



## Multilocus sequence analyses reveal several unnamed *Mesorhizobium* genospecies nodulating *Acacia* species and *Sesbania sesban* trees in Southern regions of Ethiopia

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### ABSTRACT

Leguminous trees play an important role in agroforestry in Ethiopia, but studies of their rhizobial symbionts are scarce. In earlier studies, we surveyed natural nodulation of native leguminous trees growing in different agro-ecological zones in Southern Ethiopia, isolated 400 rhizobia, and characterized them based on different phenotypic and genotypic methods. In the present study we characterized 18 strains belonging to the genus *Mesorhizobium*, isolated from nodules of *Acacia abyssinica*, *A. senegal*, *A. tortilis* and *Sesbania sesban*. Phylogenetic analysis of nearly full-length 16S rRNA gene grouped the test strains into three distinct clades separated from all currently recognized *Mesorhizobium* species. Three divergent strains formed separate branches while the other 15 strains formed three distinct groups, genospecies I–III. Grouping of the isolates under study based on the house-keeping genes *recA*, *gyrB*, *rpoB* and *gltA* were consistent and in agreement with that of 16S rRNA. Similarly phylogenetic relationships based on the symbiosis-related genes *nodC*, *nodA* and *nifH* were generally similar to those shown by the core genes, suggesting that these *Acacia* and *Sesbania* symbionts have a long history of separate evolution within *Mesorhizobium*. Cross inoculation experiments demonstrated a large variation in the ability of the test strains to elicit effective nodules. The *Sesbania* isolates, occupying a distinct clade in the *nodC* phylogenetic tree, formed effective nodules only with this host legume. The study strongly suggests that this collection of *Mesorhizobium* strains comprises several new species, and also indicates the role of the symbiotic genes in determining the host range of these bacteria.

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### Introduction

The importance of acacias and other leguminous trees in the rural economy of Africa cannot be overemphasized; they are sources of fuel, timber, gum, tannins and forage [54], they tolerate harsh environmental conditions such as drought and can grow in alkaline soils. Their capacity to restore and maintain soil fertility makes them a useful component of traditional agroforestry throughout dry lands of Africa [31]. *Acacia* is one of the largest genera in the Leguminosae, which comprises about 1250 species, including 134 indigenous species only in Africa [36]. Like most legumes, acacias form nitrogen-fixing symbiosis with rhizobia, a biological property that enables them to colonize and grow on poor low-N soils. Some *Acacia* species are considered fast growing trees and have records of good symbiotic nitrogen fixation capacities [10,55]. Different species of *Rhizobium*, *Sinorhizobium*,

*Mesorhizobium* and *Bradyrhizobium* have been isolated from nodules of various *Acacia* species [7,8,26]. The ecological distribution and abundance of the different *Acacia* species is mainly explained by altitude and rain fall gradients. Most *Acacia* species, including *A. nilotica*, *A. seyal*, *A. senegal* and *A. tortilis*, are abundant in low lying dry savanna regions, while others such as *A. lahai* and *A. abyssinica* grow at higher altitude and hillsides [54]. A subspecies of the latter, *A. abyssinica* Benth. subsp. *abyssinica* is native only to Ethiopia [36] and is an important upper storey shade tree to young coffee plants in the traditional low-input agroforestry practices, where it plays a useful role in nutrient cycling [24]. However, its rhizobial symbionts are not explored at all.

There are about 50 species within the legume genus *Sesbania*, which are widespread in the warmer latitudes of both hemispheres of the world [3]. Many species are annual or short-lived perennials which, because of their fast growth, are used in agriculture as green manure or fallow crops. *Sesbania sesban* (L.) Merrill (synonym: *S. aegyptiaca*), a tree that grows to 8 m height, is native of Africa and found widely distributed in semi-arid to sub-humid regions throughout the continent. Like *Acacia* spp., it is an important

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multipurpose tree and widely used in tropical agroforestry [13,15]. *S. sesban* is reported to fix 500–600 kg N/ha/year and is particularly promoted for soil fertility replenishment through ‘improved fallow’ agroforestry practice in East and Southern Africa [23,42]. Although rhizobia nodulating *S. sesban* were considered to have a restricted host range and to be specific to the genus *Sesbania*, they are nevertheless distributed, on the basis of the 16S rRNA homologies, on most of the major phylogenetic branches of the family *Rhizobiaceae* [3,28]. Young and Haukka [60] also reported that *Sesbania* species are able to form beneficial nitrogen-fixing symbiosis with fast-growing rhizobia which, in the current taxonomic status of the family *Rhizobiaceae*, could fall in any of the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium*, and *Azorhizobium*.

“Rhizobia” is a functional term used to designate soil bacteria that fix nitrogen (diazotrophy) after becoming established inside the root nodules of legumes. This provides nitrogen to the soil through leaf fall and root/nodule turnover, thus improving the fertility of the soil and its physical properties. Rhizobia are taxonomically diverse and have been found in many species and genera of the *Alphaproteobacteria* [38]. During later years symbiotic, nitrogen fixing bacteria associated with legumes have also been found which belong to the *Betaproteobacteria* [5] and *Gammaproteobacteria* [39]. Despite a record as center of origin and diversity for many legumes [32] as well as the potential of legumes for improving the nitrogen status of agricultural soils, investigations on legume microsymbionts of Ethiopia are scarce. This lack of knowledge prompted us to start a systematic collection and isolation of rhizobia from nodules of a large number of herbaceous and woody legume species growing in different agro-ecological regions of Southern Ethiopia [59]. Our biobank now consists of over 400 rhizobial strains, of which 198 strains [57] were previously characterized using several molecular biological techniques (AFLP, PCR-RFLP of the *rrn* operon genes, partial 16S rRNA gene sequence analyses) and BIOLOG<sup>®</sup> metabolic profiling. The results revealed a large taxonomic diversity in our collection, with several of the isolates showing metabolic and genetic profiles that were not related to reference species [57,59]. In the present study we further investigated 18 strains which, according to our previous analyses, grouped with members of the genus *Mesorhizobium*. The strains had all been isolated from three *Acacia* spp. and *Sesbania sesban* trees from soils at five different locations in Southern Ethiopia. In an effort to obtain a more reliable picture of their taxonomic positions, we applied multilocus sequence analyses (MLSA) in which five core genes (full-length 16S rRNA gene and the house-keeping genes, *recA*, *gyrB*, *rpoB* and *gltA*) were included. The *nodA*, *nodC* and *nifH* loci were also studied to compare core and symbiotic gene phylogenies. Furthermore, the symbiotic properties of the strains were evaluated by cross-inoculation to eleven different legume species. Such information is needed to improve the current understanding of the role of symbiotic genes in determining the host range of different rhizobia. From a practical point of view, it will also guide the selection of strains that are potentially interesting for development of inoculants.

## Materials and methods

### Bacterial strains and growth conditions

Strains used in this study are listed in Table 1. The 18 rhizobial strains from *S. sesban* and three *Acacia* spp. (*A. abyssinica*, *A. tortilis* and *A. senegal*) were obtained as part of an earlier study [59]. All the strains were kept in 15% (v/v) glycerol at –80 °C and cultured in 10 ml YMB at 28 °C for 5–6 days.

### DNA preparation

Total genomic DNA was isolated from 2 × 1.5 ml cultures using the procedure described previously by Boom et al. [4] with a slight modification using diatomaceous earth or Celite analytical filter aid as a DNA binding solid support [46].

### PCR amplification and gene sequencing

The following genes were studied. 16S rRNA, internal fragments of protein coding chromosomal genes (*recA*, *gltA*, *rpoB* and *gyrB*), and the symbiosis related genes (*nodA*, *nodC* and *nifH*). 16S rRNA gene was amplified and sequenced using primers fd1 and rd1 as described previously [53]. PCR amplification and sequencing of partial *recA* gene were undertaken according to [12] and *gltA* gene amplification and sequencing were carried out as previously described by Martens et al. [21]; while *rpoB* and *gyrB* gene amplification and sequencing were carried out following the PCR condition specified previously [22]. Partial sequences of the symbiotic genes *nodC* were carried out using the previously described primers (*nodCF* and *nodCI*) and PCR conditions as recommended by Laguerre et al. [19]. *nodA* gene amplification and sequencing were carried out as described previously [15]. For amplification and sequencing of partial *nifH* gene, primers and PCR conditions specified by Rivas et al. [34] were used.

### Phylogenetic analysis

The qualities of our sequences are evaluated by using sequence scanner software v1.0 in applied biosystem when needed. Nucleotide alignments for the test strains and the reference species in the genus *Mesorhizobium* were carried out using CLUSTAL W program from the MEGA version 4.0 software packages [45] and the same software packages were used to build neighbour-joining trees using Kimura's 2-parameter distance correction. Maximum likelihood analyses with a fully estimated GTR model were performed using the PhyML software [14]. The robustness of the tree topology was calculated from bootstrap analysis with 1000 replications of the sequences for neighbour-joining, and with 100 for maximum likelihood. Sequence similarity was calculated for each gene and each genospecies using pair-wise sequence alignments by Kimura-2 parameter distance correction, as implemented in MEGA version 4.0.

### Cross-inoculation test

All the strains were tested for infectiveness on eleven legume species (Table S1). Axenically pre-germinated seeds of each plant species were transplanted in triplicates to autoclaved Leonard jars containing sand and N-free nutrient solution, and inoculated with late lag-phase culture following [48]. Seedlings were grown under natural sunlight and temperature, harvested six weeks after inoculation and scored for nodulation as described in Table S1.

## Results

In this study, a total of 163 new sequences were generated and deposited in GeneBank (Table 1). Sequence data for nearly full-length 16S rRNA, the housekeeping genes *recA*, *rpoB*, *gyrB* and *gltA* and the symbiotic genes *nodA*, *nodC* and *nifH* were obtained for all 18 test strains. The only exceptions were four strains (AC99a, AC99b, AC99c, AC99e), isolated from *S. sesban*, for which the *nodA* primers implemented for other strains, failed to amplify this gene. In addition to the test strains, we sequenced the *rpoB*, *gyrB* and *gltA* fragments for a number of *Mesorhizobium* reference species for which such sequences were not previously available in the database (Table 1). For each gene, sequence data were aligned and used

**Table 1**  
Strains used, host of isolation, geographical origins and GenBank accession numbers for different genes generated in this study.

Strain	Host plant	Geographic origin	PCR-RFLP genotype <sup>a</sup>	AFLP <sup>b</sup>	Geno-species <sup>c</sup>	16S rRNA	recA	gyrB	rpoB	gltA	nifH	nodC	nodA
AC21a2	<i>Acacia tortilis</i>	Nazret	72	8	d	GQ847887	GQ848015	GQ847942	GQ847874	GQ847908	GQ847960	GQ847992	GQ847978
AC21c2	<i>Acacia tortilis</i>	Nazret	72	16	d	GQ847888	GQ848017	GQ847943	GQ847875	GQ847909	GQ847961	GQ847993	GQ847979
AC28c2	<i>Acacia tortilis</i>	RFC	68	4	I	GQ847889	GQ848016	GQ847944	GQ847876	GQ847910	GQ847962	GQ847994	GQ847980
AC39a	<i>Acacia abyssinica</i>	Chofa	68	10	I	GQ847890	GQ848018	GQ847945	GQ847877	GQ847911	GQ847963	GQ847995	GQ847981
AC39c1	<i>Acacia abyssinica</i>	Chofa	68	10	I	GQ847891	GQ848019	GQ847946	GQ847879	GQ847912	GQ847964	GQ847996	GQ847982
AC39d	<i>Acacia abyssinica</i>	Chofa	68	4	d	AY776192	GQ848020	GQ847959	GQ847878	GQ847913	GQ847965	GQ847997	GQ847983
AC39e1	<i>Acacia abyssinica</i>	Chofa	68	10	I	GQ847892	GQ848021	GQ847947	GQ847880	GQ847914	GQ847966	GQ847998	GQ847984
AC39e2	<i>Acacia abyssinica</i>	Chofa	68	10	I	GQ847893	GQ848022	GQ847948	GQ847881	GQ847915	GQ847967	GQ847999	GQ847985
AC98a	<i>Acacia abyssinica</i>	Wondogenet	71	15	III (b)	GQ847894	GQ848023	GQ847949	GQ847882	GQ847916	GQ847968	GQ848000	GQ847986
AC98b	<i>Acacia abyssinica</i>	Wondogenet	71	15	III (b)	GQ847895	GQ848010	GQ847950	GQ847865	GQ847917	GQ847969	GQ848001	GQ847987
AC98c	<i>Acacia abyssinica</i>	Wondogenet	71	15	III (b)	GQ847896	GQ848011	GQ847951	GQ847866	GQ847918	GQ847970	GQ848002	GQ847988
AC98e	<i>Acacia abyssinica</i>	Wondogenet	71	15	III (b)	GQ847897	GQ848012	GQ847952	GQ847867	GQ847919	GQ847971	GQ848003	GQ847989
AC99a	<i>Sesbania sesban</i>	Wondogenet	69	12	II	GQ847898	GQ848024	GQ847953	GQ847868	GQ847920	GQ847972	GQ848004	-
AC99b	<i>Sesbania sesban</i>	Wondogenet	69	12	II	GQ847899	GQ848025	GQ847954	GQ847869	GQ847921	GQ847973	GQ848005	-
AC99c	<i>Sesbania sesban</i>	Wondogenet	69	24	II	GQ847900	GQ848026	GQ847955	GQ847870	GQ847922	GQ847974	GQ848006	-
AC99e	<i>Sesbania sesban</i>	Wondogenet	69	24	II	GQ847901	GQ848027	GQ847956	GQ847871	GQ847923	GQ847975	GQ848007	-
AC100c	<i>Acacia senegal</i>	Leku	67	15	III (a)	GQ847902	GQ848013	GQ847957	GQ847872	GQ847924	GQ847976	GQ848008	GQ847990
AC100e	<i>Acacia senegal</i>	Leku	67	15	III (a)	GQ847903	GQ848014	GQ847958	GQ847873	GQ847925	GQ847977	GQ848009	GQ847991
<i>M. tianshanense</i> (HAMB1 1870 <sup>T</sup> )	<i>Glycyrrhiza uralensis</i>	Xinjiang	-	-	-	AF041447	AJ294368	GQ847931	GQ847885	GQ847904	-	-	-
<i>M. mediterraneum</i> (HAMB1 2096 <sup>T</sup> )	<i>Cicer arietinum</i>	Spain	-	-	-	L38825	AJ294369	GQ847939	FJ393284	GQ847905	-	-	-
<i>M. amorphae</i> (LMG 18977 <sup>T</sup> )	<i>Amorpha fruticosa</i>	Beijing	-	-	-	AF041442	AM076369	GQ847930	FJ393285	GQ847906	-	-	-
<i>M. septentrionale</i> (HAMB1 2582 <sup>T</sup> )	<i>Astragalus adsurgens</i>	Liaoning	-	-	-	AF508207	EF639843	GQ847940	GQ847884	GQ847907	-	-	-
<i>M. huakuii</i> (HAMB1 1674 <sup>T</sup> )	<i>Astragalus sinicus</i>	Nanjing	-	-	-	D12797	EU429341	GQ847936	GQ847883	AY094147	-	-	-
<i>M. thioanganeticum</i> (SJT <sup>T</sup> )	<i>Clitoria ternatea</i>	India	-	-	-	AJ864462	AM040610	GQ847933	GQ847886	-	-	-	-
<i>M. plurifarium</i> (LMG 11892 <sup>T</sup> )	<i>Acacia senegal</i>	Senegal	-	-	-	Y14158	AY494824	GQ847926	FJ393278	HM991863	-	-	-
<i>M. tarimense</i> (HAMB1 2973 <sup>T</sup> )	<i>Lotus frondosus</i>	Xinjiang	-	-	-	EF035058	EF549482	GQ847927	FJ393288	-	-	-	-
<i>M. gobiense</i> (HAMB1 2974 <sup>T</sup> )	<i>Oxytropis glabra</i>	Xinjiang	-	-	-	EF035064	EF549481	GQ847928	FJ393289	-	-	-	-
<i>M. caraganae</i> (HAMB1 2990 <sup>T</sup> )	<i>Caragana microphylla</i>	Liaoning	-	-	-	EF149003	EU249394	GQ847929	FJ393287	-	-	-	-
<i>M. chacoense</i> (LMG 19008 <sup>T</sup> )	<i>Prosopis alba</i>	Argentina	-	-	-	AJ278249	AM076370	GQ847932	FJ393294	-	-	-	-
<i>M. albizae</i> (LMG 23507 <sup>T</sup> )	<i>Albizia kalkora</i>	Sichuan	-	-	-	DQ100066	EU249396	GQ847934	FJ393286	-	-	-	-
<i>M. loti</i> (HAMB1 1129 <sup>T</sup> )	<i>Lotus corniculatus</i>	New Zealand	-	-	-	X67229	AM182156	GQ847935	FJ393277	-	-	-	-
<i>M. huakuii</i> (HAMB1 1674 <sup>T</sup> )	<i>Astragalus sinicus</i>	Nanjing	-	-	-	D13431	EU255219	GQ847938	FJ393283	-	-	-	-
<i>M. ciceri</i> (HAMB1 1750 <sup>T</sup> )	<i>Cicer arietinum</i>	Spain	-	-	-	U07934	DQ441457	GQ847937	FJ393282	-	-	-	-
<i>M. temperatum</i> (HAMB1 2583 <sup>T</sup> )	<i>Astragalus adsurgens</i>	Liaoning	-	-	-	AF508208	FJ619310	GQ847941	FJ393281	-	-	-	-
<i>M. shangrilense</i> (CCBAU 65327 <sup>T</sup> )	<i>Caragana bicolor</i>	China	-	-	-	EU074203	EU672501	-	-	-	-	-	-
<i>M. alhagi</i> (HAMB1 3019 <sup>T</sup> )	<i>Alhagi sparsifolia</i>	China	-	-	-	EU169578	-	-	-	-	-	-	-
<i>M. australicum</i> (LMG 24608 <sup>T</sup> )	<i>Biserrula pelecinus</i> L.	Australia	-	-	-	AY601516	-	-	-	-	-	-	-
<i>M. robiniae</i> (HAMB1 3082 <sup>T</sup> )	<i>Robinia pseudoacacia</i>	France	-	-	-	EU849582	GQ856501	-	-	-	-	-	-
<i>M. opportunistum</i> (WSM 2075 <sup>T</sup> )	<i>Biserrula pelecinus</i> L.	Australia	-	-	-	AY601515	-	-	-	-	-	-	-
<i>M. metallidurans</i> (STM 2683 <sup>T</sup> )	<i>Anthyllis vulneraria</i>	France	-	-	-	AM930381	AM930380	-	-	-	-	-	-

<sup>a</sup> *rrs* types as defined by PCR-RFLP of 16S rRNA [59].

<sup>b</sup> AFLP groupings from [56,57].

<sup>c</sup> Genospecies defined based on present study.

<sup>d</sup> Single strains possibly representing new genospecies.

to infer phylogenetic trees and to compare the placement of the test strain with respect to all currently designated *Mesorhizobium* species as references. Besides performing single-gene analyses, the gene sequences from the core genes were concatenated, and the composite tree and similarity values thus derived were used for further evaluation of the phylogenetic positions of the new isolates.

16S rRNA gene sequence analysis

Pair-wise comparisons of the 16S rRNA gene sequences revealed sequence similarities ranging from 95.7% to 99.3% when the 18 test strains were compared with the respective reference strains for recognized *Mesorhizobium* species (Table 2). In this analysis we also constructed the phylogenetic tree by including at least two reference species (up to five strains of *M. plurifarium*, owing to the heterogeneity of strains in this species). Similarity indices between 99.0% and 99.3% were found in only four of these cases, all of them were between test strains and *M. plurifarium* or *M. amorphae*. Pair-wise comparison between the test strains showed similarity values of 97.0–99.3%, with only two values being  $\geq 99.0\%$ . In the 16S rRNA gene phylogenetic tree, the test strains grouped into three distinct clades within the genus *Mesorhizobium*, two of these representing a well-supported new monophyletic clade that excluded any described species in the genus (Fig. 1a). Except for the three divergent strains AC21a2, AC21c2 and AC39d, which represented single branches, the other 15 test strains were distributed over the three clades, forming three tightly clustered distinct groups. These are in the following referred as genospecies I–III for clarity. Within genospecies III two subgroups (a and b) are represented.

Strains isolated from *A. abyssinica* at two different locations represented genospecies I and III subgroup b, and shared 99.3% and 98.0% sequence similarity with *M. plurifarium* (strain LMG 11892<sup>T</sup>) and *M. amorphae* (strain ACCC 19665<sup>T</sup>), respectively. Genospecies III subgroup a represented two strains isolated from *A. senegal* at another location and shared sequence similarity of 99.0% with *M. amorphae*. Genospecies II comprised a tightly clustered group isolated from *S. sesban* at Wondogenet, the same location where genospecies III subgroup b from *A. abyssinica* was obtained, and shared sequence similarity of 98.3% with both *M. amorphae* and *M. plurifarium*. The three strains AC21a2, AC21c2 and AC39d occupied single branches in the 16S rRNA gene phylogenetic tree (Fig. 1a) and shared sequence similarities varying from 97.3% to 99.0% when compared to the genospecies defined here (Table 2).

Analysis of individual housekeeping genes

The length of the alignment was 403 bp for *recA*, 616 bp for *gyrB*, 854 bp for *rpoB* and 650 for *gltA*. The resulting nucleotide sequences were translated into amino acid sequences. Based on the result obtained from this, some of the sequences do possess indels (2–11 indels) containing gap number that is not divisible by 3 for example Accession numbers GQ848017 and GQ848022 of *recA* gene, GQ847865 and GQ847866 of *rpoB* gene, GQ847926 of *gyrB*, GQ847906 and GQ847907 of *gltA*. Sequences of the same gene, within each genospecies defined, were highly similar, ranging from 99.3% to 100% for *recA*, 99.5% to 100% for *rpoB*, 99.9% to 100% for *gyrB* and 99.7% to 100% for *gltA*. In contrast, on a pair-wise comparison, a large gap in sequence similarity range was apparent between the different genospecies and the recognized *Mesorhizobium* reference species (4.7% to 14.6% for *recA*, 3.7% to 13.8% for *rpoB*, 7.8% to 68% for *gyrB* and 5.0% to 36% for *gltA*) (data not presented).

The phylogenetic trees of the individual housekeeping genes (Fig. 1b–e), generated from sequence alignments of the test strains and the described reference species, revealed tight clusters for all the different genospecies and were in agreement with groupings based on 16S rRNA gene (Fig. 1a), where the different genospecies

Table 2 Estimates of evolutionary identity between 16S rRNA sequences of the test strains (Table 1) and recognized *Mesorhizobium* species, based on pair-wise analysis of 23 sequences using Kimura's-2-parameter distance correction.

No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23			
1	GSI	≈100																								
2	GSI	97.8																								
3	GSI (a)	97.5	97.0																							
4	GSI (b)	97.9	97.2	97.1																						
5	AC21a2	98.0	97.6	97.0	97.4																					
6	AC21c2	98.9	98.8	97.6	97.6	98.4																				
7	AC39d	99.0	98.7	97.3	97.3	98.1	99.3																			
8	<i>M. loti</i>	97.0	97.2	97.0	97.3	97.0	97.0	100																		
9	<i>M. tianshanense</i>	97.7	97.1	97.0	97.1	97.1	98.2	98.3	100																	
10	<i>M. plurifarium</i>	99.3	98.3	97.6	97.6	98.0	98.8	99.1	97.9	98.9	100															
11	<i>M. amorphae</i>	99.1	98.3	99.0	98.0	97.8	98.8	98.7	98.4	99.3	99.5	100														
12	<i>M. septentrionale</i>	98.9	97.4	97.8	97.8	97.6	98.6	98.4	98.2	99.1	99.3	97.7	100													
13	<i>M. mediterraneum</i>	97.3	97.4	97.0	97.2	97.2	98.2	98.3	98.5	99.5	98.7	99.0	98.8	100												
14	<i>M. temperatum</i>	97.3	97.3	97.0	97.2	97.2	98.2	98.3	98.5	99.5	98.7	99.0	98.8	99.9	100											
15	<i>M. chacoense</i>	97.0	97.2	97.0	97.3	96.2	97.4	97.5	98.1	98.4	97.9	98.4	98.2	98.1	98.1	100										
16	<i>M. gobiense</i>	97.8	97.7	97.1	97.2	97.2	98.3	98.5	98.6	99.9	98.1	98.8	98.6	99.9	98.1	100										
17	<i>M. tarimensense</i>	97.8	97.5	97.1	97.2	97.2	98.3	98.5	98.6	99.9	98.1	98.8	98.6	99.9	99.9	100										
18	<i>M. metallidurans</i>	97.8	97.6	97.1	97.2	97.2	98.3	98.5	98.6	99.9	98.1	98.8	98.6	99.9	99.9	100										
19	<i>M. albizae</i>	97.0	97.1	96.8	97.0	96.8	97.9	98.0	98.5	97.5	97.9	97.7	99.0	98.7	98.1	98.6	100									
20	<i>M. huakuii</i>	98.1	97.4	97.2	97.8	97.3	97.9	98.1	96.3	97.5	98.7	98.9	97.6	97.6	97.1	97.6	97.6	100								
21	<i>M. thioangenicum</i>	96.2	96.3	96.3	96.1	95.7	96.5	97.3	96.1	96.1	97.3	97.2	97.0	96.8	96.9	96.8	96.8	97.2	100							
22	<i>M. caraganae</i>	97.5	97.2	97.1	97.0	97.2	98.1	98.1	99.5	97.8	98.3	98.1	99.4	99.4	97.9	99.9	99.9	98.7	98.1	100						
23	<i>M. ciceri</i>	97.0	97.2	97.2	97.1	97.0	97.1	97.0	99.9	98.3	97.8	98.2	98.0	98.5	97.5	98.5	98.5	98.5	98.8	98.5	98.5	97.4	95.9	98.7	100	

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**Fig. 1.** Phylogenetic trees based on 16S rRNA (a), *gyrB* (b), *recA* (c), *rpoB* (d), *gltA* (e) genes and the concatenated sequences (f) showing the relationships among the different *Acacia* spp. and *S. sesban* microsymbionts (shown in bold) and recognized species of the genus *Mesorhizobium*. Accession numbers are given in parentheses. Trees were constructed by the neighbour-joining method using MEGA version 4 [45] and maximum likelihood with PhyML [14]. Bootstrap values over 50% (based on 1000 replications) are shown at each node. Bars, % estimated substitutions. A: Azorhizobium; M: Mesorhizobium; B: Bradyrhizobium; P: Phyllobacterium.

were placed into separate clades within *Mesorhizobium*. The tree topologies for all the housekeeping genes, and thus the placement of individual single genotypes within the trees, were consistent for all the housekeeping genes except for a few discrepancies regarding the single strain genotypes AC21a2, AC21c2 and AC39d.

In the analysis of *gyrB*, the most discriminative housekeeping gene in this study, the *A. abyssinica* isolates in genospecies I were placed into a well resolved monophyletic clade, with only 92.2% sequence similarity to the closest phyletic branch, *M. plurifarium* (Fig. 1b). Similarly, the phylogenetic analyses based on *recA* and

*rpoB* genes (Fig. 1c and d) identified *M. plurifarium* to be the most closely related reference species to genospecies I, with sequences similarity of 93.6% and 96.3%, respectively. In contrast, strains in genospecies III subgroup b, also isolated from *A. abyssinica*, displayed close relatedness to *M. septentrionale* (92.2%) for *gyrB* while its *recA* and *rpoB* genes were most closely associated with *M. huakuii* and *M. plurifarium*, with sequence similarities of 96.0% and 95.5%, respectively. However, unlike genospecies I, genospecies III subgroup b showed *recA* sequence similarity of 95.3% to a number of reference species including *M. temperatum*, *M. tianshanense*, *M. caragane* and *M. ciceri*. Genospecies III subgroup a represented strains isolated from *A. senegal* and were consistently found in the same clade as genospecies III subgroup b isolated from *A. abyssinica*. Subgroup a in genospecies III shared sequence similarity of 91.2% with *M. septentrionale* for *gyrB* while both its *recA* (95.6%) and *rpoB* (95%) sequences were most closely associated to *M. huakuii*. Genospecies II, which comprised only the *S. sesban* isolates, was the most divergent group representing only 90.3% *gyrB* sequences similarity to *M. plurifarium* as its closest relative. On the basis of *recA* and *rpoB*, genospecies II was closely related to *M. caragane* (94.7%) and *M. plurifarium* (96.2%). The single strains (AC21a2, AC21c2 and AC39d) displayed low sequence similarity and large sequence divergence among each other, as well as to genospecies I–III and to the described *Mesorhizobium* reference species with respect to all the housekeeping genes considered (Fig. 1b–e), thus, indicating that these genotypes also represent new genospecies. However, their placement on the phylogenetic tree varied depending on the different housekeeping genes considered. For instance, strain AC21a2 was closely associated with *M. plurifarium* on the basis of both *gyrB* (92.8%) and *recA* (96.9%), while its *rpoB* gene was identical to that of AC21c2 and related to *M. huakuii* with sequence similarity of 95.5% for the same gene (Fig. 1b–e). Horizontal gene transfer and subsequent recombination may be a possible explanation for the inconsistent grouping with this strain. Hence, the inclusion of more strains would improve their exact phylogenetic positions with respect to genospecies I–III and described *Mesorhizobium* reference species.

#### Analysis of concatenated gene sequences

The topologies of the single gene phylogenetic trees for the housekeeping genes, and including the 16S rRNA gene, were similar in the placement of the three genospecies and the recognized *Mesorhizobium* references (Fig. 1a–e). The aberrant clustering on trees based on single housekeeping genes trees only occurred for genotypes represented by single strains (AC21a2, AC21c2 and AC39d). Phylogenetic trees constructed from the concatenation of the three housekeeping genes, including the 16S rRNA gene, and with or without aberrant sequences from the single genotypes, resulted in nearly identical tree topologies supported by high boot strap (BT) values (Fig. 1f, S1 and S2).

In line with the single-gene sequence characteristics (Fig. 1a–e), the concatenated genes (Fig. 1f) displayed high sequence similarity within each of the three genospecies defined (sequence divergence ranging only from 0% to 1%). In contrast, sequence divergence was clearly higher at the interspecies level, between our genospecies and the *Mesorhizobium* reference species, ranging from 3% to 16%. In agreement with the single-gene phylogeny, the phylogenetic analysis of concatenated sequences grouped the test strains into three well-supported (100% BT) monophyletic clades, with only *M. plurifarium* distantly placed along with genospecies II in one of the branches (Fig. 1f). However, in contrast to most of the results based on single-gene phylogeny, the Ethiopian genospecies were relatively closely associated to three or more recently described *Mesorhizobium* reference species from China (Table S2). For instance, genospecies I, while sharing 97% aver-

age nucleotide identity (ANI) with *M. plurifarium*, was also closely related to *M. caragane*, *M. gobiense*, *M. temperatum*, *M. septentrionale* and *M. huakuii*, showing an overall sequence similarity of 95.1–96%. Genospecies III subgroup a, which was related to *M. septentrionale* (94.6% ANI), also shared 94–94.3% sequence similarity to *M. caragane*, *M. huakuii*, *M. ciceri* and *M. plurifarium*. Genospecies III subgroup b was even more irresolute, showing 95% ANI to all three references, *M. huakuii*, *M. septentrionale* and *M. plurifarium*.

#### Phylogenetic analysis of symbiosis-related genes

Sequence alignments of 910 bp, 600 bp and 400 bp were used for analyses of *nodC*, *nodA* and *nifH* genes, respectively. The phylogenetic tree of *nodC* placed all the Ethiopian strains into two well-supported monophyletic clades, on which all the *Acacia* isolates formed several sub-clusters on one of the clades (Fig. 2b). Interestingly, strains in genospecies I and III, which clustered alike in the respective core genes, also grouped into separate sub-clusters on this clade. The *nodC* sequences of the genotypes represented by single strains (AC21c2 and AC39d), unlike their divergence in core genes, closely associated with genospecies I and III subgroup b. Strains of *M. huakuii* and *M. amorphae* isolated from China were the closest possible neighbours found in the database to these *Acacia* isolates and shared only less than 81% sequence similarity. The *nodC* of *S. sesban* strains in genospecies II, despite being isolated from the same site as genospecies III subgroup b, represented a well resolved phyletic branch. Strains of *Phyllobacterium* sp., *Rhizobium leguminosarum* and *Mesorhizobium* sp. were their closest neighbours found in the database and shared only 80% sequence similarity. These were isolated from nodules of *Ononis tridentata* L., a shrub legume highly prized for the revegetation of gypsum soils in semiarid Mediterranean areas [33].

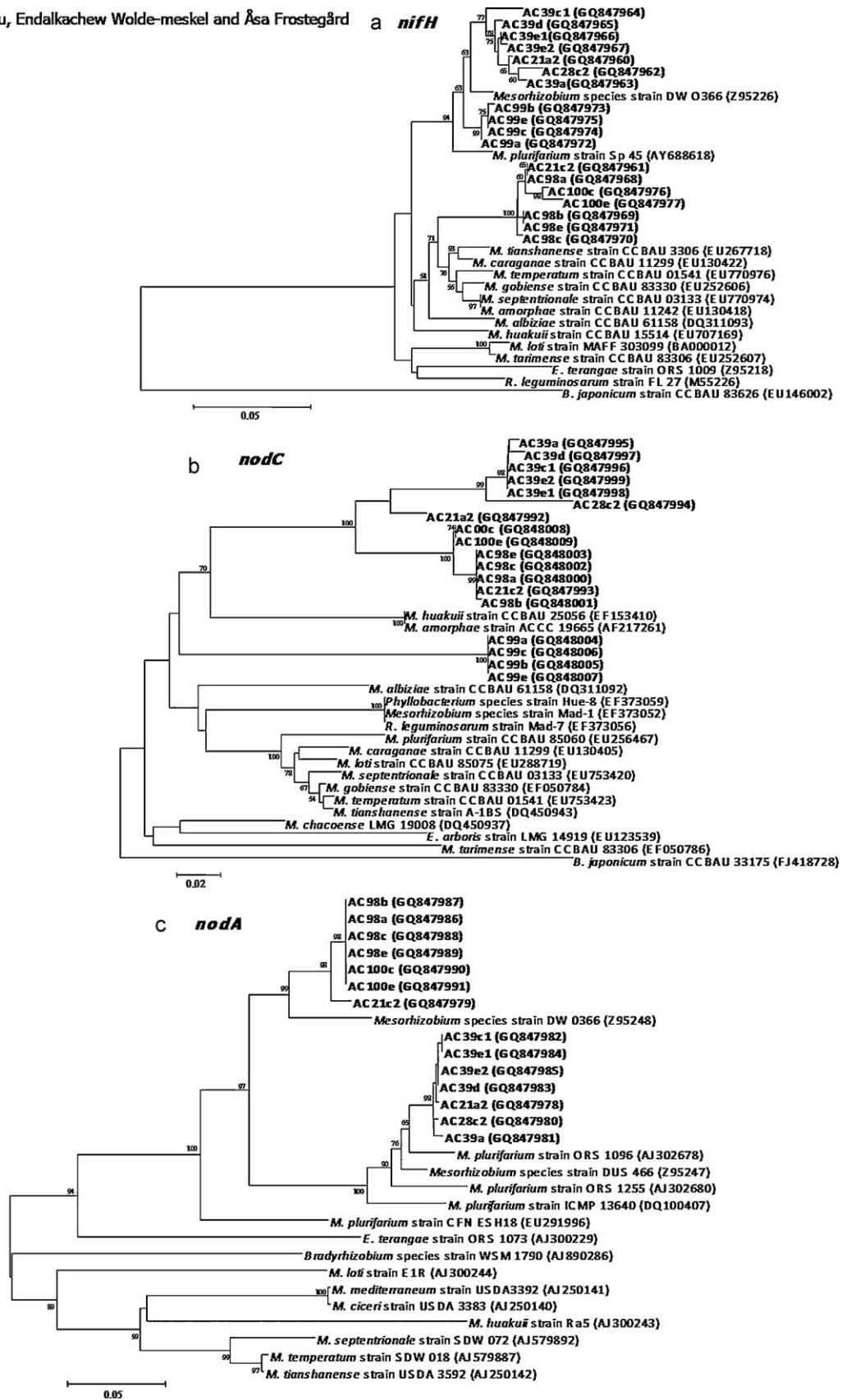
Sequence analysis of *nodA* gene grouped all isolates from the three *Acacia* spp. into two distinct clusters (Fig. 2c). The cluster which comprised genospecies I, together with AC21a2 and AC39d, showed low intrastrain sequence divergence (0–1%), and closely related to *nodA* of *M. plurifarium* nodulating *A. tortilis* in Africa (95% similarity). In contrast to their differences in *nodC*, genospecies III subgroup a and b shared identical *nodA* sequences and were closely related (with sequence similarity of 93%) to an undescribed *Mesorhizobium* sp. isolated from *A. polyacantha* in Kenya.

The phylogenetic tree based on *nifH* had similar topology as trees constructed with *nodC* and core genes (Figs. 2a and 1b–e). Genospecies II formed an independent lineage distantly related to the strains from *Acacia* spp. (genospecies I and III) and showed 95% sequence similarity to undescribed *Mesorhizobium* sp. nodulating *A. polyacantha*, also in Kenya and *M. plurifarium* from *Sesbania punicea* in Venezuelan wetlands [50]. The *nifH* of the *Acacia* strains (genospecies III subgroup a and b) occupied two different clades, in agreement with the grouping of the other symbiotic genes. *M. amorphae* and *M. septentrionale* strains nodulating legumes in China were the closest sequences in the database and displayed 95% similarity to genospecies III.

#### Infectiveness and symbiotic characteristics of test strains

Test strains representing various new genospecies, as defined in the present work, were different in their ability to infect and elicit effective nodules on 11 legume host species (nine tropical trees and two crop legumes) (Table S1). Genospecies III subgroup b, isolates from *A. abyssinica* at Wondogenet, were broad-host range and induced root nodules on 7–9 of the host species tested, followed by genospecies I, also *A. abyssinica* symbionts from another location, which elicited nodules on 5–6 legumes. As expected, genospecies II (the *S. sesban* isolates) was found to have a narrow host range, being able to induce effective nodules on hosts belonging to only *Sesbania*.

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**Fig. 2.** Phylogenetic trees based on *nifH* (a) (400 bp), *nodC* (b) (910 bp) and *nodA* (c) (600 bp) genes, showing the relationships among the different *Acacia* spp. and *S. sesban* isolates (shown in bold) and recognized species of the genus *Mesorhizobium*. Accession numbers are given in parentheses. Trees were constructed by the neighbour-joining method using MEGA version 4 [45] and maximum likelihood with PhyML [14]. Bootstrap values over 50% (based on 1000 replications) are shown at each node. Bars, % estimated substitutions expected number of changes per site. M: *Mesorhizobium*; B: *Bradyrhizobium*; E: *Ensifer*; R: *Rhizobium*.

Interestingly, while *S. sesban* and *A. seyal* were nodulated by most of the strains (14–15 of the 18 test strains), *P. juliflora* and *A. nilotica* were found to be poorly nodulating legumes in this study. It is also to be noted that genospecies III subgroup b and the two single strain genotypes from *A. tortilis* (AC21a2 and AC21c2) failed to nodulate a different provenance of homologous host species. None of the test strains were able to induce nodules in crop legume species tested.

## Discussion

### *Acacia* sp. and *S. sesban* microsymbionts represent new *Mesorhizobium* genospecies

In earlier investigations, in which we characterized a large number of rhizobial populations from native and exotic legumes using PCR - RFLP and partial 16S rRNA gene sequence analyses, we showed that these 18 test strains form separate branches in *Mesorhizobium* [56,57,59] (Table 1). In the present undertaking, comparisons of an almost full-length 16S rRNA of the test strains to all 21 currently recognized *Mesorhizobium* species, including the recently reported new genospecies from China [52], clearly indicated that the Ethiopian strains may represent several unique taxonomic positions in this genus (Fig. 1a, Table 2). The ribosomal gene is an extensively used marker in microbial ecology to differentiate bacteria, for rapid grouping of new isolates, and for defining bacterial genera and species [19]. Its merit as universal marker for taxonomic identification of bacteria allows phylogenetic studies and comparisons between different laboratories. However, it is highly conserved and frequently shows limited divergence, providing poor resolution at and below the genus level [17]. Recently the analysis of multiple protein-encoding housekeeping genes (Multilocus sequence analyses, MLSA) has become a widely applied tool for the investigation of taxonomic relationships [21,22,25,51]. The housekeeping genes have a higher level of sequence divergence than 16S rRNA, sufficiently conserved to retain phylogenetic signals [41], and were also successfully used to elucidate taxonomic relationships among species of *Mesorhizobium* [47] *Bradyrhizobium* [27,35] and *Ensifer* [21,22] in *Rhizobiaceae*. In this study, the taxonomic positions of the Ethiopian isolates was further explored by comparing their *recA*, *gyrB*, *rpoB* and *gltA* gene sequence alignments with all currently recognized *Mesorhizobium* reference species. The phylogenetic tree constructed from the different gene alignments consistently placed the test strains in tight clusters of the respective monophyletic branches that exclude any described species (Fig. 1b–e), demonstrating that the Ethiopian strains represent several distinct new taxonomic groups in *Mesorhizobium*.

Currently, DNA–DNA reassociation is a “benchmark” for species definition [41]. A set of strains to qualify for the same species, in addition to phenotypic similarities, should show 70% or greater relatedness. However, DNA–DNA hybridization is technically challenging and difficult to reproduce, and can be a cause for conflicting reports between different laboratories (consider the 39% relatedness reported for *E. xinjiangensis*/*E. fredii* [29] to 74–89% by [22]). Recently, several reports indicated that by concatenating sequences of carefully selected multiple protein-encoding loci, the MLSA approach provides a much improved way of defining species within a genus [22]. Konstantinidis et al. [18] demonstrated from a whole genome comparison study that the MLSA could surpass the precision of DNA–DNA hybridization in predicting genome relatedness; and further they indicated that the classical cut-off 70% DNA–DNA relatedness correspond to 96% ANI value. Martens et al. [22], in a study involving seven concatenated genes, deduced an ANI value of 97.3% as cut point for species delineation in *Ensifer*. By the phylogenetic analysis of the concatenated four genes studied here, a

more reliable determination of the phylogenetic positions of our test strains was possible. Accordingly, the Ethiopian isolates were grouped into three distinct genospecies (I–III) and the three single-strain genotypes (each represented by one single branch) (Fig. 1f). The concatenated tree, while consistent with single gene trees and more robust (supported by higher BT values), further highlighted the potential of housekeeping genes to delineate species in *Mesorhizobium*.

In this study, pair-wise comparison of the composite sequences for some *Mesorhizobium* reference species showed relatively low divergence, notably *M. tianshanense* with *M. gobiense* and *M. tarimense*; *M. gobiense* with *M. tarimense*; but also *M. tianshanense* with *M. temperatum* and *M. mediteranum*; *M. loti* with *M. ciceri* (Table S2). This was also true for the different single core loci (data not shown). While this may be due to the inclusion of only one strain for each species in our analyses and it remains to be seen if these species are distantly related on other housekeeping loci, we support the suggestion by Wei et al. [52] that the nature of species in *Mesorhizobium* needs to be evaluated by thorough population genetics principles, as carried out for *Bradyrhizobium* [49] and for *Ensifer* [21,22]. In contrast, all the different genospecies delineated in this study, including the genotypes represented by single strains, were more distant than or as distantly related as other *Mesorhizobium* reference species recognized at present.

Strains AC21a2 and AC21c2, despite their isolation from same host (*A. tortilis*) at the same site (Nazret) (Table 2), showed consistent differences in their *rrs* and all housekeeping genes analyzed in the present study. This is in agreement with earlier findings where they showed different AFLP profiles [56,59]. Specific to these strains was that their grouping consistently changed depending on housekeeping genes under consideration (Fig. 1a–e). While it is outside the scope of the present study to provide an explanation to this phenomenon, similar observations are often tentatively explained by lateral gene transfer and subsequent recombination [6], or as arising from random gene lineage sorting due to coalescence in populations [20].

### Native host species harbor diverse rhizobia

The legume-rhizobium symbiosis can be affected by environmental factors such as low soil pH or moisture [11] as well as by the compatibility of the symbionts, which is controlled by a series of molecular interaction between the two partners before nodules are formed [9]. It is also known that long association, in space and time, allows differentiation and more diversity in compatible rhizobia at the center of origin and diversity of the host. For example, the diversity of *Rhizobium galegae* matched the diversity of the hosts in the Caucasus, the center of origin of *Galega* plants [2]; soybean nodulating bacteria appear to be more diverse in Asia, where soybean originated [62]; for example the recent study by Vinuesa et al. [51] that isolation of *Bradyrhizobial* strains affiliated to different species in the genus and the investigation of a novel *Bradyrhizobium* lineage from Soybean; and the diversity of rhizobia nodulating *Phaseolus* is found to be higher in the Americas, where beans originated, than elsewhere [1]. On the contrary, legumes that are introduced to new geographical areas apparently recruit their symbiotic partners from only a segment of the *in situ* pool of rhizobia. This is the case with peanut introduced to China [61], and *Leucaena*, *Gliricidia*, and *Calliandra* introduced to Ethiopia, all of which are nodulated only by rhizobia with distinct genomic profiles [57].

*Acacia abyssinica* Benth. subsp. *abyssinica* is native to Ethiopia [36]. Along with other tree species including *Albizia gummifera* (Gmel.) and *Milletia ferruginea*, it forms the upper storey tree cover in the dry evergreen mountain forest ecosystem in the highlands

where coffee plants, also native to Ethiopia, grow understory [44]. In the present study, out of the three *Mesorhizobium* genospecies defined, genospecies I and III subgroup b were isolated from this legume in soils at two different sites (Table 1) located only 80 km apart, indicating that the bacterial populations associated with this plant are taxonomically diverse. We showed in earlier investigations that rhizobia associated with native tree legumes including *A. abyssinica* represented a large number of genomically distinct groups, most of which were not related to reference species [59]. The relative diversity in the chromosomal background of the isolates from this native legume, as evidenced by two *Mesorhizobium* genospecies only from few isolates, is in line with the previous findings and might be related to the long term co-existence and gradual differentiation of the bacteria. The two sites where strains of the two genospecies were isolated represent different agro-climatic regions and markedly vary in soil texture, amount of annual rainfall and land use [58], which may also play a role in selection and differentiation of rhizobial strains compatible to the host *in situ*.

#### Phylogenetic analysis of symbiosis-related genes

The common nodulation genes *nodABC* determine the core structure of the Nod factors [30]. Among these, *nodC* is one of the components of host-specific nodulation (*hsn*) and encodes for N-acetyl-glucosaminyltransferase which is used for the first step in Nod factor assembly [30]. The *nifH* gene encodes the nitrogenase complex. It usually exists as a single copy in *Mesorhizobium* [15], and its phylogeny is in general coincident with that of the 16S rRNA. The phylogenetic analysis in this study clearly demonstrated that grouping of the test strains based on *nifH*, *nodC* and *nodA* were generally congruent, and almost similar to the phylogenetic relationships obtained based on core genes (Figs. 1a–f and 2a–c). The correspondence of these distinct clades of nodulation genes with distinct genospecies (I–III), while indicating vertical parental transfer, suggests that these *Acacia* and *Sesbania* symbionts may have a long history of separate evolution within *Mesorhizobium*. In *Mesorhizobium*, the *nod* and *nif* genes are closely linked and located on transmissible elements such as plasmids or transposon-like symbiotic islands [43]. Interestingly, despite isolation of the strains of genospecies III subgroup b and genospecies II from the same soil at a particular location (Wondogenet), no horizontal gene transfer could be detected between these genospecies. Also, the differences in host range that these genospecies displayed (Table S1) could be a reflection of the distinct nature of their *nod* and *nif* genes.

The reason for the failure to amplify the *nodA* gene in all the *S. sesban* isolates (genospecies II) was not clear. However, in view of the distinct *nodC* lineage in this group, divergent *nodA* and sequence differences at primer annealing sites could be a possible reason. Compared to most of the *Acacia* isolates, which grouped into other genospecies, isolates in genospecies II displayed a narrow host range and induced effective nodules only on *Sesbania*. The *nodC* sequences of genospecies II were related, although distantly, to undescribed members of *Rhizobiaceae* associated with the gypsophyte *Ononis tridentata* L., a shrub legume highly prized for re-vegetation of gypsum soils [33]. This may bear an ecological implication and gives a clue to innovative research aiming at formulating suitable inocula to recover semiarid ecosystems.

The *nodC* sequences of the strains isolated from all the different *Acacia* species were separately placed in one well supported clade with 100% BT value (Fig. 2b), demonstrating that *Acacia* species stringently selected the rhizobial symbiotic genotypes rather than their genomic backgrounds. The cross-nodulation result, showing that most of the strains isolated from *Acacia*, were (irrespective of their genospecies groupings) able to infect most of the different *Acacia* species tested here, reflect this phenomenon.

Overall, the *nodC* genes of *Acacia* species and *S. sesban* symbionts formed well-supported, distinct clades that did not share close sequence similarity to any of the previously reported sequences in the database. An interesting property of these distinct sequences is that they have diverged so much from other known sequences, and yet have little within-group variation. This is probably indicative of isolated symbiotic co-evolution with legumes native to Ethiopia. The existence of diverse *nod* lineages has been explained as a consequence of co-evolution of the host and *nod* sequences in the microsymbiont, and lateral transfer of *Sym* genes [19]. The genotypes represented by single strains (AC21a2 and AC21c2), despite their distinct core genes which are divergent from all the genospecies defined here (Fig. 1a–f), carried *nod* and *nif* gene sequences similar to the other *Acacia* isolates in genospecies I and III reflecting history of lateral gene transfer from common ancestors in the past.

#### Symbiotic properties of the strains and host species

The use of N fixing leguminous trees is a promising alternative to fertilizer in agroforestry systems in developing countries [37]. However, its benefit is realized only if the trees are nodulated with effective rhizobia. All the 18 strains tested here nodulated at least two of the hosts and were markedly different in their ability to induce effective nodules (Table S1). The two single genotypes (AC21a2 and AC21c2) isolated from *A. tortilis* and genospecies III subgroup a from *A. senegal* were unable to nodulate homologous host species from a different provenance suggesting, in view of this and other reported findings, that strain specificity at provenance level may occur in *Acacia* species. Sniezko and Stewart [40] have observed differences in the extent to which west and east African provenances of *Faidherbia albida* (syn. *Acacia albida*) nodulated. The phenomenon was commonplace also for provenances of *A. tortilis*, *A. seyal* and *A. nilotica* [55,57]. Interestingly, the results from cross-inoculation experiments show that *S. sesban* was the most promiscuous host (nodulating by 15 of the 18 strains tested) followed by *A. seyal* and *S. aculiata* which were nodulated by 14 and 13 strains, respectively (Table S1). These experiments were done by inoculating axenic seedlings with one single strain of rhizobia. In contrast to these results, *S. sesban* was specific in its nodulation with a narrow host-range group of strains; genospecies II, when trapping experiments were performed in which the seedlings were grown in non-sterile soil [57]. Earlier studies based on trapping (and/or isolation from excavated nodules), also conclude that rhizobia nodulating *S. sesban* have a restricted host range and are specific to the genus *Sesbania* [28]. This implies that, although *S. sesban* has the capacity to produce nodules in association with a wide variety of microsymbionts, only a narrow range of these will infect *S. sesban* in presence of a large diversity of rhizobia found in soil. Still, isolates from *S. sesban* have been shown to be genetically diverse, with isolates represented in the genera *Rhizobium*, *Mesorhizobium*, *Sinorhizobium* and *Azorhizobium* [3,60]. Bala et al. [3] suggested that this may be due to the similarity of the accessory genes under different genome background that governs the nodulation behavior of *S. sesban*. Analysis of *nodC* gene in the present investigation shows that this symbiotic gene grouped the isolates from *S. sesban* into one separate and well-defined clade in the phylogenetic tree (Fig. 2b). No *nodA* amplification products could be obtained for genospecies II, which may be a further indication of unique organizations and sequences of symbiotic genes in these strains. Thus, despite the diverse symbiotic genes identified and nodulation by almost all strains tested here in axenic cultures, the unique nature of *S. sesban* nodulation when growing in non-sterile soil perhaps relates to its inherent capacity to produce specific compounds of flavonoids which specifically stimulate certain rhizobial strains. Information in the literature is

inconclusive and further investigations would be interesting in this regard.

In conclusion, in the context of the cross nodulation information obtained here, three different strategies could be adopted to produce inoculants to promote N-fixation in target agro-ecosystem; (i) Selection of narrow host range strains (genospecies II) specific in nodulation, targeting few individual host species such as *S. sesban*. (ii) Selection of broad-host range strains such as genospecies I and III subgroup a, which are effective on wide range of different host species. (iii) Production of mixed inoculants which combines both types to cover a vast spectrum of host species. The latter has been shown to be an effective practice on legume crops [16].

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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.2010.09.006.

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