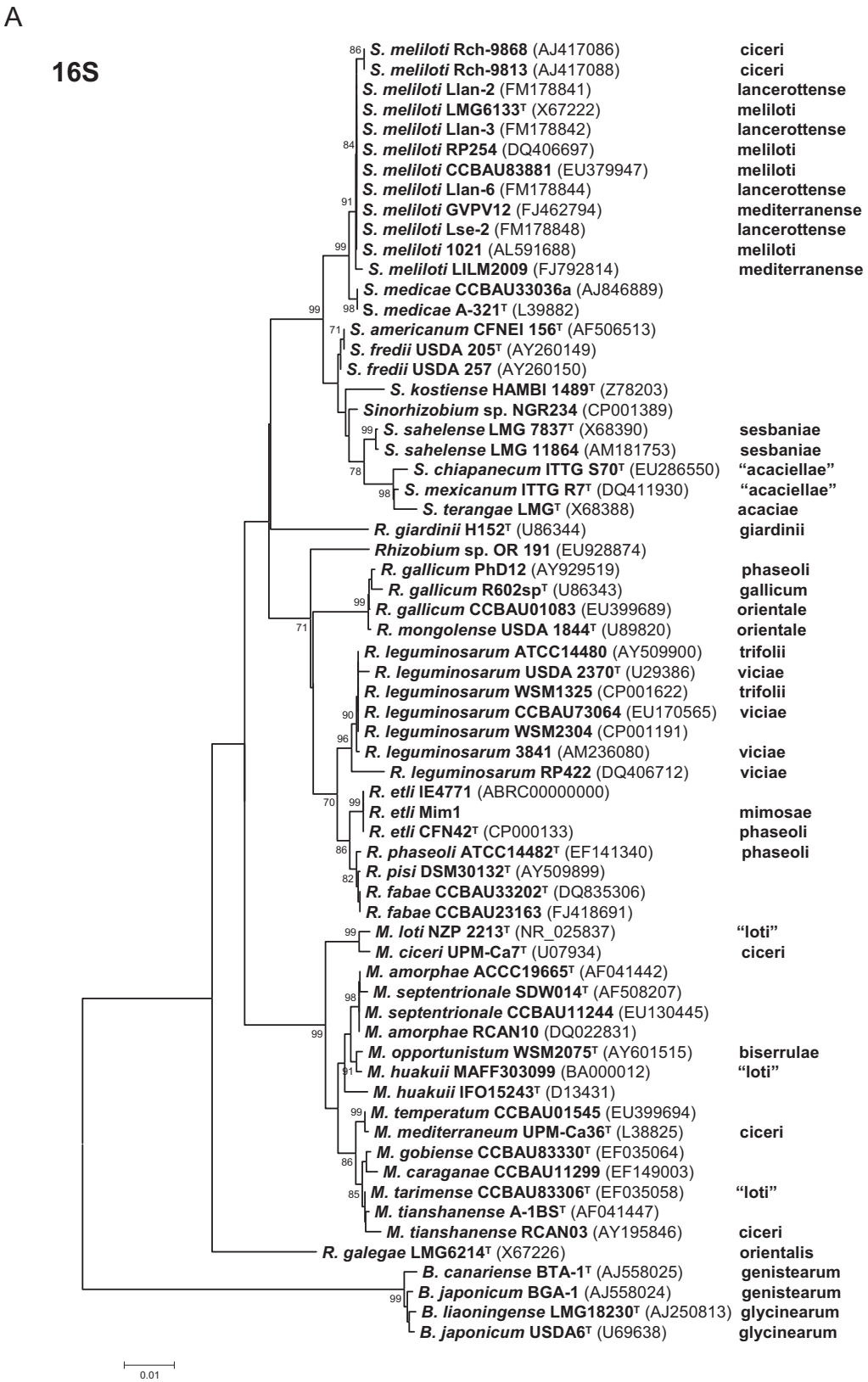

Fig. 1. Maximum likelihood phylogenies (A) 16S gene tree of rhizobial species with assigned biovars. Type strains are indicated with a superscript T. Biovars are indicated in the right column, (B) *nodC* gene tree, (C) *nifH* gene tree. Biovars names are indicated with brackets.

a *nodC* gene sequence as that found in bv. phaseoli [19]. Additionally when the *R. leguminosarum* type strain was similarly analyzed, there were some surprises: not all type strains from different collections were the same. DSM 30132 supposedly corresponding to *R. leguminosarum* type strain was different from other *R. leguminosarum* isolates and from the synonymous USDA 2370 type strain. Therefore DSM 30132 strain was assigned to a novel species *R. pisi* nodulating pea and other legumes [60] and its biovar is viciae

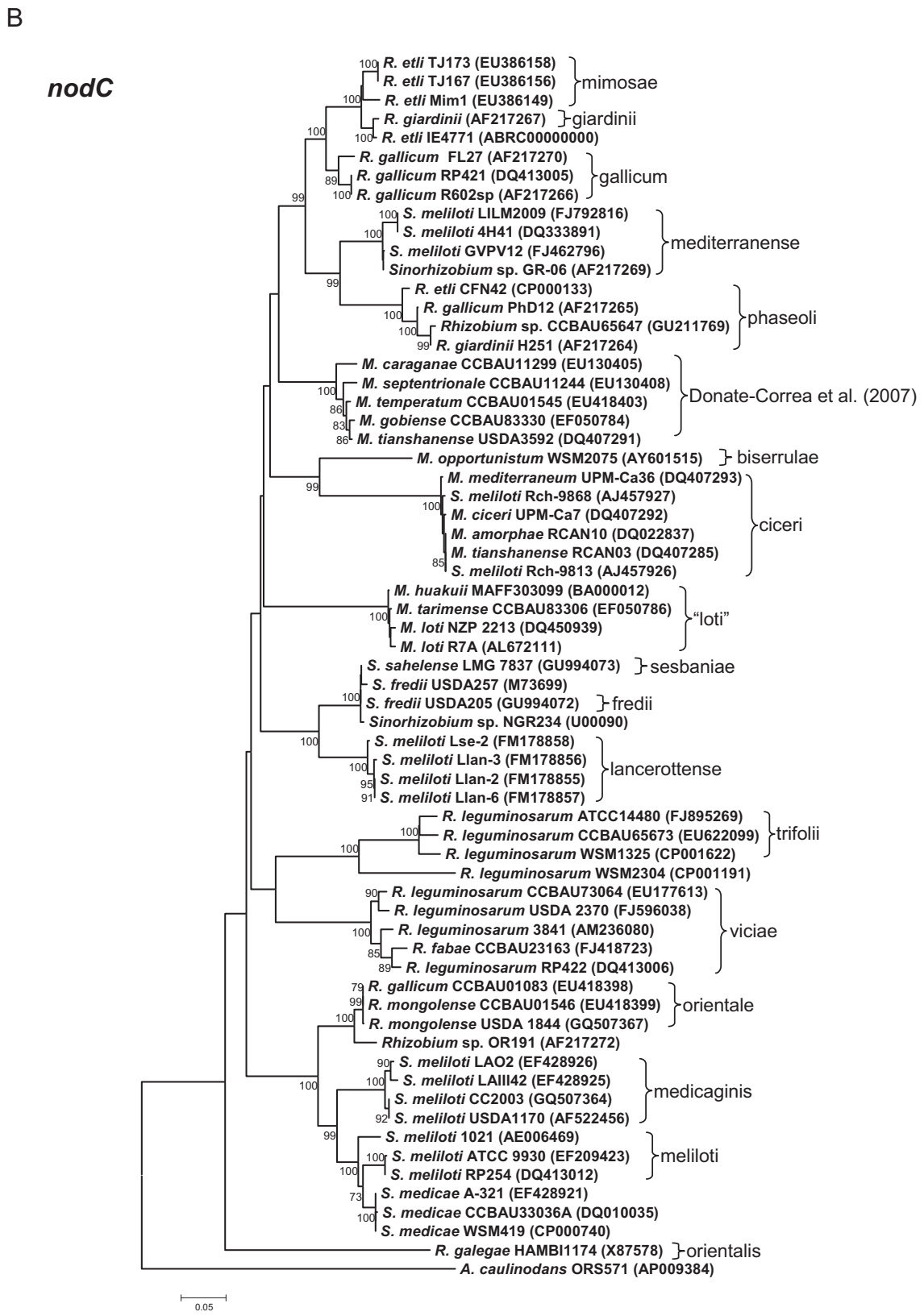

Fig. 1. (*Continued*)

(Velazquez, personal communication). This is also the biovar in the closely related *R. fabae* that was obtained from *Vicia faba* nodules in China and is capable of nodulating pea [71] with *nodC* genes similar to those found in bv. viciae (Fig. 1B), and related *nodA* genes as well (our own unpublished data).

A large number of isolates from *P. vulgaris* bean nodules in Spain corresponded to *R. leguminosarum* bv. phaseoli, the most frequently isolated species from *P. vulgaris* bean in that region [19]. A new revision of *Trifolium* nodulating strains based on the sequence of some genes and DNA–DNA hybridization showed that they should

C

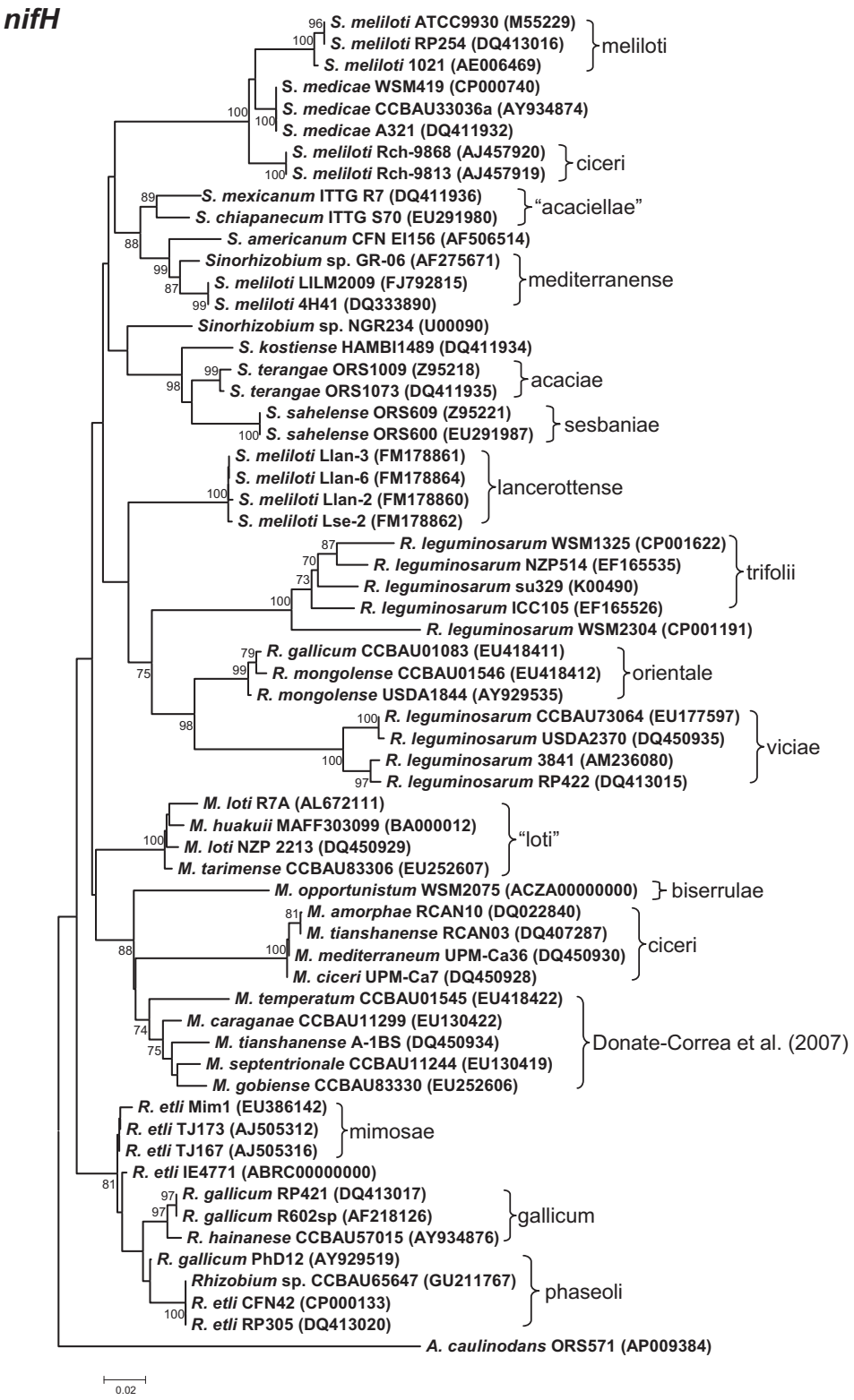

Fig. 1. (*Continued*).

be considered *R. leguminosarum* [60] and their biovar is bv. trifolii [60]. Comparisons of the gene sequences from the genomes of *R. leguminosarum* bv. viciae strain 3841 [83] isolated from pea in fields in England [26] and *R. leguminosarum* bv. trifolii strain WSM1325 (isolated from an annual clover, *Trifolium* sp. in Greece, R. Yates, PhD thesis, Murdoch University, 2008) shows their belonging to a single species, their different specificities (*Trifolium* versus *Pisum*) strongly supports the concept of biovars. However *R. leguminosarum* bv. trifolii strains WSM1325 and WSM2304 (isolated from the perennial *Trifolium polymorphum* in Uruguay [61]) do not

Table 1
Biovars in different rhizobial species and host legumes.

Biovar	Rhizobial species	Legume host	References
acaciae	*S. terangae*	*Acacia*	[37]
	S. sahelense	*Acacia*	[22]
	S. meliloti	*Acacia tortilis*	[5]
acaciellae	*S. chiapanecum*	*Acaciella angustissima*	This work
	S. mexicanum	*Acaciella angustissima*	This work
biserrulae	*M. opportunistum*	*Biserrula pelecinus*	[50]
ciceri	*M. amorphae*	*Cicer arietinum*	[63]
	M. tianshanense	*Cicer arietinum*	[63]
	M. ciceri	*Cicer arietinum*	[50]
	M. mediterraneum	*Cicer arietinum*	[51]
	S. meliloti	*Cicer arietinum*	[38]
gallicum	*R. gallicum*	*Phaseolus vulgaris*, *Leucaena leucocephala*	[2]
	R. giardinii	*Phaseolus vulgaris*, *Leucaena leucocephala*	[2]
genistearum	*B. japonicum*	*Genisteae*, *Loteae*	[77]
giardinii	*R. giardinii*	*Phaseolus vulgaris*, *Leucaena leucocephala*	[2]
glycinearum	*B. japonicum*	*Glycine*	[77]
lancerottense	*S. meliloti*	*Lotus lancerottense*	[34]
medicaginis	*S. meliloti*	*Medicago laciniata*	[76]
mediterranense	*S. fredii*	*Phaseolus vulgaris*	[45]
	S. meliloti	*Phaseolus vulgaris*	[45]
meliloti	*S. meliloti*	*Medicago sativa*, *Medicago truncatula*	[76]
mimosae	*R. etli*	*Phaseolus vulgaris*, *L. leucocephala*, *Mimosa affinis*	[79]
officinalis	*R. galegae*	*Galega officinalis*	[59]
orientalis	*R. galegae*	*Galega orientalis*	[58]
orientale	*R. mongolense*, *Rhizobium* spp.	*Medicago ruthenica*, *Phaseolus vulgaris*	[66]
phaseoli	*R. gallicum*	*Phaseolus vulgaris*	[2]
	R. giardini	*Phaseolus vulgaris*	[2]
	R. leguminosarum	*Phaseolus vulgaris*	[28]
	R. etli	*Phaseolus vulgaris*	[64]
	R. phaseoli	*Phaseolus vulgaris*	[60]
sesbaniae	*S. terangae*	*Sesbania*	[37]
	S. sahelense	*Sesbania*	[37]
	Agrobacterium sp.	*Sesbania*	[12]
trifolii	*R. leguminosarum*	*Trifolium*	[28]
viciae	*R. leguminosarum*	*Vicia sativa*	[28]
	R. fabae	*Vicia faba*	[71]
	R. pisi	*Vicia sativa*	[60]

have the same specificity and nodulate and fix nitrogen in different *Trifolium* species from distinct geographical origin [81]. In theory they should not correspond to the same biovar. Only WSM 1325 corresponds to bv. trifolii, then WSM 2304 should deserve a distinct biovar. In agreement *nodD* genes (that recognize plant flavonoids and regulate the expression of other *nod* genes) were more divergent than chromosomal genes and *nolR* was found only in WSM1325 and not in WSM2304; probably they produce different Nod factors [81, Yates, PhD thesis, Murdoch University, 2008].

Diversity of *R. leguminosarum* bv. viciae was determined by the plant [14,24,49]. Furthermore, not all *R. leguminosarum* bv. viciae strains exhibited the same host range when tested with different legumes [49] suggesting that biovars may be more complex than we think; probably subtle genetic differences may have specificity effects. However the species designation of such isolates should be revised considering the novel related species recently described.

Rhizobium gallicum bv. gallicum and bv. phaseoli were found in *P. vulgaris* bean nodules in Europe [2]. Later *R. gallicum* bv. gallicum was also found in *Phaseolus coccineus* and in few *P. vulgaris* nodules in Mexico [65] and in Tunisia [44]. *R. gallicum* was reported from other legumes such as *Oxytropis* and *Onobrychis* in Canada [32] and in other sites, reviewed in [65]. Biovars gallicum and phaseoli have different *nod* gene sequences and host ranges, biovar gallicum has a broad host range including *Leucaena* while bv. phaseoli strains do not form nodules in this host.

Biovar gallicum and biovar giardinii *nod* genes are related to those found in *Mimosa* nodulating bacteria including bv. mimosae from *R. etli* and also *R. tropici* [42]. *R. giardinii* bv. giardinii and bv. phaseoli were found as well in *P. vulgaris* bean nodules and were distinguished by their different specificities [2], they seem to be less efficient for nitrogen fixation in *P. vulgaris* bean than other rhizobia.

Biovar phaseoli is found in *R. etli*, *R. phaseoli*, *R. leguminosarum*, *R. gallicum* and in *R. giardinii* (Supplementary Fig. 1) with conserved *nodC* and *nifH* genes [31]. The phaseoli symbiotic plasmid of *R. etli* strains is well conserved in nucleotide sequences as well as in gene content, as shown in a recent comparative genomic study [20]. It remains to be established if the symbiotic plasmid is also conserved in the other species harboring bv. phaseoli.

In addition to bv. phaseoli, a new biovar was described in *R. etli*, biovar mimosae, for *Mimosa* nodulating bacteria, [79], that is considered the ancestral biovar in *R. etli*. Our recent analysis of the genome of *R. etli* bv. mimosae Mim1 showed that there is an extensive identity to *R. etli* bv. phaseoli strains CFN42 and CIAT 652 in the chromosome and in some of the plasmids (supporting their belonging to the same species) but not in the symbiotic plasmids. The conserved plasmids but not the symbiotic plasmid corresponded to the recently defined chromids [21]. Differences in the overall gene content of the bv. phaseoli and bv. mimosae symbiotic plasmids were observed in addition to differences in the *nod* and *nif* gene sequences. Genes involved in the biosynthesis of sulfated Nod factors were found in bv. mimosae but not in bv. phaseoli (Rogel et al. unpublished). *R. etli* strain IE4771 that was considered as corresponding to bv. mimosae [66] seems to correspond to bv. giardinii (Fig. 1B). Although bv. giardinii and bv. mimosae are closely related in *nodC* gene phylogenies (Fig. 1B), the genomic comparison of the respective symbiotic plasmids in Mim1 and IE4771 showed that they are significantly different (unpublished) suggesting different evolutionary histories. If *nodB* and *nifH* gene sequences are indica-

tive of overall differences in symbiotic plasmids, then there seems to be some heterogeneity inside bv. phaseoli [65,66]. Different alleles of *nodC* genes are known as well [19]. Additionally different Nod factors have been reported among *R. etli* bv. phaseoli strains [55] as well as differences in the regulation of nitrogen fixation, reviewed in [40]. A characteristic of biovar phaseoli is the multiple (three) copies of the *nif* operon [57] (bv. gallicum carries a single *nifH* copy, and bv. mimosae 2 *nifH* gene copies). A *nodA* gene separated from the common *nod* operon was observed in bv. phaseoli strains [74] but such *nod* gene organization is not observed in *R. etli* bv. mimosae. Different *nifH* gene hybridization patterns exist in bv. phaseoli strains [39]. Although *nifH* genes do not determine host specificity, they represent characteristic markers of symbiotic plasmids and they clearly allow the distinction and grouping of biovars (Fig. 1C).

Rhizobium mongolense was isolated from *Medicago ruthenica* from Mongolia [73]. It was argued that *R. mongolense* may be considered as *R. gallicum sensu lato* [66] and as synonymous with *R. gallicum* (E. Velázquez, personal communication). Interestingly *R. mongolense nodB* genes resemble the respective genes from sinorhizobia that nodulate *Medicago* and biovar orientale was proposed [66]. In contrast *R. mongolense nifH* genes resembled *R. leguminosarum* bv. viciae. In spite of the similarities in *nodB* genes, the existence of biovar orientale in both *R. mongolense* and in *R. yanglingense* [66] is doubtful due to the differences in legume specificities.

Rhizobium galegae strains nodulate the legumes *Galega orientalis* and *G. officinalis* from the Caucasus and biovars orientalis and officinalis were proposed in relation to host nodulation and differences in sequences of *nod* genes were related to the legume species [59]. Later the bacteria from the two distinct biovars were found to correspond to different groups in AFLP analysis and it was suggested that *R. galegae* was diverging into subspecies perhaps driven by host specificity [70]. Consequently no evidence of recombination was detected between the biovars [4].

R. huautlense, a related species to *R. galegae*, that forms nodules in *Sesbania* has *nodA* genes similar to sinorhizobia biovar sesbaniae [62], meaning that there are peculiar genetic determinants for *Sesbania* nodulation [36]; however, symbiosis genes in *Azorhizobium* and in mesorhizobia nodulating *Sesbania* are not related. Interestingly *nodA* and *nifH* genes in *Agrobacterium* strain IRBG74 nodulating *Sesbania* are similar to those from bv. sesbaniae of *Sinorhizobium* [12] and probably the biovar in IRBG74 is bv. sesbaniae. *R. huautlense* and *Mesorhizobium plurifarium* from *Sesbania* in South America did not equally nodulate distinct *Sesbania* species and other legumes and it was suggested that different biovars may exist in both species [78].

Biovars in *Sinorhizobium*

Sinorhizobium sahelense (corrected name of *S. saheli*) and *S. terangae* were isolated in Africa from *Acacia* and *Sesbania* trees and were found to be closely related in 16S rRNA and *nifH* gene phylogenies. Biovars sesbaniae and acaciae were described in both *Sinorhizobium sahelense* and *S. terangae* [7]. Different Nod factors are produced by the different biovars [35,36]. *nodA* genes of the biovar acaciae from the different species *S. terangae*, *S. sahelense*, and *S. arboris* are similar [62].

S. meliloti is the best studied rhizobial species in regard to the molecular mechanisms involved in plant nodulation. It is well known for its capacity to nodulate alfalfa (*Medicago sativa*) plants but also forms nodules in *Trigonella* and *Melilotus*. Novel biovars, bv. acaciae [5], bv. medicaginis [76] and bv. mediterranense [45] besides bv. meliloti and probably bv. ciceri [38] were recognized in *Sinorhizobium meliloti* (Table 1, Figs. 1 and Supplementary Fig. 1). *S. meliloti* bv. acaciae was obtained from *Acacia tortilis* nodules and produces a Nod factor similar to that produced by *S. terangae* bv. acaciae and by other rhizobia and mesorhizobia nodulating acacias [5]. *S. meliloti* strains that effectively nodulated *P. vulgaris* beans corresponded to bv. mediterranense, their *nodC* and *nifH* genes were not related to those of bv. meliloti nor to bv. phaseoli but were more closely related to those of Mediterranean *Sinorhizobium fredii* strains nodulating *Phaseolus vulgaris*; they are salt tolerant.

Isolates from *Medicago laciniata* that also nodulate *M. sauvagei* but not *M. truncatula* were classified as *S. meliloti* by chromosomal characteristics such as 16S rRNA genes and DNA–DNA hybridization but different host range, sequence of *nodA* and RFLP patterns of *nifDK* genes justified its designation as a novel biovar, medicaginis.

Additionally, another biovar, lancerottense, has been reported in *S. meliloti* with distinct symbiotic genotypes and effectively nodulating *Lotus lancerottensis* [34]. These isolates did not nodulate *Medicago* and this was the first time that *S. meliloti* was described as symbiont of *Lotus*; the isolates seem to be the preferred symbionts of *L. lancerottensis*. The *Lotus* isolates were tolerant to salinity and alkaline conditions [34].

nodC genes from bv. medicaginis and bv. meliloti are related (Fig. 1B). It is interesting to note that bv. mediterranense *nodC* gene cluster is related to the cluster bv. phaseoli and those genes from bv. lancerottense are more similar to those from *S. fredii* USDA 257 and NGR234. This similarity was not observed with *nifH* genes: the *nifH* phylogeny showed no close relationship between bv. mediterranense and bv. phaseoli (Fig. 1C), nor between *S. fredii* and *S. meliloti* bv. lancerottense [34]. *nif* and *nod* gene phylogenies are not congruent in some cases (Fig. 1A and B) meaning that recombination has had a role in the evolution of biovars. Genetic rearrangements have been observed in *R. etli* biovar phaseoli [18]. In addition to recombination, gene loss and gain may be responsible for generating particular gene assemblies that eventually determine biovars. Horizontal gene transfer and recombination drive the diversity of sinorhizobia associated with *Medicago* [6]. Although NGR234 clusters with bv. fredii by *nodC* gene sequences (Fig. 1), its remarkably broader host range and its lack of soybean nodulation would place NGR234 out of biovar fredii.

Biovar mediterranense was designated in *S. fredii* for strains with specificity for *Leucaena leucocephala* and *P. vulgaris* and unable to nodulate soybean [45]. This biovar was also identified in *S. meliloti* as described above. It was argued that bv. mediterranense was not the bv. in *Sinorhizobium mexicanum* or *S. chiapanecum* in spite of the similarities in *nod* gene sequences because bv. mediterranense strains did not efficiently nodulate *Acaciella angustissima*, the original host for *S. mexicanum* and *S. chiapanecum* [62]. *S. mexicanum* or *S. chiapanecum* have the same host specificity (for *Acaciella*) and very similar symbiosis genes, their corresponding biovar should be named bv. acaciellae.

Biovars in *Mesorhizobium*

Mesorhizobium amorphae biovar ciceri and *M. tianshanense* biovar ciceri nodulate chickpea [63], whereas the originally described species do not nodulate chickpea. *M. amorphae* nodulates *Amorpha fruticosa* [80] and *M. tianshanense* nodulates various legumes native to arid China [9], thus the different biovar determines alternative specificity. *M. ciceri* [52] and *M. mediterraneum* [51] were described as species nodulating chickpeas (*Cicer arietinum*). In *M. ciceri* two biovars were described, biserrulae and ciceri distinguished by different *nodA* and *nifH* gene sequences and by host specificities [50]. Isolates corresponding to bv. biserrulae do not nodulate chickpea and those from bv. ciceri do not nodulate *Biserrula pelecinus*. Do chickpea nodulating bacteria share symbiosis genes? A conserved *nodC* gene [33] was found in all species nodulating chickpea (Fig. 1B).

Both *Mesorhizobium huakuii* and *M. huakuii* bv. renge [48] form nodules in milkvetch (*Astragalus sinicus*) that is used as green manure in rice crops. By nodulating the same host, bv. renge does not meet the criterium to be considered as a different *M. huakuii* biovar. Differences in 16S rRNA gene sequences and sensitivity to bacteriophages led to the proposal of subspecies in *M. huakuii*. Strains corresponding to bv. renge were found to belong to one of the subspecies further supporting their misclassification as bv. renge [53].

The *Mesorhizobium* strain MAFF 303099 formerly considered as *Mesorhizobium loti* has been classified as belonging to *M. huakuii* [72]. A biovar loti having the capacity to nodulate *Lotus* seems to be contained in different mesorhizobial species [34].

Different *Mesorhizobium* species that nodulate the shrub *Anagyris latifolia* have similar symbiosis genes (Fig. 1B and C). All these species probably share a single novel biovar in mesorhizobia [15].

Biovars in *Bradyrhizobium*

Biovar genistearum was found in *B. canariense* and in *B. japonicum* meaning that both species shared the capacity to nodulate genistoid plants, brooms [77]. Biovar glycinearum was described in *B. japonicum* and *B. liaoningense* strains that nodulate soybean. It is conceivable that symbiotic islands could be exchanged among related *Bradyrhizobium* species as has been described in *Mesorhizobium* strains [68,69]. In *Bradyrhizobium* genomic islands have been identified [25] but up to now symbiotic plasmids have not been found although other plasmids exist in this genus [13].

Is the term biovar adequate to define symbiotic capabilities in rhizobia?

In *Agrobacterium* three biovars were recognized for a long time. They now correspond to different species [54] and even to different genera [67,75]. The term biovar as used in *Agrobacterium* and in other bacteria has not the same connotation in rhizobia. The term biovar in *Rhizobium* as reviewed here has been used to refer to the symbiotic capabilities and it would be more adequate or appropriate to use the term symbiotic variant (abbreviated symbiovar) as a parallel term to pathovar in pathogenic bacteria. A revision to the International Standards for naming pathovars of phytopathogenic bacteria [17] was published in 1991. Pathovars are defined "on the basis of distinctive pathogenicity to one or more plant hosts." "Usually pathovars are distinguished in terms of proved differences in host range. Clear differences in symptomatology on the same plant species can warrant different pathovar designations." Similarly symbiovars should be defined on the basis of the symbiotic capabilities in plant hosts distinguished by the differences in host range. If different plant effects (symptomatology in the case of pathogens) would be taken into account, then the efficiency in nitrogen fixation should also be evaluated and considered. As symbiotic gene sequences are commonly analyzed in rhizobial studies then the proposal of a biovar should be additionally supported with sequence data of symbiosis genes. Gene sequence data would be particularly useful when dealing with promiscuous hosts. Symbiovars would reflect a successful assembly of genes (some maybe yet unknown) that provide suitable host specificity. A symbiovar is determined by a symbiotic plasmid or island but may be conditioned as well by other replicons (chromosome or plasmids) carrying symbiotic determinants. A particular symbiovar may be maintained in different diverging bacteria lineages (it seems that preferentially in related species) by lateral transfer of symbiotic information.

Differences in one or few symbiosis genes may have specificity effects as has been clearly shown in *B. japonicum* [29,46]. The genetic basis of host specificity needs to be further studied in plants and in rhizobia and will provide a better understanding of symbiovars that will also derive from genomic studies. An extensive analysis of bradyrhizobial specificity and *nodA* genes in relation to the presence of other different *nod* genes that modify Nod factors has been published [46].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.syapm.2010.11.015.

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