Comparison of nucleotide diversity and symbiotic properties of *Rhizobium meliloti* populations from annual *Medicago* species

B. Brunel a,b,c,*, S. Rome a,d, R. Ziani a, J.C. Cleyet-Marel a

a Laboratoire de Recherche sur les Symbiotes des Racines, Ecole Nationale Supérieure Agronomique de Montpellier, Institut National de Recherche Agronomique, 2 place Pierre Viala 34 060, Montpellier Cedex 1, France
b Unité de Formation et de Recherche de Science du Sol, 2 place Pierre Viala 34 060, Montpellier Cedex 1, France
c Centre d’Analyse Moléculaire de la Biodiversité, Université Claude Bernard Lyon I, 69 622 Villeurbanne Cedex, France
d Laboratoire d’Ecologie Microbienne des Sol, Centre National de la Recherche Scientifique URA 1977, Université Claude Bernard Lyon I, 69 622 Villeurbanne Cedex, France

Received 14 June 1995; revised 12 October 1995; accepted 13 October 1995

Abstract

Forty-three isolates of *Rhizobium meliloti* were trapped from soil with five annual species of *Medicago* (M. polymorpha, M. truncatula, M. rigidula, M. orbicularis and M. minima) and one perennial species of *Medicago* (M. sativa). The annual species were growing naturally near the soil sampling site, and the commonly studied perennial species was used for comparison. Each *R. meliloti* was characterized by PCR-RFLP methods applied to two DNA regions nested between 16S rRNA and 23S rRNA genes and between *nifD* and *nifK* genes. They fell into two highly divergent groups (groups I and II), separated at a genetic distance of 0.024 by rDNA-amplified pattern analysis (profiles R1 and R2) and at 0.029 by *nif*-amplified pattern analysis (profiles N1-N2 and N3). These two groups were consistent with some cross-nodulation and -fixation results: rhizobia with the R1 genetic background elicited rudimentary nodules and could not fix nitrogen on *M. polymorpha*, while they were able to nodulate the five other species of *Medicago*. In contrast, rhizobia with an R2 profile were highly effective on *M. polymorpha* and poorly nodulated *M. rigidula* species, but were able to nodulate efficiently the other species. The striking phenotypic traits on *M. polymorpha* were also shared by reference strains: strains genetically close to R2 type triggered typical and efficient nodules on *M. polymorpha* while those close to R1 type elicited rudimentary and non-efficient ones. Our results suggest that the presence of *R. meliloti* with R2 genetic backgrounds could be favoured by the distribution of *M. polymorpha* species.

Keywords: *Rhizobium meliloti*; *Medicago*, annual; Cross-inoculation group; PCR-RFLP; rDNA; *nif*DK genes

1. Introduction

Annual *Medicago* species (or medics) are well adapted and native to areas in the Mediterranean basin [1]. They are excellent candidates for use in sustainable agriculture systems such as forage and cover crops, and are promising for rangeland man-
agement and pasture reseeding in the French Mediterranean area [2]. For this region (Provence-Côte d’Azur, Languedoc-Roussillon and Corse), five main and ubiquitous medic species (Medicago polymorpha, M. truncatula, M. rigidula, M. orbicularis M. minima) were recorded and selected for their suitability for pasture improvement [3]. Medics are more widely used in Australia where ley farming (a cereal pasture rotation) is practiced; they increase soil nitrogen, improve soil structure and control soil erosion [4,5]. Since the use of nitrogenous fertilizers is not appropriate in these extensively planted areas, medics are enhanced by adapted Rhizobium-legume symbiosis and can thus supply nitrogen to other plants through their association with appropriate Rhizobium spp. To take advantage of this biological nitrogen input in semi-natural systems with economic and environmental benefits, suitable rhizobia must be present in the soil and must be able to establish an efficient symbiosis with medics. If not, efficient strains should be selected for introduction into soil as inoculant. But the establishment of inoculant strains can be hindered by the presence of ineffective populations of R. meliloti which can cause parasitic nodulation. Thus, to improve growth of medics, it is essential to characterize the native R. meliloti populations and the factors which influence their composition and dynamics.

Medics are associated with rhizobia belonging to the R. meliloti species. Their symbiotic interaction appears to be very specific; all R. meliloti strains are not able to nodulate all the Medicago species, and when they are, they do not always fix nitrogen. Eventually, when fixation does occur, the levels may vary. Brockwell and Hely [6,7] defined three R. meliloti groups based on their ability to elicit nodules on Medicago, and eight based on their similarities in fixing nitrogen on Medicago species. For instance, M. minima can form effective associations with a great number of R. meliloti strains whereas M. rugosa, M. rigidula or M. laciniana have very specific requirements [7–9]. More recently, R. meliloti populations from annual and perennial Medicago were examined by multilocus enzyme electrophoresis (MLEE) and classical restriction fragment length polymorphism (RFLP) analyses on rRNA genes [10]: R. meliloti originating from medics were divided into two highly diverging groups, which could coincide with different host-microsymbiont affinities.

To define the properties of indigenous R. meliloti populations occurring in southern France, we collected rhizobia by trapping with the most ubiquitous medics and then applied an approach in two steps for characterizing isolates. Screening all isolates by cross-inoculation methods and classifying rhizobia on the basis of their ability to nodulate and fix nitrogen is laborious and could be difficult to interpret properly, i.e. variations and inconsistencies were already noted by Brockwell and Hely [7]. Consequently, before examining isolate characters by plant tests, we first identified all isolates by the PCR-RFLP typing which provides an efficient and rapid method for classifying soil bacteria and rhizobia [11–13]. Specific DNA regions are first amplified by polymerase chain reaction (PCR), then the PCR products digested by restriction endonucleases are separated by gel electrophoresis to reveal restriction fragment length polymorphism (RFLP). Universal primers were chosen in rRNA and nif genes because prior studies had shown that different portions of the genes could produce genetic and phylogenetic polymorphism [11–15]. The nodulation and nitrogen fixation specificity was then examined by cross-inoculation among each of the different PCR-RFLP groupings. We expected differences in genetic groupings to reveal consistent symbiotic traits. Moreover, one commercial perennial Medicago sativa species was used since medics or lucerne could preferentially trap some types of rhizobia. Comparisons were also made with reference R. meliloti strains.

2. Materials and methods

2.1. Description of the site

A 5 m × 10 m soil plot, where five species of annual spontaneous medics naturally coexisted, was located in the Mediterranean garrigue (Combaillaux, Hérault, France) in May 1986. Medic species were identified as Medicago polymorpha (L.), M. truncatula (Gaertn.), M. rigidula (L.), M. orbicularis (All.) and M. minima (Grubf.). Samples of surface soil from a part of the site where no legumes were growing, were collected aseptically from the top 10
of the profile; they were then mixed and stored at 4°C less than 24 h before use. The physical and chemical properties of the soil were as follows: sand 486 mg \cdot g^{-1}; silt 259 mg \cdot g^{-1}; clay 227 mg \cdot g^{-1}; total calcareous 476 mg \cdot g^{-1}; active calcareous 142 mg \cdot g^{-1}; pH (1:1 soil – water) 8.6; organic matter 28 mg \cdot g^{-1}.

2.2. Isolation of rhizobia

Seeds from the five medics collected on the site and from a commercial perennial lucerne (M. sativa cv. magali) were used as hosts to trap rhizobia. They were surface sterilized in 1% calcium hypochlorite for 2 min and washed with seven changes of sterile water [16]. They were scarified by a spot hot-shock treatment with an iron microtip of a thermoregulated soldering station (JBC, Montpellier, France) to improve seed germination, and then incubated on a 0.7% agar medium. They were germinated for 72 h in a growth chamber at 28°C. The pregerminated seeds were placed aseptically in Gibson tubes [17] containing vermiculite with a nitrogen-free nutrient plant solution [18]. Soil was homogenized by shaking for 30 min and then 20 g of the soil were added to 190 ml of sterilized water to obtain a dilution of 10^{-1}, which was vigorously shaken. One ml of the dilution was then used to inoculate eight plants grown axenically in Gibson tubes. Eight control tubes were not inoculated. The plants were grown for one month in a room with controlled environment (70% moisture), under a combination of metal halide and mercury vapor lamps that provided a photon flux density of 600 \mu mol \cdot m^{-2} \cdot s^{-1} (400 to 700 nm) over a 16-h photoperiod. Day and night temperatures were 25°C and 20°C respectively. One nodule was removed from each nodulated plant 30 days after inoculation and stored in tubes in the presence of CaCl_{2}. Bacterial strains were obtained by the crushed-nodule method [16]. One Rhizobium isolate

| Table 1 | Isolates of R. meliloti and reference strains |
|---------|--------------------------------------------|
| Bacterial isolates or strains | Obtained from | Geographical origin, source or reference |
| R. meliloti isolates | | |
| CM1I, CM2I, CM5I, CM1II, CM3II, CM5II, CM3III | M. minima | France, this study |
| CR1I, CR2I, CR4I, CR5I, CR3II, CR5III | M. rigidula | France, this study |
| CP1I, CP2I, CP4I, CP6I, CP7II, CP4III | M. polymorpha | France, this study |
| CS1I, CS2I, CS3I, CS4I, CS5I, CS5III | M. sativa | France, this study |
| CT1I, CT2I, CT4I, CT5I, CT2II, CT6II, CT3III, CT2IV | M. truncatula | France, this study |
| CO1I, CO3I, CO4I, CO5I, CO7I, CO8I, CO7II, CO2III, CO4III, CO5III | M. orbicularis | France, this study |

Reference R. meliloti strains

| | Obtained from | Geographical origin, source or reference |
| WSM533 | M. murex or M. polymorpha (undetermined) | Sardinia, [19] |
| M29 | M. rigidula | Syria, Dr. Materon. ICARDA |
| M3 | M. orbicularis | Syria, [10] |
| M104 | M. truncatula | Syria, [10] |
| 2011 | M. sativa | Australia, Dr. Rosenberg, INRA |
| L5.30 | M. sativa | Australia, Dr. Rosenberg, INRA |

ICARDA: International Center for Agricultural Research in the Dry Areas, Aleppo, Syria.
INRA: Institut National de la Recherche Agronomique, Castanet-Tolosan, France.
was obtained from each nodule. Single-colony isolates were purified on yeast extract-mannitol (YEM) agar [16]. A total of 43 isolates was obtained and stored on YEM slants at 4°C or in 50% glycerol at −80°C. These isolates and the reference _R. meliloti_ strains are listed in Table 1. Reference strains belonging to both divisions A and B [10] were included for comparative analysis.

### 2.3. Genomic DNA extraction

Small-scale preparations of total bacterial DNA were obtained by growing each strain on a tryptone-yeast (TY) medium [20] agar plate for 4 days at 28°C. Cells were scraped off in TE8 buffer (Tris-HCl 50 mM, EDTA 50 mM, NaCl 100 mM, pH 8) and pelleted in a microcentrifuge tube for 2 min (10 000 × g, 15°C). The cell pellets were washed twice with TE8 buffer and finally resuspended in 0.5 ml TE8. The bacterial suspension was lysed by passing the microtubes through liquid nitrogen and boiling water twice for 1 min each time. The samples were first extracted with phenol equilibrated with TE8 buffer and then with chloroform. DNA was precipitated by adding 1 volume of isopropanol and 0.1 volume of NaCl (1 M) and collected by centrifugation for 10 min in a microcentrifuge. The resulting DNA pellet was washed with 70% ethanol and 95% ethanol and then allowed to air-dry. The dried DNA was dissolved in 20 μl of pure water at 37°C for 1 h. This procedure routinely yielded 100 to 200 ng DNA that was sufficiently pure to be used as a template for the polymerase chain reaction (PCR).

### 2.4. PCR-RFLP analysis

PCR amplification was performed in standard 50 μl PCR mixtures containing 200 μM of each of dNTP (Pharmacia LKB Biotechnology, St Quentin en Yvelines, France), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl₂, 0.01% gelatine, 0.1 μM of each of the two primers, template DNA (1 μl), and 2.5 units Taq-polymerase (Gibco BRL, Cergy-Pontoise, France) [21]. The amplification mixture was overlaid with two drops of mineral oil (Sigma, St Quentin Fallavier, France). In all cases, the negative controls contained all compositions for PCR, excluding the template DNA. The reactions were run on a DNA thermal cycler (Integra Biosciences, Eaubonne, France) for a 35-cycle amplification series: after initial denaturation of the reaction mixture at 95°C for 3 min, each cycle included denaturation at 95°C for 1 min, reannealing at 55°C for 1 min and extension at 72°C for 1 min. The final extension was carried out at 72°C for 3 min. Amplified DNAs were then digested for RFLP analyses. DNA samples (1 to 2 μg) were digested completely (2 h) with 10 U of restriction endonucleases _CfoI_, _HaeIII_ or _MspI_ (Gibco BRL) according to the manufacturer’s instructions. The restricted fragments were separated by horizontal electrophoresis on 3% (wt/vol) NuSieve agarose (Tebu, Le Perray en Yvelines, France) gels containing 1 μg ml⁻¹ ethidium bromide. A 123-bp DNA ladder (Gibco BRL) was used as standard for molecular size determinations. The gels were run in TBE buffer (89 mM Tris (pH 8.3), 89 mM boric acid, 2.5 mM EDTA, pH 8.2) for 4 h at 3 V cm⁻¹. The gels were photographed under 312 nm ultraviolet light with Polaroid type Ilford 667 films (Polaroid Corp., Cambridge, MA) or Kodak negative films.

### 2.5. Oligonucleotide primers

One DNA segment containing the IGS (intergenic spacer) between the 16S _rrn_ (gene encoding 16S rRNA) and 23S _rrn_ genes (approximately 2.7 kb) was amplified from each _Rhizobium_ isolate. The two oligonucleotide sequences used as primers were: 5'-GGA GAG TTA GAT CTT GGC TC-3' (FGPS6) and 5'-CCG GGT TTC CCC ATT CGG-3' (FGPS132') characterized according to Normand et al. [22]. A fragment containing only sequences of 16S _rrn_ was specifically amplified using primer FGPS6, described above, and primer PGPS1509', 5'-AAG GAG GTG ATC CAG CCG CA-3'. The 16S rDNA sequence amplified between primers FGPS6 and PGPS1509' is 1478 nucleotides long according to two _R. meliloti_ 16S rDNA sequences [23,24]. Another DNA fragment (approximately 1.35 kb) containing only sequences of the IGS (intergenic spacer) between the _nifD_ and _nifK_ was amplified from the following two primers: 5'-CAC TGC TAC CGG TCG ATG AA-3' (FGPD807) [11] and 5'-GTG GCT GCC CAC GAA GAA GCT TGG NGT GTG-3' (FGPK597'). Synthesized and purified oligonucleotides were from Bioprobe Systems (Montreuil sous bois, France).
2.6. Data analysis

To allow a quantitative comparison of the different PCR-RFLP analyses, restriction fragment patterns from *R. meliloti* isolates and references were compared pairwise for the presence or absence of restriction fragments. The proportion of shared fragments (*F*), termed Dice’s similarity coefficient, was calculated as

\[
F = 2 \frac{n_{xy}}{n_x + n_y},
\]

where \(n_{xy}\) is the number of common fragments between isolate \(x\) and \(y\), with the three restriction endonucleases used and \(n_x\) and \(n_y\) the numbers of fragments from isolates \(x\) and \(y\), respectively [25]. Nucleotide diversity, \(\Pi\), the average number of nucleotide differences between isolates, was calculated as

\[
\Pi = \left[ -\ln F / r \right],
\]

where \(r\) is the number of nucleotide base pairs in the restriction endonuclease recognition site [25]. All restriction endonucleases used in this study to digest amplified DNA recognize sites of \(r = 4\). An unweighted pair-group method using arithmetic average (UPGMA) dendrograms of nucleotide diversities was constructed by the method of Sneath and Sokal [26]. A phylogenetic dendrogram was also constructed by the neighbor-joining method [27] with the Restsite software program developed by Miller [28]. The observed frequencies of PCR-RFLP patterns were compared with expected values using chi-square (\(\chi^2\)) tests (when expected numbers were at least five in each class).

2.7. Symbiotic effectiveness

Isolates were tested by cross-inoculation for their ability to form nodules and to efficiently fix nitrogen on the six species of *Medicago*. Surface-sterilized seedlings of *Medicago* were grown axenically as described above. One ml of each rhizobial suspension from an early stationary-phase culture in YEM broth (containing \(10^{-6}-10^{-7}\) cfu \(\cdot\) ml\(^{-1}\)) was inoculated in each seedling in Gibson tubes [17]. Eight replicates were done for each isolate and reference strain. Eight uninoculated plants and eight plants supplemented with nitrogen (10 mM of Ca(NO\(_3\))\(_2\)) were used as controls. The experiments were conducted in the same growth conditions as described above. The plants were harvested after 50 days, dried at 70°C for 48 h and then weighed to estimate the nitrogen fixation efficiencies. The experiments were repeated at least twice. Shoot dry matter data were subjected to an analysis of variance [29]. The data being numerous, ANOVA was done in two steps. First, variance analyses were performed within each group of Combaillaux isolates originated from one *Medicago* species in order to assay intra-species variations. Secondly, the dry matter weight averages of each isolate were compared between themselves in order to assay inter-species variations, and compared with the dry matter data of reference strains and controls.

3. Results

The isolates we had obtained at Combaillaux were first subjected to PCR-RFLP analysis on ribosomal and *nif* DNA regions. Then a subsample of 33 rhizobia was chosen among each of the different PCR-RFLP groupings and each of the six plant origins in order to determine their nodulation and fixation abilities.

3.1. PCR-RFLP analysis

Electrophoretic analysis of uncut PCR products indicated that all amplified fragments were similar in overall size: approximately 1.5 kb and 2.7 kb for the ribosomal 16S rDNA and 16SrDNA + IGS regions respectively, and 1.35 kb for the *nif* D region (data not shown). The amplified 16S rDNA region size was in agreement with those of 1478 bp estimated from the *R. meliloti* 16S rDNA sequences available in databases. Amplified DNA regions were then digested by three four-base cutting endonucleases to detect polymorphism by RFLP.

Among the 43 Combaillaux isolates, two profiles R1 and R2 were identified on the amplified DNA region nested between the 16S rDNA and 23S rDNA + IGS regions (Fig. 1). Two isolates (CP4I and CP4III) trapped from *M. polymorpha* (profile R2) were indeed distinguishable from the other 41 isolates (profile R1) which showed the same patterns for each of the three restriction enzymes tested. The reference strains M29 and WSM533 presented two ribosomal DNA region patterns identical to the Combaillaux isolates (profiles R1 and R2 respectively). In contrast, the other reference strains (M3, M104, 2011...
Fig. 1. PCR-RFLPs of amplified 16S rDNA+IGS regions digested by three restriction endonucleases which are noted above the gel. Lanes 1, 6 and 10: profile R1 (all 43 *R. meliloti* isolates except CP41 and CP4III, plus the strain M29); lanes 2, 7 and 11: profile R2 (CP41, CP4III, WSM533); lanes 3, 8 and 12: profile R3 (2011, L5.30); lanes 4, 7 and 11: profile R4 (M3); lanes 5, 9 and 13: profile R5 (M104); L: 123 bp-ladder.

and L5.30) gave three new patterns named profiles R3, R4 and R5 (Fig. 1). The rhizobial diversity detected in the ribosomal region was mainly due to the IGS ribosomal fragment, as the DNA segment amplified from the primers located on the 16S rRNA gene revealed no polymorphism (data not shown).

Among the Combaillaux isolates, slightly more polymorphism was detected when the DNA region nested between nifK and nifD was used (Fig. 2): the 43 isolates grouped into three RFLP patterns named N1, N2 and N3. Of the 41 R1 profile isolates, 32 were assigned to N1 pattern (CM2I, CM5I, CM3II, CM5II, CR1I, CR2I, CR4I, CR5I, CR5II, CP1I, CP2I, CP6I, CS1I, CS2I, CS3I, CS5I, CS5III, CT1I, CT2I, CT4I, CT5I, CT2II, CT6II, CT3III, CT2IV, CO1I, CO3I, CO4I, CO5I, CO2III, CO4III, CO5III) and nine to N2 pattern (CM1I, CM3II, CR3II, CS4I, CP7II, CO7I, CO8I, CO7II). The two remaining isolates which were identified by R2 profile, owned a specific N3 pattern (CP4I and CP4III). Among the six references, three (M3, WSM533, M104) also showed a N3 pattern while two others (L5-30, 2011) fell in the N2 grouping. One additional pattern (N4) was obtained by characterizing the reference strain M29.

Of the 20 possible combinations between the five ribosomal and the four nif profiles, three were recovered from Combaillaux bacterial populations and four additional ones were found among reference strains which originated from different geographic locations. Pattern R1N1 representing 74% of all isolates, predominated in each sample from one plant species. Its frequency did not differ significantly (*P* < 0.05) between the six *Medicago* species (*x*² = 4.49; 5 degrees of freedom). The two other patterns R1N2 and R2N3 represented 21% and 5% of the total sampling respectively. The R1N1 and R1N2 distributions according to the six trapping plants were statistically similar.

Dendrograms A and B were generated by UPGMA clustering from the data sets obtained with ribosomal and nif typings, respectively (Fig. 3). In ribosomal dendrogram A, rhizobia grouped at 0.024 of diversity and could be divided into two distinct clusters (AI and AII). Branch AI contains the R1 profile isolates and the strains, M29, L5-30 and 2011 while branch AII is constituted of the R2 profile isolates and strains WSM533, M3 and M104. Dendrogram B, obtained by analysis of the four nif patterns, has three branches. branches BI and BII defined at 0.029 of diversity, and a more divergent one corresponding to the N4 pattern represented by the single strain M29. Except for M29, rhizobial populations from the AI cluster belonged to the BI cluster and those from the AII cluster recovered the BII cluster (Fig. 3). The tree inferred by the neighbor-joining method agreed with the UPGMA dendrogram topography (data not shown). With the same
method, the strain M29, which has a highly divergent nif profile, clustered in branch I of the rDNA dendrogram A while it was excluded from branch I of the nif dendrogram B.

3.2. Symbiotic properties

A sample of 33 R. meliloti isolates and five references were assayed for both nodulation and nitrogen fixation efficiency in symbiosis by cross-inoculation with five annual species and one perennial species of Medicago (Table 2).

The isolates nodulated the five other Medicago species from which they were not issued. However, we observed variations in nodulation efficiencies on M. rigidula and in nodule aspects on M. polymorpha. On M. rigidula, two isolates (CP4I and CP4III) originating from M. polymorpha and the WSM533 strain could form only a few nodules since only 2 to 5 tubes on 10 were positively-nodulated ($\overline{x} = 0.2 \pm$

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Fig. 3. Dendrograms (UPGMA) of genetic relationships between the 43 isolates and the six reference R. meliloti strains estimated on the basis of PCR-RFLP profiles from ribosomal-amplified DNAs (A; R1: R1 profile isolates (see Fig. 1), M29; R2: R2 profile isolates, WSM533; R3: 2011, L5.30; R4: M3; R5: M104) and from nif-amplified DNAs (B; N1: N1 profile isolates (see Fig. 2); N2: N2 profile isolates, 2011, L5-30; N3: N3 profile isolates, M3, WSM533, M104; N4: M29).

Fig. 4. M. polymorpha plants inoculated with: (A) a R1 profile isolate; (B) the R5 profile reference strain M104 and (C) a profile R2 isolate. Arrows indicate rudimentary non efficient nodules (A) and typical mature nodules (B and C). Black bars indicate 10 mm and white bars indicate 1 mm.
Table 2

Symbiotic effectiveness of 33 isolates of *R.* meliloti and five reference strains

| Strains | minima | rigida | polymorpha | sativa | truncatula | orbicularis |
|---------|--------|--------|------------|--------|------------|-------------|
|         | DM (mg/plant ± S.D.) |        |            |        |            |             |
| CM1I    | 13.2 ± 1.1 b | 29.1 ± 4.7 b | 3.8 ± 0.4 b | 57.8 ± 6.6 b | 18.0 ± 1.8 b | 10.7 ± 2.8 bc |
| CM2I    | 14.7 ± 1.9 b | 14.4 ± 2.4 b | 3.6 ± 0.8 b | 28.7 ± 8.6 b | 22.0 ± 2.2 b | ND          |
| CM5I    | 12.5 ± 1.2 b | 18.3 ± 5.3 b | 3.8 ± 0.3 b | 33.1 ± 8.7 b | 27.2 ± 5.9 b | ND          |
| CM1I    | ND      | 27.8 ± 8.5 b | 3.6 ± 0.2 b | ND      | 18.6 ± 1.5 b | 8.3 ± 1.6 bc |
| CR1I    | 8.3 ± 1.7 b | 18.1 ± 5.7 b | 2.7 ± 0.4 b | 35.3 ± 10.4 b | 23.1 ± 2.3 b | 11.4 ± 1.6 bc |
| CR2I    | ND      | 3.7 ± 0.6 b | 76.2 ± 7.8 b | ND      | 19.1 ± 1.5 b | ND          |
| CR4I    | 12.8 ± 1.6 b | 22.7 ± 9.0 b | 4.3 ± 0.7 b | 58.7 ± 12.7 b | 27.9 ± 2.7 b | ND          |
| CR5I    | 11.3 ± 1.0 b | 28.2 ± 5.1 b | 1.7 ± 0.7 b | 43.3 ± 8.4 b | 18.5 ± 1.0 b | ND          |
| CR3I    | 14.3 ± 1.6 b | 25.0 ± 9.0 b | 4.1 ± 0.5 b | 47.3 ± 6.9 b | 25.6 ± 2.3 b | ND          |
| CR5II   | 12.2 ± 1.7 b | 31.7 ± 5.9 b | 3.2 ± 0.4 b | ND      | 22.6 ± 4.0 b | 10.4 ± 1.0 bc |
| CP4I    | 10.2 ± 1.2 b | 7.2 ± 0.5 c | 93.7 ± 11.7 c | ND      | 44.7 ± 11.0 b | 21.8 ± 2.6 b | 6.7 ± 1.5 bc |
| CP4III  | 9.6 ± 1.1 b | 6.4 ± 0.6 b | 91.5 ± 18.4 c | ND      | 20.9 ± 2.7 b | 7.4 ± 0.9 bc |
| CP1I    | 11.3 ± 2.1 b | 28.2 ± 7.8 b | 4.4 ± 1.1 b | 62.0 ± 12.4 b | 16.0 ± 3.4 b | ND          |
| CP2I    | 10.5 ± 0.7 b | 18.1 ± 5.3 b | 2.9 ± 0.8 b | 46.5 ± 4.3 b | 24.5 ± 2.6 b | ND          |
| CP6I    | 8.4 ± 0.9 b | 30.2 ± 7.1 b | 3.5 ± 0.5 b | 30.2 ± 7.8 b | 20.8 ± 2.1 b | 6.6 ± 0.8 bc |
| CP7II   | 13.1 ± 1.6 b | 29.9 ± 9.4 b | 2.7 ± 0.2 b | ND      | 15.7 ± 1.5 b | 15.8 ± 3.0 bc |
| CS1I    | 10.6 ± 1.6 b | 34.2 ± 9.4 b | 3.7 ± 0.9 b | 44.3 ± 14.9 b | 17.6 ± 3.2 b | 10.0 ± 1.6 bc |
| CS2I    | 17.0 ± 1.5 b | 40.1 ± 7.8 b | 2.7 ± 0.3 b | 47.3 ± 10.8 b | 25.5 ± 2.2 b | ND          |
| CS3I    | 17.0 ± 3.4 b | 30.5 ± 6.2 b | 2.1 ± 0.1 b | 40.2 ± 8.1 b | 25.0 ± 2.1 b | ND          |
| CS4I    | 18.9 ± 2.7 b | 36.2 ± 8.4 b | 3.0 ± 0.6 b | 52.8 ± 8.4 b | 23.9 ± 4.0 b | ND          |
| CS5III  | 10.7 ± 1.0 b | 36.1 ± 7.1 b | 3.6 ± 0.7 b | 48.9 ± 11.0 b | 21.9 ± 3.9 b | 13.9 ± 2.9 bc |
| CT1I    | 10.3 ± 1.3 b | 36.4 ± 7.8 b | 2.5 ± 0.2 b | 57.3 ± 8.3 b | 24.7 ± 4.1 b | 10.0 ± 1.8 bc |
| CT2I    | 13.0 ± 1.0 b | 35.3 ± 6.8 b | 2.4 ± 0.3 b | 44.7 ± 10.1 b | 31.9 ± 2.8 b | ND          |
| CT4I    | 10.2 ± 0.9 b | 28.5 ± 5.5 b | 2.2 ± 0.5 b | 67.1 ± 10.7 b | 32.2 ± 4.4 b | 11.1 ± 3.3 bc |
| CT6I    | 11.1 ± 0.9 b | 29.2 ± 9.1 b | 2.5 ± 0.2 b | 37.3 ± 8.3 b | 32.6 ± 3.9 b | ND          |
| CT3III  | 10.5 ± 1.3 b | 28.8 ± 8.8 b | 2.1 ± 0.2 b | 49.9 ± 12.4 b | 30.0 ± 4.1 b | 7.3 ± 1.2 bc |
| CO1I    | 16.7 ± 1.7 b | 29.4 ± 6.7 b | 3.7 ± 0.4 b | 44.3 ± 13.7 b | 27.6 ± 3.0 b | 7.2 ± 1.1 bc |
| CO3I    | 9.5 ± 1.5 b | 26.0 ± 7.0 b | 5.1 ± 1.4 b | 52.4 ± 7.9 b | 19.8 ± 2.2 b | 9.2 ± 2.0 bc |
| CO4I    | 13.0 ± 1.2 b | 32.2 ± 7.9 b | 2.4 ± 0.4 b | 57.4 ± 14.4 b | 24.1 ± 2.2 b | ND          |
| CO5I    | 15.7 ± 1.5 b | 29.2 ± 4.3 b | 3.5 ± 0.5 b | 54.2 ± 6.9 b | 21.5 ± 3.0 b | ND          |
| CO2III  | 13.2 ± 2.1 b | 42.4 ± 6.8 b | 3.4 ± 0.3 b | 36.7 ± 8.4 b | 24.3 ± 3.8 b | ND          |
| 2011    | 13.2 ± 2.3 b | 19.8 ± 2.8 b | 2.4 ± 0.3 b | 34.5 ± 10.9 b | 22.4 ± 2.0 b | 8.5 ± 4.6 bc |
| M29     | ND      | 21.1 ± 7.3 b | 4.6 ± 1.0 b | 31.7 ± 8.7 b | 21.6 ± 2.9 b | 6.8 ± 3.1 bc |
| M104    | ND      | ND      | 11.4 ± 4.2 bc | ND      | ND          | ND          |
| M3      | ND      | ND      | 27.5 ± 15.2 c | ND      | ND          | ND          |
| WSM533  | 14.7 ± 5.8 b | 42.7 ± 15.2 de | ND      | ND      | ND          | ND          |
| Control with N (10 mM) | 48.0 ± 1.8 c | 107.1 ± 6.3 d | 103.6 ± 13.5 f | 65.0 ± 17.0 b | 185.5 ± 12.0 c | 17.4 ± 1.4 c |
| Uninoculated control | 3.9 ± 0.9 d | 6.5 ± 2.7 c | 4.6 ± 1.2 b | 5.1 ± 1.2 c | 10.1 ± 1.9 d | 4.0 ± 1.7 b |

* DM: dry matter of *Medicago* species.

* a: in mg/plant ± S.D. (eight replicates).

ND: not determined.

Letters (b to f) indicate significant differences (*P < 0.01*) between data from a given *Medicago* species in each column.
6.7 nodules per plant). In contrast, the four other M. polymorpha isolates could elicit, with high regularity a higher number of nodules (x = 23.2 ± 38.3 nodules per plant). On M. polymorpha, differences in the aspect of nodules were observed: all the isolates, except CP4I and CP4III, formed rudimentary and white (Fig. 4A) nodules which we counted positively as they provide evidence of infection. In contrast, CP4I and CP4III isolates elicited cylindrical and typical efficient nodules (Fig. 4C). This striking phenotypic trait was also shared by reference strains: strains M3, M104 (Fig. 4B) and WSM533 triggered typical nodules while M29 and 2011 elicited rudimentary ones.

The shoot dry matter data are given in Table 2. Among the 33 Combaillaux isolates, two significant differences were detected: (i) on M. rigidula, dry matter was lower (P < 0.001) for two isolates (CP4I and CP4III) than for all other R. meliloti isolates and the two reference strains (2011 and M29); and (ii) on M. polymorpha, two isolates (CP4I and CP4III) trapped from M. polymorpha and two reference strains (WSM533 and M3) strains were able to develop an efficient nitrogen fixation (P < 0.001). The 29 other Combaillaux isolates and two other reference strains (2011 and M29) elicited non-effective and rudimentary nodules on M. polymorpha, with no significant difference in biomass over the uninoculated control. The fifth reference strain M104 tested on M. polymorpha showed plant biomass data slightly higher than the uninoculated control but not significantly different. However, plants in symbiosis with M104 were obviously greener and more vigorous (Fig. 4B) than uninoculated plants or plants associated to non-efficient Combaillaux isolates like CP6I (Fig. 4A). Probably, M104 is a strain with a low N2-fixation ability that we cannot reveal with the dry plant biomass method.

3.3. Comparison between molecular groupings and symbiotic properties

The most relevant symbiotic properties of Combaillaux isolates were noted on M. polymorpha plants: isolates with a R1 ribosomal profile elicited rudimentary and non-efficient nodules on M. polymorpha whereas the two isolates with a R2 profile (CP4I and CP4III) triggered typical and efficient ones (Fig. 4A,C). Such properties were extended to five R. meliloti reference strains: strains M29 and 2011 close to R1 type, belonged to branch I and elicited rudimentary and non-efficient nodules on M. polymorpha while strains WSM533, M3 and M104, genetically close to R2 type, belonged to branch II and triggered cylindrical and mature ones. These typical nodules formed on M. polymorpha were efficient except for M104 which appears to be a low symbiotic N2-fixer (Fig. 4B). Moreover, rhizobia isolates with a R1 profile seem to nodulate more poorly M. rigidula plants than isolates identified by a R2 profile. The correlation with the same symbiotic properties can be recovered from groups I and II depicted by nif pattern analysis except for the strain M29. In contrast, the subdivision within branch I (i.e. N1 and N2 groupings) could not be related to the cross-nodulation and -fixation results.

4. Discussion

Two markedly divergent R. meliloti groups, which we designated groups I and II, were found in a restricted soil area: a dominant one (I) was trapped by the six Medicago species and a minor one (II) by M. polymorpha. Our classification is based on the analysis of two chromosomal and plasmidic markers and of the symbiotic properties of different isolates and strains.

Our analysis of a chromosomal region containing the 16S rDNA plus the IGS nested between 16S and 23S rRNA genes allows an easy differentiation among Rhizobium isolates from the same geographical origin. Because the restriction patterns of amplified DNAs harbouring only the 16S rRNA gene were identical for all the rhizobia tested, divergence between isolates and strains laid on the IGS part analysis. This result is not surprising as 16S rDNA sequences are highly conserved within a species: two divergent R. meliloti strains differed only by a single nucleotide on a 260-bp 16S rDNA segment [30] and differences between 16S rRNA sequences may be insufficient to give satisfactory resolution within bacterial genera [31]. Thus, we confirmed that intergenic and non-coding rDNA regions are excellent targets to discriminate closely related species among rhizobia and relatives [13,32]. Groups I and II were
already found by two other chromosomal analyses with isoenzymes [10] and with repetitive DNA dispersed in the genome [33]. Our results compare well with those just mentioned as we used some identical strains. The two ribosomal groups I and II we detected, should correspond to divisions A and B, as defined by Eardly et al. [10]; indeed, division A represented by strains M29, L5-30 and 2011, parallels ribosomal group I, whereas division B including strains M3 and M104, matches ribosomal group II. The classification of *R. meliloti* in two groups is therefore validated by a combination of different techniques as required for a polyphasic taxonomic approach. However, groups I and II will be formally stated as individual species only when differences by DNA/DNA hybridization and by phenotypic traits are well documented [34].

Although chromosomally encoded characteristics are crucial for establishing bacterial taxonomy, symbiotic performances which are plasmid-encoded among *R. meliloti* [35] are important for a practical use of rhizobia. So it was interesting to chose a pSym marker since plasmidic DNA could evolve differently than chromosomal DNA. A plasmidic marker might have been more informative than a chromosomal one for screening symbiotic properties. Nevertheless, the discrimination power between the two molecular markers was slightly better with the amplified ribosomal intergenic region than those of *nif* KD genes (five versus four profiles), and divisions I and II detected by *nif*K-D amplified DNA analysis matched those established by ribosomal PCR-RFLPs, except for one strain M29 which had a highly divergent *nif* region.

The occurrence of groups I and II was confirmed by nodulation and fixation abilities on *M. polymorpha*. Profile K2 was consistent with isolates efficient on *M. polymorpha* whereas profile R1 corresponded to isolates which elicit rudimentary and non efficient nodules. Such properties were shared by reference strains from different geographical origins: strains genetically close to R1 type were not efficient whereas those close to K2 type were efficient in fixing nitrogen on *M. polymorpha*. The strain M29, characterized by a R1 profile with a broadly divergent *nif* region, induced non-efficient nodules on *M. polymorpha* and thus behaved like the other strains belonging to group I. Yet, M29, which was selected as inoculant for *M. rigidula* [36], behaved in a particular way: its nodulation activity could be unstable after long storage at 4°C (> 3 months) (L.A. Materon, personal communication, unpublished results). Erratical nodulation on *M. rigidula* for group II rhizobia, was also noted. A similar phenomenon was obtained by Brockwell et al. [8] on one line of *M. rigidula*. This could be the result of variability due to experimental conditions used for plant tests. It may be also due to a mixture of nodulating and non-nodulating plants grown from seeds collected from local *M. rigidula* plants, as shown on other leguminous plants [37]. Further data are thus required on *M. rigidula* for determining if the low ability of nodulation is correlated to rhizobia from group II. Yet all molecular polymorphism’s detected were not related to symbiotic properties. Within group I, N1 and N2 groupings, and within group II, R2, R4 and R5, could not be related to the nodulation and fixation test results, but could correspond to other biological properties. The correlation between genotypic and symbiotic characterisations was thus consistent only when the deeper dichotomy I and II of the ribosomal and *nif* dendrograms were compared with the nodulation behaviors on *M. polymorpha*. M29 was still an exception when we considered only its *nif* grouping which was excluded from groups I and II although its ribosomal profile belonged to group I. The ribosomal profiles we have observed have probably no particular significance in the nodulation process on *M. polymorpha*, they are merely markers of genetic lineages. Therefore, it would be interesting to study nodulation processes of these two lineages as they could show differential adaptation to this medic.

Seventy-four *R. meliloti* strains originating from four of the five annual medics we used (*M. truncatula*, *M. polymorpha*, *M. orbicularis* and *M. rigidula*) were included in both divisions A and B as defined by Eardly et al. [10]. Similarly, isolates trapped with the Combaillaux *M. polymorpha* plants, belonged to the two different groups I and II. Although group II rhizobia were able to nodulate efficiently the perennial lucerne *Medicago sativa* and some other medics, they were not extracted from them. Group II rhizobia could be a lower competitor than those of group I on *M. sativa* and the medics tested except *M. polymorpha*. This suggestion of a difference in competition
ability is not in contradiction with characterization by MLEE [10] for strains collected from *M. rigidula* and *M. sativa*. When *M. rigidula* was the plant origin, 23 isolates fell into division A and ten in division B, whereas when *M. sativa* was the plant origin, the difference of distribution was more clearly marked: 93 *M. sativa* rhizobia belonged to division A while only 14 were found in division B. In contrast, isolates from *M. truncatula*, *M. orbicularis* and *M. polymorpha* were equally distributed between both divisions A and B [10]. One important point to consider for characterizing groups I and II seems to be their nodulation behaviors on medics and not only their plant origin as non effective strains could be isolated from *M. polymorpha*.

In this study, the divergence between groups I and II cannot reflect any geographic difference, as they originated from a very restricted area of ten-gram soil. They are two sympatric groups in the Combaillaux sampling site but their distribution areas could be different as their multiplication into the soil could depends on the occurrence of the plant host. The presence of *R. meliloti* with R2 backgrounds could be favoured by the distribution of *M. polymorpha* species, whereas rhizobia with R1 backgrounds could be better promoted by other medics or lucerne.

Acknowledgements

We are grateful to Dr. G. Gintzburger for helping to identify the medic species and Drs. B. Eardly, C. Rosenberg, L. Materon, and J. Howieson for supplying reference strains of *R. meliloti* to Lucette Mauré for technical assistance and to François Mazzela for photographic artwork. We thank the laboratory of Biochimie et Physiologie Végétales INRA-ENSA-CNRSURA 573 (Montpellier, France) for the use of their equipment.

References

[1] Lesins, K.A. and Lesins, I. (1979) Genus *Medicago* (Leguminosae): a Taxogenetic Study, pp. 46–53. Dr. W. Junk bv Publishers. The Hague, The Netherlands.

[2] Gintzburger, G., Rochon, J.J. and Conesa, A.P. (1990) The French Mediterranean zones: sheep rearing systems and the present and potential role of pasture legumes. In: The Role of Legumes in the Farming Systems of the Mediterranean Areas (Osman, A.E. et al., Eds.), pp. 174–194. ICARDA. The Netherlands.

[3] Gintzburger, G. and Prosperi, J.-M. (1987) D'autres luzernes anuelles. Semences fourragères, 101, 23–26.

[4] Puckridge, D.W. and French, R.J. (1983) The annual legume pasture in the cereal-ley farming system of southern Australia: a review. Agric. Ecosys. Environ. 9, 229–267.

[5] Crawford, E.J., Lake, A.W.H. and Boyce K.G. (1989) Breeding annual *Medicago* species for semiarid conditions in southern Australia. Adv. Agron. 42, 399–437.

[6] Brockwell, J. and Hely, F.W. (1961) Symbiotic characteristics of *Rhizobium meliloti* from the brown acid soils of the Macquarie region of New South Wales. Aust. J. Agric. Res. 12, 630–643.

[7] Brockwell, J. and Hely, F.W. (1966) Symbiotic characteristics of *Rhizobium meliloti*: an appraisal of the systematic treatment of nodulation and nitrogen fixation interactions between hosts and rhizobia of diverse origins. Aust. J. Agric. Res. 17, 885–899.

[8] Brockwell, J., Holliday, R.A., Daoud, D.M. and Materon, L.A. (1988) Symbiotic characteristics of *Rhizobium*: specific annual medic, *Medicago rigidula* (L.) All. Soil Biol. Biochem. 20, 593–600.

[9] Materon, L.A. (1991) Symbiotic characteristics of *Rhizobium meliloti* in west Asian soils. Soil Biol. Biochem. 23, 429–434.

[10] Eardly, B.D., Materon, L.A., Smith, N.H., Johnson, D.A., Rumbaugh, M.D. and Selander, R.K. (1990) Genetic structure of natural populations of the nitrogen-fixing bacterium *Rhizobium meliloti*. Appl. Environ. Microbiol. 56, 187–194.

[11] Jamann, S., Fernandez, M.P. and Normand, P. (1993) Typing method for *N*₂-fixing bacteria based on PCR-RFLP application to the characterization of *Frankia* strains. Mol. Ecol. 2, 17–26.

[12] Laguere, G., Allard, M.R., Revoy, F. and Amarger, N. (1994) Rapid identification of rhizobia by restriction fragment length polymorphism analysis of PCR-amplified 16S rRNA genes. Appl. Environ. Microbiol. 60, 56–63.

[13] Nour, S., Fernandez, M.P., Normand, P. and Cleyet-Marel, J.C. (1994) *Rhizobium ciceri* sp. nov. consisting of strains that nodulate chickpeas (*Cicer arietinum* L.). Int. J. Syst. Bacteriol. 44, 511–522.

[14] Jensen, M.A., Webster, J.A. and Straus, N. (1993) Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. Appl. Environ. Microbiol. 59, 945–952.

[15] Weisburg, W.G., Barns, S.M., Pelletier, D.A. and Lane, D.J. (1991) 16S ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173, 697–703.

[16] Vincent, J.M. (1970) A manual for the practical study of root-nodule bacteria. IBP handbook no. 15. Blackwell Scientific Publications Ltd., Oxford.

[17] Gibson, A.H. (1980) Methods for legumes in glasshouse and controlled environment cabinets. In: Methods for Evaluating Biological Nitrogen Fixation (Bergersen, F.J., Ed.), pp. 139–184. Wiley. Chichester and N.Y.
[18] Brunel, B., Cleyet-Maré, J.C., Normand, P. and Bardin, R. (1988) Stability of *Bradyrhizobium japonicum* inoculants after introduction into soil. Appl. Environ. Microbiol. 54, 2636–2642.

[19] Howieson, J.G., Ewing, M.A. and D’Antuono, M.F. (1988) Selection for acid tolerance in *Rhizobium meliloti*. Plant and Soil 105, 179–188.

[20] Beringer, J.E. (1974) R factor transfer in *Rhizobium leguminosarum*. J. Gen. Microbiol. 84, 188-198.

[21] Simonet, P., Grosjean, M.C., Misra, A.K., Nazaret, S., Cournoyer, B. and Normand P. (1991) *Frankia* genus-specific characterization by polymerase chain reaction. Appl. Environ. Microbiol. 57, 3278–3286.

[22] Normand, P., Cournoyer, B., Simonet, P. and Nazaret, S. (1992) Analysis of a ribosomal RNA operon in the actinomycete *Frankia*. Gene 111, 119–124.

[23] Willems, A. and Collins, M.A. (1993) Phylogenetic analysis of rhizobia and agrobacteria based on 16S rDNA gene sequences. Int. J. Syst. Bacteriol. 43, 305–313.

[24] Yanagi, M. and Yamasato, K. (1993) Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. FEMS Microbiol. Lett. 107, 115–120.

[25] Nei, M. and Li, W.H. (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. USA 76, 5269-5273.

[26] Sneath, P.H.A. and Sokal, R.R. (1973) Numerical Taxonomy. The Principles and Practice of Numerical Classification, pp. 223–234. W.H. Freeman and Co., San Francisco.

[27] Saitou, N. and Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

[28] Miller, J.C. (1992) RESTSITE: a phylogenetic program that sorts restriction data. J. Heredity 32, 262–263.

[29] Dagnelie, P. (1970) L’analyse de la variance à deux critères, p. 153–209. In: Théorie et Méthodes Statistiques. Applications Agronomiques, Vol.2. Les Presses Agronomiques de Gembloux, Gembloux, Belgium.

[30] Eardly, B.D., Young, J.P.W. and Selander, R.K. (1992) Phylogenetic position of *Rhizobium* sp. strain OR191, a symbiont of both *Medicago sativa* and *Phaseolus vulgaris*, based on partial sequences of the 16S rRNA and *rifH* genes. Appl. Environ. Microbiol. 58, 1809–1815.

[31] Fox, G.H., Wisotzkey, J.D. and Jurtshuk, Jr. (1992) How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. Int. J. Syst. Bacteriol. 42, 166–170.

[32] Ponsonnet, C. and Nesme, X. (1994) Identification of *Agrobacterium* strains by PCR-RFLP analysis of pTi and chromosomal regions. Arch. Microbiol. 161, 300–309.

[33] De Bruijn, F.J. (1992) Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. 58, 2180–2187.

[34] Graham, P.H., Sadowsky, M.J., Keyser, H.H., Barnet, Y.M., Bradley, R.S., Cooper, J.E., De Ley, D.J., Roslycky, E.B., Strijdom, B.W. and Young, J.P.W. (1991) Proposed minimal standards for the description of new genera and species of root- and stem-nodulating bacteria. Int. J. Syst. Bacteriol. 41, 582–587.

[35] Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. and Kondorosi, A. (1981) Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *R. meliloti*. Mol. Gen. Genet. 184, 318–325.

[36] ICARDA (International Center for Agricultural Research in the Dry Areas) (1988) Pasture, Forage and Livestock Program. Annual report, Aleppo, Syria.

[37] Cregan, P.B., Keyser, H.H. and Sadowsky, M.J. (1989) Host plant effects on nodulation and competitiveness of the *Bradyrhizobium japonicum* serotype strains constituting serocluster 123. Appl. Environ. Microbiol. 55, 2532–2536.