

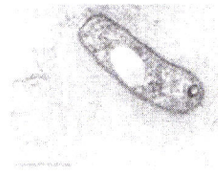

GENETIC DIVERSITY AND SYMBIOTIC EFFECTIVENESS OF CHICKPEA RHIZOBIA STRAINS

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Dissertation presented to obtain the Ph.D. degree in Biology at the Universidade de Évora

Universidade de Évora
Évora, 2006

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This thesis does not include the critics and suggestions made by the juri.

To Gui and Nini

"...the way in which I approach microbiology...can be concisely stated as the study of microbial ecology, i. e., of the relation between environmental conditions and the special forms of life corresponding to them."

Beijerinck, 1905

ACKNOWLEDGEMENTS

The present study was carried out at the Soil Microbiology Laboratory from the Institute of Mediterranean Agrarian Sciences (ICAM) and the Department of Biology of the University of Évora, to which I want to thank for providing the necessary conditions to perform this work.

Thanks are also due to the "Fundação para a Ciência e a Tecnologia" for funding both a PhD fellowship (SFRH/BD/6772/2001) and two research projects PRAXIS-(PRAXIS/P/AGR/11129/1998) and SAPIENS (POCTI/BME/44140/2002).

Furthermore, this work would not have been possible without some people to whom I want to express my gratitude:

To Professor Solange Oliveira, my supervisor, for believing in me from the beginning. She was always most demanding and at the same time most supportive, allowing me to grow up as a scientist. This is the result of her excellent guidance and invaluable friendship.

To Professor Peter Young, my co-supervisor, for receiving me in his lab and for having accepted the co-supervision of this thesis. He provided valuable help in critical moments, and was always available for insightful email discussions.

To Doctor Jorge Machado from the INSA, for having kindly received me in his lab, where I performed the 16S rDNA gene sequencing.

To Prof. Encarna Velázquez of the University of Salamanca for her collaboration, especially in the sequencing of symbiotic genes.

To Doctor Isabel Duarte, from the ENMP; for providing the chickpea seeds and some useful information about the grain legume-chickpea, and also for her friendship.

To Prof. Peter Young, Prof. Encarna Velázquez, Prof. Esperanza Martínez-Romero, Prof, Kristina Lindström, Prof. William Broughton, Prof. Tomas Ruíz-Argüeso and Dr. Julie Weaver for kindly providing the rhizobia reference and type strains.

To Dr. Tânia Tenreiro from ICAT for her help with the BioNumerics software.

To Dr. Ivo Chelo from ICAT for his help with the PAUP software.

To Engº Luís Alho, the head of our lab, for his help during the first times in the lab, but also for his support and friendship.

To Gertrudes Mariano, our lab technician, for her precious helps in preparing some of the material and lending a hand in the chamber trials and strain maintenance.

To Ana Alexandre, with whom I have worked in the lab for the last three and a half years, for her priceless help, and above all for her friendship.

To all the other people with whom I have shared the bench, among other things, throughout my years in the lab: Rute Rodrigues, Cristina Branco, Rebeca Soares, Carla Rodrigues, Catarina Campos and Clarisse Brígido.

To my dear friends and colleagues in science, particularly, Rosário Félix, José Manuel Martins, Joana Cardoso, Matilde Piteira, Carla Varanda, Vera Valadas, Elsa Lamy and Paulo Vieira thank you for your valuable helps and discussions (not always scientific ones!) and for making life fun.

To my family, for their support, in particular to Luís, and to my children, Gui and Nini.

THESIS ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which are listed by chronological order of publication. Furthermore, some unpublished results are presented.

1. **Laranjo, M., Rodrigues R., Alho L. and Oliveira, S.** 2001. Rhizobia of chickpea from southern Portugal: symbiotic efficiency and genetic diversity. J. Appl. Microbiol. 90: 662-667.

2. **Laranjo, M., Branco, C., Soares, R., Alho, L., Carvalho, M. and Oliveira, S.** 2002. Comparison of chickpea rhizobia isolates from diverse Portuguese natural populations based on symbiotic effectiveness and DNA fingerprint. J. Appl. Microbiol. 92: 1043-1050.

3. **Laranjo, M., Machado, J., Young, J. P.W. and Oliveira, S.** 2004. High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. FEMS Microbiol. Ecol. 48: 101-107.

4. **Alexandre, A., Laranjo, M. and Oliveira, S.** 2006. Natural populations of chickpea rhizobia evaluated by antibiotic resistance profiles and molecular methods. Microb. Ecol. 51: 128-136.

5. **Velázquez, E., Rivas, R., Laranjo, M., Mateos, P. F., Oliveira, S. and Martínez-Molina, E.** Strains of *Mesorhizobium amorphae* and *M. tianshanense* carrying symbiotic genes of common chickpea endosymbiotic species constitute a novel biovar (ciceri) of these species able to nodulate *Cicer arietinum*. *Submitted to Letters in Applied Microbiology* (LAM-2006-0954).

6. **Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W. and Oliveira, S.** Phylogenetic analysis of symbiotic genes supports lateral gene transfer within the genus *Mesorhizobium*. *Manuscript in preparation.*

7. **Laranjo, M., Alexandre, A., Alho, L., Young, J. P. W. and Oliveira, S.** A new species of *Mesorhizobium* supported by a multilocus sequence approach. *Manuscript in preparation.*

OTHER PUBLICATIONS

1. **Rodrigues, C., Laranjo, M. and Oliveira, S.** 2006. Effect of heat and pH stress in the growth of chickpea mesorhizobia. Curr. Microbiol. 53: 1-7.

2. **Laranjo, M. and Oliveira, S.** 2006. Rhizobial Strain Improvement: Genetic Analysis and Modification. In "Microbial Biotechnology in Agriculture and Aquaculture" (Editor: R. C. Ray). Science Publisher Inc, Enfield, New Hampshire, USA. Vol II, pp. 225-260.

3. **Brígido, C., Alexandre, A., Laranjo, M. and Oliveira, S.** Moderately acidophilic mesorhizobia isolated from chickpea. Lett. Appl. Microbiol. *Accepted for publication* (LAM-2006-0709).

4. **Alexandre, A., Brígido, C., Laranjo, M., Rodrigues, S., Alho, L. and Oliveira, S.** Assessment of chickpea rhizobia species diversity in Portugal: beyond the two known microsymbionts. *Manuscript in preparation*.

ABSTRACT

Rhizobia are soil bacteria able to establish nitrogen-fixing symbioses with leguminous plants inside special root tissues, the nodules. These symbioses are of extreme importance in agriculture allowing many plant crops to be cultivated without the need for chemical fertilisers.

In the present thesis work, the genetic diversity, phylogeny and symbiotic effectiveness (SE) of native rhizobia, able to nodulate chickpea (*Cicer arietinum* L.), have been addressed.

Forty-one chickpea rhizobia isolated from four different Portuguese soils (Beja, Elvas, Elvas- ENMP and Évora) were phenotypically evaluated. Twenty-one isolates were further analysed and assigned to the genus *Mesorhizobium* based on their 16S rDNA sequences. Highly diverse *Mesorhizobium* strains were identified, belonging to six different species groups: *M. ciceri* and *M. mediterraneum*, the expected known chickpea symbionts, *M. loti*, *M. tianshanense*, and two probably new species.

A correlation was found between 16S rDNA species groups and origin of individual isolates.

Rhizobial diversity of the forty-one isolates from natural populations was assessed by molecular methods, namely 16S rDNA restriction fragment length polymorphism (RFLP) analysis, plasmid profiles, direct amplified polymorphic DNA (DAPD) fingerprinting and SDS–PAGE analysis of protein profiles. Plasmid number of isolates ranged from zero to six and was found to be correlated with origin and with species groups. 16S rDNA RFLP, DAPD and protein profiles generated analogous clustering of the isolates, supporting results on 16S rDNA sequence based phylogeny of the subgroup of twenty-one isolates. DAPD analysis, a newly described PCR-based approach, proved to be the most discriminating approach in strain differentiation and can be used as a fast method to screen diversity in new isolates. Evaluation of genetic diversity by the four molecular methods showed different levels of heterogeneity in the natural populations. A higher genetic diversity was found in Elvas-ENMP and Beja populations.

The SE determined for the twenty-one isolates as well as for the two chickpea microsymbionts, *M. ciceri* and *M. mediterraneum*, ranged from 4 to 84%. No correlation was depicted between SE and origin site of the isolates. However, Beja isolates show the highest mean SE, and Elvas-ENMP isolates have the lowest mean SE. We detected no significant correlation between SE and species.

A multilocus phylogenetic approach was used to confirm the molecular phylogeny of the subgroup of twenty-one chickpea rhizobia isolates. Phylogenetic analysis based on the intergenic spacer between 16S and 23S rRNA genes (ITS), the

ATP synthase (*atp*D) or the DNA recombinase A (*rec*A) sequences corroborated the 16S rDNA phylogeny and confirmed the existence of six distinct species groups among chickpea mesorhizobia. Further evidence is provided for supporting one of these evolutionary lineages as new species within the genus *Mesorhizobium*. Indeed, sequencing of another housekeeping protein coding gene, the glutamine synthetase I gene (*gln*A), from this new group of isolates, confirmed its separate position and assignment to a new species. The name *M. lusitanum* is proposed, with isolate 64b.-Beja as the type strain. All isolates from the *M. lusitanum* group showed a high symbiotic effectiveness (above 50%) and may be potentially useful field inoculants.

Chickpea has been considered a restrictive host for nodulation by rhizobia. However, the present work, as well as other recent studies, have shown that several *Mesorhizobium* species may effectively nodulate chickpea. In order to investigate the relationships between symbiosis genes from different rhizobia species able to nodulate chickpea, the *nif*H and *nod*C genes from the twenty-one Portuguese chickpea rhizobia isolates were sequenced and used for phylogenetic studies.

The phylogenies based on symbiosis genes showed that, regardless of their species affiliation, all chickpea rhizobia isolates formed a single highly supported cluster, an evidence of lateral transfer of symbiosis genes across different species. Chickpea is confirmed as a non-promiscuous host. The six different rhizobia species, that nodulate chickpea, share common symbiosis genes, suggesting recognition of only a few Nod factors by chickpea. Further analysis of symbiosis genes, namely copy number and location, performed by Southern hybridisation of plasmid profiles suggests the presence of, at least, two symbiosis plasmids in some isolates. Moreover, we provide evidence for the existence of at least two copies of the *nod*C gene in three isolates (6b.-Beja, 29-Beja and EE-29-ENMP), which is uncommon and has not been reported before in mesorhizobia.

Overall, this work has contributed to the study of diversity and evolutionary relationships among mesorhizobia and has presented further evidence of horizontal gene transfer among several species of the genus *Mesorhizobium*, being the first report on lateral gene transfer between chickpea mesorhizobia. It has changed the current view on chickpea microsymbionts, since it has revealed that several species of *Mesorhizobium* can nodulate chickpea, besides *M. ciceri* and *M. mediterraneum*. Furthermore, it has contributed to the better understanding of the symbiosis between chickpea and rhizobia at the molecular level.

RESUMO ALARGADO

Os rizóbios são bactérias do solo capazes de infectar as raízes das plantas leguminosas e estabelecer simbioses fixadoras de azoto dentro de tecidos especiais da raiz, os nódulos.

A simbiose entre rizóbios e plantas leguminosas é um caso especial de uma associação entre organismos procariotas e organismos eucariotas, que tem sido intensivamente estudada no decorrer das últimas décadas por desempenhar um papel importante na agricultura.

Presentemente, a fixação simbiótica do azoto pelos rizóbios é um sistema bem estudado e é um modelo conveniente das interacções planta-micróbio e da sua evolução.

A maior vantagem da fixação biológica do azoto em leguminosas de grão e forrageiras é o aumento potencial dos rendimentos sem o recurso a fertilizantes químicos azotados e a consequente diminuição da poluição. Esta simbiose entre rizóbios e leguminosas é o mecanismo biológico mais importante para fornecer azoto ao sistema planta-solo, o que justifica a importância ecológica da cultura de leguminosas, não só para melhorar a reserva de azoto para outras culturas, tais como pastagens e cereais, mas também na remediação do solo.

O estabelecimento da simbiose requer uma interacção molecular complexa e dinâmica entre os dois parceiros simbióticos. Os nódulos formam-se como resultado de troca de sinais complexos entre os dois parceiros simbióticos. Este diálogo químico é responsável pela especificidade da simbiose. Os microssimbiontes podem ter um espectro de hospedeiros estreito ou largo e, do mesmo modo, as leguminosas hospedeiro podem ser específicas ou promíscuas.

Os flavonóides parecem ser os exsudados da planta mais importantes para a simbiose, pois podem direccionar a bactéria para a rizosfera por quimiotaxia e possibilitar aos rizóbios distinguir o seu hospedeiro. Os flavonóides induzem a expressão dos genes de nodulação, indução esta que também requer a participação de uma proteína bacteriana activadora da transcrição, NodD.

O controlo genético da nodulação pelos rizóbios envolve genes de nodulação que determinam a produção de lipo-quito-oligossacáridos, conhecidos por factores Nod. Os factores Nod comportam-se como moléculas sinal específicas para as leguminosas e induzem respostas no hospedeiro, tais como, deformação do pêlo radicular e divisão celular cortical. São indispensáveis para a nodulação e são reconhecidos por receptores das leguminosas para os quais têm elevada afinidade.

Na região mediterrânica as leguminosas de grão, e o grão-de-bico em particular, constituem importantes culturas alternativas aos cereais, uma vez que são uma fonte de proteína essencial para a alimentação humana e animal. Em Portugal, o grão-de-bico é tradicionalmente uma cultura de Primavera destinada à alimentação humana. É cultivado praticamente em todo o país, mas a principal área produtiva está localizada no Alentejo. No

entanto, as variedades tradicionais de Primavera, adaptadas às condições de seca, apresentam valores de produção muito reduzidos. Ainda assim, desde os finais do século XX, a área de cultivo de grão-de-bico tem vindo a ser reduzida, devido aos baixos rendimentos, entre outros factores. Cultivares melhoradas de grão-de-bico possibilitaram o seu uso como uma cultura de sementeira antecipada de Outono/Inverno com rendimentos potencialmente mais elevados, quando comparados com as variedades tradicionais de Primavera.

A avaliação da diversidade rizobiana é essencial para a identificação e selecção de estirpes inoculantes apropriadas para uma determinada cultura.

O estudo das populações rizobianas pode ser conseguido usando abordagens fenotípicas e moleculares. Vários métodos têm sido usados para diferenciação de estirpes que permitem avaliar as estirpes de rizóbio a diferentes níveis, nomeadamente DNA, RNA, características fenotípicas, função e expressão de proteínas, ácidos gordos e marcadores quimiotaxonómicos. A grande variedade de técnicas disponível conduziu à proposta de uma abordagem polifásica em taxonomia bacteriana que inclua resultados obtidos com diferentes métodos.

A maioria das espécies de rizóbios possuem plasmídeos grandes que podem variar em número e peso molecular. Estes nem sempre se encontram associados à simbiose, podem conter genes essenciais ao metabolismo celular e alguns podem ser crípticos.

Recentemente, tem vindo a ser recomendada a análise de vários genes para constituir uma base de dados de rizóbio que inclua sequências de genes essenciais, como DNAr 16S, *dna*K (chaperonina molecular de 70 kDa), *gln*A e *gln*II (glutamina sintetases I e II), *atp*D (ATP sintase β), *rec*A (DNA recombinase A), e *glt*A (citrato sintase) utilizados em filogenia molecular.

Apesar da filogenia baseada na sequência do gene do RNA ribosomal 16S se ter tornado geralmente aceite, é óbvio que a história evolutiva das bactérias só poderá ser melhor compreendida através de uma filogenia baseada nas sequências de vários genes.

No trabalho da presente tese, a diversidade genética, a filogenia e a eficiência simbiótica de isolados de rizóbio nativos, capazes de nodular grão-de-bico (*Cicer arietinum* L.), foram estudadas.

Quarenta e um rizóbios de grão-de-bico isolados de quatro solos portugueses diferentes (Beja, Elvas, Elvas-ENMP e Évora) foram analisados fenotipicamente e atribuídos ao género *Mesorhizobium* com base nas suas sequências de DNAr 16S. Foram identificadas estirpes de *Mesorhizobium* muito diversas, pertencentes a seis espécies diferentes: *M. ciceri* e *M. mediterraneum*, os simbiontes de grão-de-bico já conhecidos, *M. loti*, *M. tianshanense*, e duas prováveis novas espécies.

A diversidade rizobiana das quatro populações naturais foi estimada por métodos moleculares, nomeadamente análise de fragmentos de restrição do DNAr 16S, perfis de plasmídeos, padrões de DAPD ("Direct Amplified Polymorphic DNA") e análise por SDS-

PAGE de perfis de proteínas. O número de plasmídeos dos isolados varia de zero a seis e parece não estar correlacionado com a origem e a espécie. A análise de RFLP do DNAr 16S, os perfis DAPD e os perfis de proteínas geraram um agrupamento análogo dos isolados, suportando os resultados da filogenia baseada na sequência do DNAr 16S de um subgrupo de vinte e um isolados. A análise DAPD, uma abordagem baseada em PCR descrita recentemente, provou ser a abordagem mais discriminativa na diferenciação de estirpes e pode ser usada como um método rápido para avaliar diversidade de novos isolados. A avaliação da diversidade genética pelos quatro métodos moleculares mostrou diferentes níveis de heterogeneidade em populações naturais. Foi encontrada uma maior diversidade genética nas populações de Elvas-ENMP e Beja.

A eficiência simbiótica determinada para o subgrupo de vinte e um isolados, bem como para os dois microssimbiontes de grão-de-bico, *M. ciceri* e *M. mediterraneum*, foi de 4 a 84%. Não se detectou correlação entre eficiência e local de origem dos isolados. Os isolados de Beja mostram a maior eficiência simbiótica média, e os isolados de Elvas-ENMP têm a eficiência simbiótica média mais baixa. Não foi detectada qualquer correlação significativa entre eficiência simbiótica e espécie.

Uma abordagem filogenética com vários genes foi usada para corroborar a filogenia molecular de um subgrupo de vinte e um isolados portugueses de rizóbio de grão-de-bico. Análises filogenéticas baseadas nas sequências do espaçador intergénico entre os genes do RNA ribosomal 16S e 23S (ITS), da ATP sintase β (*atp*D) e da recombinase A (*rec*A) corroboram a filogenia baseada no DNAr 16S e confirmam a existência de, pelo menos, seis grupos distintos de espécies de mesorizóbios de grão-de-bico.

Com o objectivo de determinar se existiam mais isolados pertencentes às duas novas espécies suportadas pela filogenia baseada em cinco diferentes loci, foi realizada a sequenciação parcial do gene do DNAr 16S de mais nove isolados que se encontravam no mesmo grupo na árvore de RFLP do DNAr 16S. Foram encontrados mais cinco isolados pertencentes a uma nova espécie e mais dois isolados pertencentes a outra nova espécie. Para além disto encontrou-se um isolado, 87-Évora, que parece pertencer à espécie *M. huakuii*.

Apresenta-se ainda evidência adicional que suporta uma desta linhas evolutivas como uma nova espécie dentro do género *Mesorhizobium*. De facto, a sequenciação de outro gene essencial codificante para proteínas, o gene da glutamina sintetase I (*gln*A), deste novo grupo de isolados, confirmou a sua inclusão numa nova espécie. O nome *M. lusitanum* é proposto, com o isolado 64b.-Beja como a estirpe tipo. Isolados do grupo de *M. lusitanum* mostraram uma elevada eficiência simbiótica e podem ser úteis como inoculantes de campo.

O grão-de-bico tem sido considerado um hospedeiro restritivo à nodulação por rizóbios. No entanto, o presente estudo, bem como outros estudos recentes, mostraram que várias espécies de *Mesorhizobium* podem nodular efectivamente o grão-de-bico. Para investigar as

relações evolutivas entre estas diferentes espécies com a capacidade de nodular o mesmo hospedeiro, os vinte e um isolados portugueses de rizóbio de grão-de-bico foram analisados. Os genes simbióticos *nif*H e *nod*C foram sequenciados e usados para estudos filogenéticos.

As filogenias baseadas em genes simbióticos mostraram que, independentemente da sua afiliação em termos de espécie, todos os isolados de rizóbio de grão-de-bico formavam um único grupo bem suportado, evidenciando a transferência lateral de genes simbióticos entre diferentes espécies. O grão-de-bico é confirmado como hospedeiro não promíscuo. Apesar de ser nodulado por muitas espécies diferentes, estas partilham os mesmos genes simbióticos, o que sugere o reconhecimento de poucos factores Nod pelo grão-de-bico. A análise mais aprofundada dos genes simbióticos, nomeadamente do número de cópias e da localização pelo método de hibridação Southern sugere a presença de, pelo menos, dois plasmídeos simbióticos nalguns isolados. Para além disso, apresenta-se evidência para a existência de pelo menos duas cópias do gene *nod*C em três isolados, o que não é comum e ainda não tinha sido relatado para mesorizóbios. Este é o primeiro relato sobre transferência lateral de genes entre espécies de *Mesorhizobium* de grão-de-bico.

Neste estudo foram descritas pela primeira vez estirpes de *M. loti* e *M. tianshanense* capazes de nodular grão-de-bico. É também a primeira descrição de estirpes de *M. tianshanense* isoladas em solos europeus. Este é também o primeiro trabalho em filogenia molecular de bactérias do género *Mesorhizobium* que utiliza uma abordagem baseada na análise da sequência de diferentes loci.

Este trabalho mudou o actual panorama sobre microssimbiontes de grão-de-bico e contribuiu para o conhecimento da filogenia de rizóbios em geral. Variações nas associações entre genes essenciais e genes simbióticos podem constituir um problema para a classificação dos rizóbios. Até agora as descrições de novas espécies de rizóbios têm sempre incluído importantes características acessórias, como as propriedades simbióticas. No entanto, estas são codificadas por genes que são bastante susceptíveis de serem lateralmente transferidos, e este estudo reforça a noção de que rizóbios com diferentes contextos cromossomais podem ser portadores de genes simbióticos similares. Esta é uma possível explicação para o facto de uma planta leguminosa, que é um hospedeiro restritivo para a nodulação, como o grão-de-bico, seja nodulada por diferentes espécies de rizóbio.

No geral, este trabalho contribuiu para o estudo da diversidade e relações evolutivas entre mesorizóbios e apresentou evidência adicional de transferência horizontal de genes entre espécies do género *Mesorhizobium*, sendo o primeiro relato de transferência lateral de genes entre mesorizóbios de grão-de-bico. Revelou que diferentes espécies de *Mesorhizobium* podem nodular grão-de-bico, para além de *M. ciceri* e *M. mediterraneum*. Para além disto, contribuiu para o conhecimento da simbiose entre grão-de-bico e rizóbios ao nível molecular.

ABBREVIATIONS

16S	16S ribosomal RNA
23S	23S ribosomal RNA
*atp*D	ATP synthase β
bp	base pair
BSA	bovine serum albumin
bv.	biovar
DAPD	direct amplified polymorphic DNA
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
FAME	fatty acids methyl esters
g	gravity acceleration
*gln*A	glutamine synthetase I
IAR	intrinsic antibiotic resistance
kb	kilobase
LMW	low molecular weight
Mb	megabase
min	minutes
MLEE	multilocus enzyme electrophoresis
mM	milimolar
MPN	most probable number
n.d.	not detectable
n.dt.	not determined
NDW	nodules dry weight
NN	number of nodules
PAGE	polyacrilamide gel electrophoresis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
p*Sym*	symbiosis plasmid
rDNA	ribosomal RNA gene
*rec*A	DNA recombinase A
RDW	root dry weight
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid

RNase	ribonuclease
rRNA	ribosomal RNA
SDS	sodium dodecyl sulphate
SDW	shoot dry weight
SE	symbiotic effectiveness
sec	seconds
sp./spp.	species
SSC	sodium chloride/sodium citrate
TBE	Tris/borate/EDTA buffer
TE	Tris/EDTA buffer
TEMED	N, N, N', N'-tetrametylenediamine
Tris	tris hydroximethyl aminomethane
tRNA	transfer RNA
U	unit
v/v	volume/volume
w/v	weight/volume

NUCLEOTIDE BASES

A	Adenine
T	Thymine
G	Guanine
C	Cytosine

Y	pYrimidine	(C or T)
R	puRine	(A or G)
W	Weak	(A or T)
S	Strong	(C or G)
K	Keto	(T or G)
M	aMino	(C or A)
N	any base	(A, T, G or C)

TABLE OF CONTENTS

1. GENERAL INTRODUCTION

1.1. Background

The symbiosis between rhizobia and legumes is a special example of an association between prokaryotic and eukaryotic organisms (Perret *et al.*, 2000) and plays an important role in agriculture, having thus been intensively studied over the recent years.

The main advantage of biological nitrogen fixation, in grain and forage legumes, is the increase in yield potentials without the use of chemical nitrogen fertilisers and with the consequent decrease in pollution (Freiberg *et al.*, 1997; Stephens & Rask, 2000). This symbiosis rhizobia-legume is the most important biological mechanism for providing nitrogen to the soil/plant system, which justifies the ecological importance of legume crops not only in the improvement of nitrogen nutrition to other crops, such as pasture or cereals, but also in land remediation.

In the late nineteenth century, Hellriegel and Wilfarth (1888) explained the principles of biological nitrogen fixation. Their work made way to all the subsequent studies in nitrogen fixation and legume-bacteria symbioses and, at present, symbiotic nitrogen fixation by rhizobia is the best studied system (Young & Johnston, 1989) and a convenient model of plant-microbe interactions and their evolution (Quispel, 1988; Sprent, 1994).

Although many legumes are important crops for human and animal nutrition, cereals are still the major food crops all over the world. Therefore, the long-term ambitious goal of biological nitrogen fixation research has been to design strategies to extend the nitrogen-fixing symbioses to non-nodulated cereal plants, such as rice and wheat. Recently, endophytic associations of nitrogen-fixing organisms with non-legumes have been reported: rhizobia with rice (Reddy *et al.*, 1997; Yanni *et al.*, 2001), wheat (Webster *et al.*, 1997) and maize (Gutierrez-Zamora & Martinez-Romero, 2001); and *Azoarcus* with various grasses and rice (Reinhold-Hurek *et al.*, 1993).

Rhizobia are soil bacteria able to infect the roots of leguminous plants, promoting the formation of nitrogen-fixing nodules. These are produced as result of complex signal exchanges between the symbiotic partners. This chemical dialog is responsible for the specificity of the symbiosis: microsymbionts can have a narrow or broad host range and, similarly, host legumes are either specific or promiscuous (Dénarié *et al.*, 1996; Fisher & Long, 1992; Spaink, 1995; van Rhijn & Vanderleyden, 1995).

Table 1 shows the rhizobial species that usually nodulate some of the most cultivated legumes in the world.

Table 1. Rhizobial species that commonly nodulate legumes used for food and forage.

Legume		Rhizobial Species	Reference
Common Name	**Species**		
Chickpea	*Cicer arietinum*	*Mesorhizobium ciceri*	(Nour et al., 1994a)
		M. mediterraneum	(Nour et al., 1995)
Common bean	*Phaseolus vulgaris*	*Rhizobium etli*	(Segovia et al., 1993)
		R. gallicum	(Amarger et al., 1997)
		R. giardinii	(Amarger et al., 1997)
		R. leguminosarum bv. *phaseoli*	(Frank, 1879)
		R. mongolense	(van Berkum et al., 1998)
		R. tropici	(Martínez-Romero et al., 1991)
Faba bean	*Vicia faba*	*R. leguminosarum* bv. *viciae*	(Frank, 1879)
Soybean	*Glycine max*	*Bradyrhizobium elkanii*	(Kuykendall et al., 1992)
		B. japonicum	(Jordan, 1982)
		B. liaoningense	(Xu et al., 1995)
		S. fredii	(de Lajudie et al., 1994)
		Sinorhizobium xinjiangense	(Peng *et al.*, 2002)
Pea	*Pisum sativum*	*R. leguminosarum* bv. *viciae*	(Frank, 1879)
Alfalfa	*Medicago* spp.	*S. medicae*	(Rome et al., 1996)
		S. meliloti	(de Lajudie et al., 1994)
Clover	*Trifolium* spp.	*R. leguminosarum* bv. *trifolii*	(Frank, 1879)

The use of rhizobial inoculants has permitted the introduction of legumes outside their native regions. Nitrogen fixation depends not only on the physiological state of the host plant, but also on the effectiveness and environmental fitness of the microsymbiont and on the interaction between the two partners. Therefore, rhizobia strains used as inoculants must be competitive with the indigenous populations for nodule occupancy and be efficient in nitrogen fixation afterwards (Bloem & Law, 2001; Carter *et al.*, 1995; Okogun & Sanginga, 2003; Stephens & Rask, 2000). Inoculant strains must be selected for individual cultivars of a given legume, and also preferably for particular soil and environmental conditions; they should show high tolerance to climatic and edaphic stress conditions, such as extreme temperatures, adverse pH, and drought (Stephens & Rask, 2000). Another fundamental characteristic is its persistence in the soil over time (Carter *et al.*, 1995; Howieson, 1995).

At the recommended inoculation rates (Brockwell & Bottomley, 1995; Somasegaran & Hoben, 1994), inoculant strains often dominate in nodulation in the first year of a newly introduced crop (Singleton & Tavares, 1986). Furthermore, upon nodule senescence, a large number of viable rhizobia are released in the soil (McDermott *et al.*, 1987). Several studies have shown that inoculant strains still predominate in nodules 5 to 15 years after initial inoculation (Lindström *et al.*, 1990), confirming that they are effective soil saprophytes persisting in soil for many years in the absence of their host (Sanginga *et al.*, 1994).

A good inoculant strain can be modified to have a few essential characteristics, such as increased symbiotic effectiveness, tolerance to adverse environmental and soil conditions and, eventually,

extended nodulation range to new hosts. Thus, the study of the biodiversity of indigenous rhizobia populations and the description of new rhizobial species will provide the necessary background information for strain modification with the purpose of inoculant development.

1.2. The symbiosis rhizobium-legume

Root infection by rhizobia is initiated by pre-infection events in the rhizosphere. Rhizobia act in response to plant root exsudates by positive chemotaxis and move towards localised sites on legume roots. Rhizobia are attracted by low concentrations of excreted compounds, known as flavonoids, such as isoflavones, chalcones, flavonols, flavones, anthocyanidins, etc (van Rhijn & Vanderleyden, 1995).

Environmental conditions can act as limiting factors to the establishment of the symbiosis and to nitrogen fixation. Different limiting or stress factors can be distinguished: salt and osmotic stress; soil moisture deficiency; temperature extremes and stress; soil acidity and sodicity; nutrient deficiency; soils amendment and amelioration (Zahran, 1999).

In general during root nodule formation, infection and nodule organogenesis occur simultaneously. In order to infect the root, rhizobia first attach to the root hair, and then induce root hair deformation and curling followed by infection thread formation. At the same time, root cortical cells dedifferentiate and enter division resulting in the formation of a nodule primordium. The infection thread full of rhizobia grows from the root hair towards the nodule primordium localised in the inner cortex. Here, rhizobia are released into the cytoplasm of the host cells, surrounded by the peribacteroid membrane. At this stage, rhizobia differentiate into their endosymbiotic form, the bacteroids, which together with the peribacteroid membrane are called symbiosome. After infection, the primordia develop into nitrogen fixing root nodules. This process as described is typical of legumes from temperate regions, such as chickpea, that develop indeterminate nodules (Lee & Copeland, 1994). These contain a persistent meristem which results in cylindrical-shaped structures and transport fixed N_2 as amides. Both determinate and indeterminate nodules have a similar tissue organisation, a central tissue where bacteria are hosted surrounded by several peripheral tissues. These include the nodule cortex, the endodermis and the nodule parenchyma, which harbours the nodule vascular bundles.

The reduction of N_2 to NH_4^+ is catalysed by nitrogenase within rhizobial cells. This enzymatic complex is composed by two proteins: component I or molybdenum-iron (MoFe) protein and component II or iron (Fe) protein (Peters *et al.*, 1995). The production of H_2 by nitrogenase is a wasteful reaction, which reduces the efficiency of the enzyme in about 25%. The Fe-protein is a homodimer with a molecular weight of 60 to 64 kDa and the MoFe-protein is a tetramer of 220 kDa molecular mass. The nitrogenase complex is very fast and irreversibly denaturated by O_2, but the ATP

required for nitrogenase is mainly derived from O_2 dependent oxidative phosphorylation within the rhizobial cell. Thus the enzyme is only functional in low O_2 environmental concentrations. *Leg*hemoglobin facilitates the diffusion of available oxygen through the plant cell cytoplasm to the bacterial cells at appropriate concentrations (Appleby, 1984; Vance & Heichel, 1991).

1.3. Bacterial symbiosis genes

The bacterial symbiosis genes, which are responsible for the establishment of an effective symbiosis, can be divided into two categories: nitrogen fixation genes (*nif* and *fix*) (Kaminski *et al.*, 1998) that are involved in atmospheric nitrogen fixation, and nodulation genes (*nod*, *nol* and *noe*) (Downie, 1998), which are responsible for nodulation (Freiberg *et al.*, 1997; van Rhijn & Vanderleyden, 1995). Other genes involved in the process of symbiosis are listed in Table 2.

Table 2. Other rhizobial genes involved in symbiotic nitrogen fixation.

Genes	Function	Species	Reference
exo	exopolyssacharide production	*S. meliloti*	(Glazebrook & Walker, 1989)
hup	hydrogen uptake	*B. japonicum*	(Maier, 1986)
gln	glutamine synthase	*R. phaseoli*	(Carlson *et al.*, 1987)
dct	dicarboxylate transport	*R. leguminosarum* *S. meliloti*	(Jiang *et al.*, 1989)
nfe	nodulation efficiency and competitiveness	*S. meliloti*	(Sanjuan & Olivares, 1989) (Garcia-Rodriguez & Toro, 2000)
ndv	β-1,2 glucans synthesis	*S. meliloti*	(Breedveld *et al.*, 1994)
lps	lipopolysaccharide production	*R. phaseoli*	(Carlson *et al.*, 1987)

The symbiosis genes can be located on a symbiosis plasmid (p*Sym*), as in *Mesorhizobium amorphae* (Wang *et al.*, 1999a), or on a symbiosis island inside the chromosome, like in *Mesorhizobium loti* (Sullivan *et al.*, 1995) and *B. japonicum* (Kaneko *et al.*, 2002a; Nakamura *et al.*, 2004), although they also have plasmids. At present, very little is known about the possible location of symbiosis genes in other mesorhizobia (Xu & Murooka, 1995). The chromosomal location has also been suggested by Cadahía and colleagues (1986) for chickpea rhizobia.

Several studies have shown that relatively few genes are required for nodulation of legumes (Long, 1989; van Rhijn & Vanderleyden, 1995).

The different rhizobial lineages probably diverged before the appearance of legumes (Turner & Young, 2000). Thus nodulation capacity may have been acquired in a single lineage after the

diversification of bacteria and, consequently, symbiosis genes may have spread by lateral gene transfer among the different rhizobia (Hirsch *et al.*, 2001). This hypothesis is supported by recent data obtained from the fully sequenced plasmids and genomes (Galibert *et al.*, 2001; Kaneko *et al.*, 2002a; Kaneko *et al.*, 2000a; Sullivan & Ronson, 1998; Sullivan *et al.*, 2002).

Complex events of gene recruitment combined with duplication and lateral gene transfer contributed to the evolution and spread of symbiotic abilities among rhizobia (Moulin *et al.*, 2004).

Plant genes involved in symbiosis have been identified by Expressed Sequence Tags (ESTs) sequencing in *Medicago truncatula* (Journet *et al.*, 2002) providing the means to study the interaction between macro- and microsymbiont, at the level of gene networks, in the symbiotic nitrogen fixation process.

1.3.1. Nodulation genes

Nodule formation requires a complex and dynamic molecular interaction between the two symbiotic partners. The onset of symbiosis is mediated by an exchange of diffusible signals (Caetano-Anollés, 1997).

Flavonoids seem to be the most important plant exudates for the symbiosis (Perret *et al.*, 2000) since they direct the bacteria to the rhizosphere by chemotaxis and enable the rhizobia to distinguish their host (Hirsch *et al.*, 2001). The flavonoids or isoflavonoids induce the expression of nodulation (*nod*) genes. However this induction also requires the participation of the transcriptional-activator protein NodD (van Rhijn & Vanderleyden, 1995).

The genetic control of nodulation by rhizobia involves nodulation genes that determine the production of lipo-chitooligosaccharides (LCOs) known as Nod factors.

Nod factors act as specific morphogenic signal molecules on legume hosts and induce early host responses such as root hair deformation and cortical cell division (Cullimore *et al.*, 2001). They are indispensable for nodulation and are seemingly recognised by high-affinity legume receptors (Cullimore *et al.*, 2001).

Root chitinases, some of which are specifically induced during symbiosis, can degrade the lipo-chitooligosaccharide backbone and may play an important role in the regulation of the Nod factors activity (Downie & Walker, 1999).

Most nodulation genes are expressed at very low concentrations when rhizobia are grown in the absence of host plants, namely in usual laboratory media (Djordjevic *et al.*, 1987; Spaink *et al.*, 1987).

Structural nodulation genes, which encode Nod factors, can be classified into two groups, the common and the host-specific genes (*hsn*). Additionally, genotype-specific nodulation (*gsn*) genes

have also been described (Sadowsky *et al.*, 1991). These genes are responsible for the nodulation of specific plant genotypes within a single legume species.

The common *nod*ABC genes are structurally and functionally conserved and are usually part of a single operon (van Rhijn & Vanderleyden, 1995). The lipo-chitooligosaccharide backbone is synthesised under the control of *nod*A (acyl transferase), *nod*B (deacetylase) and *nod*C (N-acetylglucosaminyl transferase), all of which are present as single copy genes in all known rhizobia (Downie, 1998).

NodA varies in its fatty acid specificity, thus contributing to the rhizobial host range (Roche *et al.*, 1996). *Nod*I and *nod*J are located downstream of *nod*C and seem to be part of the same operon (van Rhijn & Vanderleyden, 1995). They encode two proteins belonging to a bacterial inner membrane transport system of small molecules (Vázquez *et al.*, 1993).

The *hsn* genes are responsible for the host range and most mutations result in alteration or extension of host range (van Rhijn & Vanderleyden, 1995). For example, *nod*F and *nod*G encode acyl carrier proteins, *nod*E encodes a β-acetoacethylsynthase (Sharma *et al.*, 1993) and *nod*P, *nod*Q and *nod*H are involved in the sulfation of the Nod factor reducing sugar in *S. meliloti* (Roche *et al.*, 1991).

Examples of *gsn* genes are *nol*C, which is involved in cultivar-specific nodulation of soybean and shares homology with a heat-shock gene (Krishnan & Pueppke, 1991) and *nol*BTUVW that regulate the nodulation of *G. max* in a cultivar-specific manner (Meinhardt *et al.*, 1993).

Most *nod* genes share a conserved promoter sequence called the *nod* box (Freiberg *et al.*, 1997).

The regulatory NodD protein is crucial in the activation of other *nod* genes. *Nod*D is the only nodulation gene that is constitutively expressed even in non-symbiotic bacteria (Long, 1989). The spectrum of flavonoid specificity of NodD correlates with the broadness of the host range (van Rhijn & Vanderleyden, 1995).

The GC content of the nodulation genes is lower than that found yet in all studied rhizobial chromosomes. These differences in G+C content of essential and accessory genes could mean that the accessory genome came from another bacterium with a distinct genomic composition. However, if such a bacterium exists, it has not been found yet (Downie & Young, 2001). The same authors also propose a less plausible explanation, which is that different parts of the genome could share the same cell and diverge in composition.

Phylogeny based on common nodulation genes does not correlate with the 16S phylogeny of rhizobia; instead it reflects the phylogeny of host plants (Dobert *et al.*, 1994).

1.3.2. Nitrogen fixation genes

There are two main types of nitrogen fixation genes: *nif* and *fix* genes. Rhizobial *nif* genes are structurally homologous to those of *Klebsiella pneumoniae*, a free-living diazotrophic bacteria (Arnold *et al.*, 1988; Drummond, 1984). Fix genes are essential to nitrogen fixation, but do not have homologous equivalents in *K. pneumoniae* (Fischer, 1994).

The nitrogenase complex, which has the catalytic function of nitrogen fixation, is encoded by the structural genes *nif*HDK. *nif*D and *nif*K code for the α and β subunits of the dinitrogenase or MoFe protein subunit. *nif*H codes for the nitrogenase reductase or Fe protein subunit of the enzyme complex. Phylogenetic studies have preferably focused on this nitrogen fixation gene. A MoFe cofactor is required for the activation of the MoFe protein and is assembled from the *nif*B, V, N and E gene products (Freiberg *et al.*, 1997).

The gene products of *nif*A and *nif*L control the regulation of all other *nif* genes: NifA is the positive activator of transcription of *nif* operons and NifL is the negative activator (Morett *et al.*, 1990).

nif gene expression is regulated by oxygen and ammonia levels in the soil. Ammonia causes *nif*L to act as negative control and prevent the activator function of *nif*A. This has been called the N control system (Sadowsky & Graham, 2000). Symbiotic nitrogen fixation is influenced by the presence of mineral nitrogen in the soil: the nodulation process may be promoted by relatively low levels of available nitrate or ammonia, higher concentrations of which almost always depress nodulation (Davidson & Robson, 1986a; Davidson & Robson, 1986b; Eaglesham, 1989). Furthermore, soil NO_3^- inhibits root infection, nodule development and nitrogenase activity (Zahran, 1999).

Nitrogenase is rapidly inactivated by oxygen, thus the enzyme must be protected from O_2. In the root nodules of legumes, protection is provided by the symbiotic synthesis of the red pigmented *leg*hemoglobin, an oxygen binding protein (Quispel, 1988). The globin portion of the protein is synthesised by the plant in response to bacterial infection, while the heme group is synthesised by the rhizobia (Appleby, 1984). *Leg*hemoglobin has a high affinity to O_2, keeping its concentration low enough to protect the nitrogenase, while providing O_2 transport for the aerobic rhizobia.

The nitrogen fixation turnover is strongly associated with the concentration of *leg*hemoglobin (Werner *et al.*, 1981).

*Leg*hemoglobins are interesting proteins as they are the only globins synthesised by plants, having genetic and structural homologies with animal oxyhemoproteins, like hemoglobin (Appleby, 1984). They occur as multigene families in legumes, and several protein forms are expressed in nodules (Long, 1989).

Rhizobia have developed an independent enzymatic complex, *Hup* (Hydrogen uptake) system with nitrogenase activity, able to oxidise all or part of the hydrogen produced by nitrogenase, allowing the recycling of hydrogen (Baginsky *et al.*, 2002).

The *fix*ABCX genes are usually located on a single operon, except in *B. japonicum* and are directly involved in the electron transfer to nitrogenase (Fischer, 1994). The FixNOPQ proteins constitute a membrane-bound cytochrome-c-oxidase complex, which supports bacteroid respiration under low oxygen conditions inside the nodule (Preisig *et al.*, 1993). The *fix*GHIS genes encode transmembrane proteins (Fischer, 1994). Other *fix* genes are associated to the development and metabolism of bacteroids (Fischer, 1994).

1.4. Rhizobial genomes

As the number of sequenced genomes increases, the richness of the bacterial world is becoming clearer. In particular, the availability of rhizobial complete genomes has opened a whole new field in the biology and genomics of these bacteria, and has allowed the molecular analysis of genes involved in symbiosis, competitiveness and stress response. This will be of major importance in planning strain improvement strategies for inoculant technology invaluable for the development of a sustainable agriculture.

1.4.1. Genome organisation

The release of rhizobia complete genome sequences has been a very important step towards comparative genomics of rhizobia and has improved our knowledge on symbiosis genes (Downie & Young, 2001). An updated summary of complete genomes and genome projects is shown in Table 3.

Most species of rhizobia carry large plasmids that may vary in number and size. These are not always associated with symbiosis; they may contain essential or housekeeping genes (Barnett *et al.*, 2001; Downie & Young, 2001; Finan *et al.*, 2001; Galibert *et al.*, 2001) and some of them are cryptic (Mercado-Blanco & Toro, 1996).

Although most housekeeping genes are located in the chromosome, it has been shown that, at least in *Sinorhizobium meliloti*, *Sym* plasmids also carry some essential genes (Barnett *et al.*, 2001; Downie & Young, 2001; Finan *et al.*, 2001; Galibert *et al.*, 2001).

The genes involved in symbiosis can be located on symbiosis plasmids (p*Sym*), as in *S. meliloti* (Barloy-Hubler *et al.*, 2000b; Finan *et al.*, 2001), *R. etli* (González *et al.*, 2003) and *M. amorphae* (Wang *et al.*, 1999a), or on a symbiosis island of low GC content in the chromosome (Downie &

Young, 2001), like in *M. loti* (Sullivan *et al.*, 1995) and *B. japonicum* (Kaneko *et al.*, 2002a). The chromosomal location has also been suggested for chickpea rhizobia (Cadahía *et al.*, 1986).

In evolutionary terms, plasmid encoded symbiosis genes have the advantage of being more easily exchangeable within populations, than genes located in the chromosome (Downie, 1997). Acquisition and loss of *Sym* plasmids is a dynamic and continuous process, which seems to play a major role in the evolution of rhizobia.

1.4.2. Complete genome sequences

There are currently two major databases that gather most information available on complete genomes of rhizobia and related bacteria: RhizoBase (http://www.kazusa.or.jp/rhizobase) and RhizoBD (http://rhizo.bham.ac.uk).

The availability of complete genome sequences has demonstrated that lateral gene transfer and recombination are essential mechanisms in the evolution of bacterial genomes (van Berkum *et al.*, 2003). Furthermore, the ability of bacteria to exploit new environments and to respond to different selective pressures is more easily explained by the acquisition of new genes by horizontal gene transfer than by gene modification through accumulation of point mutations (Francia *et al.*, 2004).

Lateral transfer has been an argument to explain the inconsistencies between phylogenetic trees derived from chromosomal or plasmid genes. This fact agrees with the hypothesis that *Sym* plasmids are a recent acquisition of soil bacteria, as it has been suggested by the analysis of the complete genome sequence of *Sinorhizobium meliloti* (Galibert *et al.*, 2001).

Work on whole genome sequence analysis of *S. meliloti* 1021 (Guo *et al.*, 2003) and *Rhizobium* sp. NGR234 (Mavingui *et al.*, 2002) has shown that the structure of rhizobial genomes can be highly dynamic generating different genomic rearrangements, through homologous recombination between reiterated DNA sequences.

The transfer of symbiosis information in soil and the formation of new symbiosis plasmids by genetic recombination could result in a heterogeneous population with high adaptive capacity to interact and nodulate different legumes (Soberón-Chávez *et al.*, 1991).

The existence of complete genome sequences has changed the whole basis upon which rhizobia can be studied. The way is now opened to the functional genomics of rhizobia: comparative genomics, transcriptomics and proteomics. Gene expression analyses include monitoring changes in the transcriptome and the proteome. In the post-genomic era, experimental approaches have moved from the targeted investigation of individual genes to the study of thousands of genes simultaneously by array techniques (Pühler *et al.*, 2004), giving us the possibility to study rhizobial genes involved in symbiosis (Bontemps *et al.*, 2005), nodule development, stress response, etc.

Table 3. Complete genomes and genome projects of rhizobia and related bacteria. *information not available.

SPECIES	STRAIN	GENOME	CHROMOSOME	PLASMIDS	*SYM* GENES LOCATION	RELEASE YEAR	REFERENCE
***Agrobacterium radiobacter* bv. II**	K84	*	2	3	-	ongoing project	-
***A. tumefaciens* bv. I**	C58	~5.67 Mb	~2.84 Mb (circular) ~2.07 Mb (linear)	543 Kb (At) 214 Kb (Ti)	-	2001	(Wood *et al.*, 2001)
***A. vitis* bv. III**	S4	~5.7 Mb	2	5	-	ongoing project	-
Bradyrhizobium japonicum	USDA110	~9.1 Mb	~9.1 Mb	-	chromosome	2002	(Kaneko *et al.*, 2002a; Kaneko *et al.*, 2002b)
B. japonicum	SEMIA5079	*	*	*	*	*	-
***B.* sp.**	BTAi1	~8.4 Mb	~8.4 Mb	-	chromosome	2006	-
***B.* sp.**	ORS278	~7 Mb	~7 Mb	-	chromosome	ongoing project	-
Burkholderia phymatum	STM815	~8.9 Mb				ongoing project	
Mesorhizobium loti	MAFF303099	~7.6 Mb	~7.0 Mb	352 Kb 208 Kb	*Sym* island	2000	(Kaneko *et al.*, 2000a; 2000b)
M. loti (symbiosis island)	R7A	-	-	-	*Sym* island	2002	(Sullivan *et al.*, 2002)
Methylobacterium nodulans	ORS2060	~7.0 Mb	*	*	*	ongoing project	-
Ralstonia taiwanensis	LMG19424	~6.4 Mb	~3.5 Mb	~2.4 Mb **~500 Kb**	p*Sym*	ongoing project	-
Rhizobium etli	CFN42	~6.5 Mb	~4.4 Mb	642 Kb 505 Kb **371 Kb** 251 Kb 194 Kb 184 Kb	p*Sym*	2006	(González *et al.*, 2006; González *et al.*, 2003)

Table 4. (continued) Complete genomes and genome projects of rhizobia and related bacteria. *information not available.

SPECIES	STRAIN	GENOME	CHROMOSOME	PLASMIDS	*SYM* GENES LOCATION	RELEASE YEAR	REFERENCE
R. leguminosarum* bv. *viciae	3841	~7.75Mb	~5.06Mb	870 Kb 684 Kb **488 Kb** 352 Kb 147 Kb 151 Kb	p*Sym*	2006	(Young *et al.*, 2006)
R. leguminosarum* bv. *trifolii	WSM1325	*	*	*	*	ongoing project	-
R. leguminosarum* bv. *trifolii	WSM2304	*	*	*	*	ongoing project	-
***R.* sp.**	NGR234	~5.6Mb	~3.5 Mb	540 Kb ~2.2 Mb	p*Sym*	1997 2004	(Freiberg *et al.*, 1997; Streit *et al.*, 2004)
R. tropici	PRF 81	*	*	*	p*Sym*	ongoing project	-
Sinorhizobium medicae	WSM419	~6.7 Mb	*	*	*	2006	-
S. meliloti	1021	~6.6 Mb	~3.6 Mb	**1,354 Kb** **1,683 Kb**	p*Sym*A p*Sym*B	2000	(Barloy-Hubler *et al.*, 2000a; 2000b; Capela *et al.*, 1999; Finan *et al.*, 2001; Galibert *et al.*, 2001)

1.4.3. Genetic modification

For many years, it has been a challenge for researchers working with rhizobia to develop an inoculant that can promote higher levels of nitrogen fixation in practical field conditions. This is the final goal of strain improvement and, nowadays, this might be achieved by molecular techniques at our reach.

Genetic modifications that have been introduced in rhizobia aimed at improving the strains used as inoculants on traits such as competitiveness with indigenous strains and nitrogen fixation capacity.

Generation of *Rhizobium* strains with improved symbiotic properties has been achieved by random DNA amplification (RDA), a new strategy for the improvement of bacterial strains through the selection of desired properties without the need to identify the genes involved in the process (Mavingui *et al.*, 1997).

The recent boom of complete genome sequences has made it possible to design pathways of rearrangements that lead to different genomic structures in *Rhizobium* with potential biological implications (Flores *et al.*, 2000). However, there is evidence of a strong selective pressure imposed by the host, which favours the maintenance of certain chromosome-plasmid associations (Silva *et al.*, 2003).

An important prerequisite for genetic improvement of any bacterial species is the availability of a highly efficient gene transfer system. Traditional transformation methods have been developed for rhizobia with little success (Bullerjahn & Benzinger, 1982; Courtois *et al.*, 1988; Kiss & Kálmán, 1982; Selvaraj & Iyer, 1981). The referred methods are all time consuming and restricted to plasmid vectors carrying the *mob* gene (Garg *et al.*, 1999). Thus, gene transfer to rhizobia has mainly been possible through conjugation with *Escherichia coli* (Johnston *et al.*, 1978), which is also time-consuming. However, a simple and rapid method, based on the freeze-thaw protocol used for *Agrobacterium* transformation, has been described very recently (Vincze & Bowra, 2006).

Electrotransformation protocols have also been developed for rhizobia (Garg *et al.*, 1999; Hayashi *et al.*, 2000). Compared to the conventional methods, electroporation yields much higher transformation efficiencies. However, the optimal electroporation conditions vary from strain to strain, making it hard to establish an efficient transformation system for rhizobial strains.

Rhizobia genetically modified to be easily traceable and/or to be improved in their expression of beneficial traits have been constructed and released with plants in a number of experimental field plots (Selbitschka *et al.*, 1995). With these releases, it has been possible to monitor the modified inoculant bacteria after their introduction in field ecosystems and to assess their impact on the resident population (Amarger, 2002; Cullen *et al.*, 1998).

Marker genes that have been introduced in rhizobia to study the survival, persistence and spread of genetically modified bacteria in the field, include transposons or genes conferring antibiotic or mercury resistance which can be used with reporter genes, such as *gus*A, *luc* or *lac*Z (Amarger, 2002).

To increase the nodulation competitiveness under field conditions two approaches have been used: the introduction of genes involved in the biosynthesis of trifolitoxin, an antibiotic to which native rhizobia are susceptible, in *R. etli*, the microsymbiont of common bean (Robleto *et al.*, 1998); and the overexpression of *put*A, a metabolic gene involved in root surface colonisation, in *S. meliloti*, associated with alfalfa (van Dillewijn *et al.*, 2002; van Dillewijn *et al.*, 2001).

Two strategies have also been developed to improve the nitrogen fixing capacity of rhizobial inoculants: modifications of the expression of *nif*A, one of the *nif* genes regulatory protein, and of dicarboxylate transport (*dct*) genes, involved in the supply of carbon and energy to the nitrogen fixation process. *S. meliloti* strains with an extra copy of *nif*A and *dct* genes were constructed to inoculate alfalfa leading to an increase in plant yields under field conditions of nitrogen limitation, low endogenous rhizobial competitors and sufficient moisture (Bosworth *et al.*, 1994). Nevertheless, other authors (Ronson *et al.*, 1990) have shown that, inoculation of two varieties of soybean with strains of *B. japonicum* with an extra copy of *nif*A did not increase soybean yield.

Overall, the mentioned studies show that it may be possible to improve symbiotic effectiveness through genetic modification of rhizobial strains. However, despite much is already known about genetics of symbiotic nitrogen fixation and strain improvement, most commercial inoculants are native selected strains, usually best fitted, rather than modified strains.

From the limited results obtained so far in engineering nitrogen-fixing plants, it seems that the improvement of the nitrogen fixation process at the level of the microsymbiont symbiotic abilities may still be the best strategy.

The opportunity to carry out large-scale analyses in functional genomics now opens the way to the identification of large sets of co-regulated genes involved in biological processes at the level of gene networks instead of individual genes (Journet *et al.*, 2002). This kind of approach is striking for a better global understanding of complex developmental programs such as those activated during interactions between plants and microorganisms.

The importance of symbiotic nitrogen fixation for developing sustainable agricultural systems is enormous and current genome projects include a diversity of legumes and rhizobial species. The application of new genomic tools to identify symbiosis genes both in model macro- and microsymbionts makes way to apply these findings to improve legume and other crops that are relevant to world agriculture.

1.5. Phylogeny and diversity of rhizobia

Beijerinck (1888) was the first to isolate a bacterium from plant root nodules, which he named *Bacillus radicicola*. In 1889, Frank (1879) named this bacterium *Rhizobium leguminosarum* and identified further species belonging to the same group. However, it was not until the next century before the taxonomic diversity of root nodule bacteria was recognised (Fred, 1932).

The term rhizobia was originally used to name bacteria belonging to the genus *Rhizobium*. However, nowadays, it includes all bacteria able to produce nodules or swellings on the roots or stems of most, but not all legumes (Sadowsky & Graham, 2000), as well as on *Parasponia*, a non-legume (Trinick, 1979).

Rhizobia are Gram-negative aerobic and non-sporulated rods that can induce the formation of specialised organs in leguminous plants, the nodules, where nitrogen fixation occurs (Elkan & Bunn, 1994; Jordan, 1984; Sadowsky & Graham, 2000). Their GC content is 57 to 65% (Elkan & Bunn, 1994; Jordan, 1984; Sadowsky & Graham, 2000). They have a particularly interesting genome, for they possess generally one chromosome (4 Mb), one or two megaplasmids (1-2 Mb) and several plasmids (Martínez *et al.*, 1990).

According to Somasegaran and Hoben (1994), two large rhizobial groups can be distinguished: fast-growing acid producers and slow-growing alkali producers. However, this classification has long been outdated and obsolete.

Presently, most taxonomic studies consist of a polyphasic approach (Vandamme *et al.*, 1996) based on different types of data, namely phenotypic, chemotaxonomic, genetic and phylogenetic. All this information is combined in order to obtain a more complete picture of a bacterium's characteristics and propose an adequate classification.

The species concept has recently been reviewed (Cohan, 2002; Lan & Reeves, 2000; Rosselló-Mora, 2003) and the current view is based on an evolutionary species concept. Furthermore, the description of a new species should include a population-based approach, which means that a sufficient number of genetically and geographically diverse isolates should be examined, whenever possible (Barraclough & Nee, 2001; Vinuesa *et al.*, 2005a; 2005b; 2005c).

According to 16S rDNA-based phylogeny (Figure 1), rhizobia can be grouped into five main genera (Sawada *et al.*, 2003; Young *et al.*, 2001): *Azorhizobium* (Dreyfus *et al.*, 1988), *Bradyrhizobium* (Jordan, 1984), *Mesorhizobium* (Jarvis *et al.*, 1997), *Rhizobium* (Jordan, 1982), which includes the former genera *Allorhizobium*, *Agrobacterium* and *Rhizobium* (Young *et al.*, 2001), *Ensifer* (Young, 2003a), formerly *Sinorhizobium* (Chen *et al.*, 1988). These genera are included in the α-*Proteobacteria*: the genus *Mesorhizobium* belongs to the family *Phyllobacteriaceae*, the genera *Rhizobium* and *Ensifer* to the *Rhizobiaceae*, and *Azorhizobium* and *Bradyrhizobium* form an

independent branch (Young *et al.*, 2001). *Methylobacterium nodulans* is a facultative methylotroph, a unique characteristic among nodule-associated bacteria (Jourand *et al.*, 2004; Sy *et al.*, 2001).

More recently, rhizobia from the β-Proteobacteria (Moulin *et al.*, 2001) and γ-Proteobacteria (Benhizia *et al.*, 2004) have also been described, changing the current rhizobial taxonomy. Benhizia and co-workers (2004) have isolated some γ-Proteobacteria from the roots of several *Heydsarum* species, however, they did not demonstrate that these bacteria (family Enterobacteriaceae) are responsible for nodule formation.

Until September 2006, 57 species of rhizobia and nodule-associated bacteria have been described or validly published in the *International Journal of Systematic and Evolutionary Microbiology* (IJSEM).

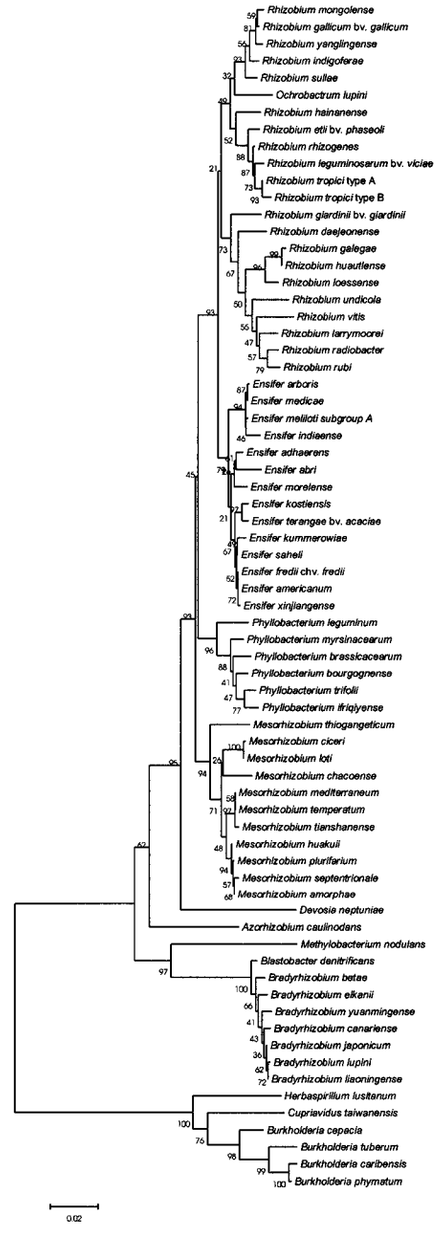

Figure 1.(on opposite page) Phylogeny of rhizobia and related genera based on 16S rDNA sequences (alignment length-1299 bp). The tree was constructed with the neighbour-joining method using published sequences.

The type species of each genus is shown in bold in Table 5. For a review see Sawada and collaborators (2003), although new species are constantly being added. For current nomenclature see J. P. Euzéby's *List of Prokaryotic Names with Standing in Nomenclature* (http://www.bacterio.cict.fr/). This list is updated with the publication of each new edition of *International Journal of Systematic and Evolutionary Microbiology*.

Table 5. Rhizobia, nodule-associated and other phylogenetically related bacteria.

Class α-Proteobacteria
Order Rhizobiales
Family Rhizobiaceae
Rhizobium (Frank, 1879)
R. daejeonense (Quan *et al.*, 2005)[1]
R. etli (Segovia *et al.*, 1993)
biovar *mimosae*
biovar *phaseoli*
R. galegae (Lindström, 1989)
biovar orientalis
biovar officinalis
R. gallicum (Amarger *et al.*, 1997)
biovar *gallicum*
biovar *phaseoli*
R. giardinii (Amarger *et al.*, 1997)
biovar *giardini*
biovar *phaseoli*
R. hainanense (Chen *et al.*, 1997)
R. huautlense (Wang *et al.*, 1998)
R. indigoferae (Wei *et al.*, 2002)
R. larrymoorei (Bouzar & Jones, 2001)[2]
R. leguminosarum (Frank, 1879)
biovar *phaseolii* (Jordan, 1984)
biovar *trifolii* (Jordan, 1984)
biovar *viciae* (Jordan, 1984)

[1] Not rhizobia.
[2] These species were part of the former genus *Agrobacterium,* are described as phytopathogenic, and they are not known to form nitrogen fixing symbiotic root nodules, with the exception of *Rhizobium rhizogenes.*

R. loessense (Wei *et al.*, 2003)

R. mongolense (van Berkum *et al.*, 1998)

R. radiobacter (Young *et al.*, 2001) [2]

R. rhizogenes (Velázquez *et al.*, 2005; Young *et al.*, 2001) [2]

R. rubi (Young *et al.*, 2001) [2]

R. sullae (Squartini *et al.*, 2002)

R. tropici (Martínez-Romero et al., 1991)

Type II A

Type II B

R. undicola (de Lajudie *et al.*, 1998a; Young *et al.*, 2001)

R. vitis (Young *et al.*, 2001) [2]

R. yanglingense (Tan *et al.*, 2001b)

Ensifer (formerly *Sinorhizobium*) (Chen *et al.*, 1988)

E. abri [3]

E. adhaerens (Wang *et al.*, 2002; Willems *et al.*, 2003b)

E. americanum (Toledo *et al.*, 2003)

E. arboris (Nick *et al.*, 1999a)

E. fredii (Chen *et al.*, 1988)

chemovar *fredii*

chemovar *siensis*

E. indiaense [3]

E. kostiensis (Nick *et al.*, 1999a)

E. kummerowiae (Wei *et al.*, 2002)

E. medicae (Rome *et al.*, 1996)

E. meliloti (de Lajudie *et al.*, 1994)

subgroup A

subgroup B

E. morelense (Wang *et al.*, 2002)

E. saheli (de Lajudie *et al.*, 1994)

E. terangae (de Lajudie *et al.*, 1994)

biovar *acaciae*

biovar *sesbaniae*

E. xianjiangense (Peng *et al.*, 2002)

Family Brucellaceae

Ochrobactrum

Ochrobactrum lupini (Trujillo *et al.*, 2005)

Family Phyllobacteriaceae

Phyllobacterium [4]

[3] These species have been published, but have not yet been included in the "Validation List" of the International Journal of Systematic and Evolutionary Microbiology.

P. bourgognense (Mantelin *et al.*, 2006)

P. brassicacearum (Mantelin *et al.*, 2006)

P. ifriqiyense (Mantelin *et al.*, 2006)

P. leguminum (Mantelin *et al.*, 2006)

P. myrsinacearum (Mergaert *et al.*, 2002; Yanagi & Yamasato, 1993) [5]

P. trifolii (Valverde *et al.*, 2005)

Mesorhizobium (Jarvis *et al.*, 1997)

M. amorphae (Wang *et al.*, 1999a)

M. chacoense (Velázquez *et al.*, 2001)

M. ciceri (Jarvis *et al.*, 1997; Nour *et al.*, 1994b)

M. huakuii (Chen *et al.*, 1991; Jarvis *et al.*, 1997)

M. loti (Jarvis *et al.*, 1982; Jarvis *et al.*, 1997)

M. mediterraneum (Jarvis *et al.*, 1997; Nour *et al.*, 1995)

M. plurifarium (de Lajudie *et al.*, 1998b)

M. septentrionale (Gao *et al.*, 2004)

M. temperatum (Gao *et al.*, 2004)

M. thiogangeticum (Ghosh & Roy, 2006) [6]

M. tianshanense (Chen *et al.*, 1995; Jarvis *et al.*, 1997)

Family Bradyrhizobiaceae

Bradyrhizobium (Jordan, 1982)

B. betae (Rivas *et al.*, 2004) [7]

B. canariense (Vinuesa *et al.*, 2005c)

B. elkanii (Kuykendall *et al.*, 1992)

B. japonicum (Jordan, 1982)

B. liaoningense (Xu *et al.*, 1995)

B. lupini (Barrera *et al.*, 1997) [3]

B. yuanmingense (Yao *et al.*, 2002)

Blastobacter

Blastobacter denitrificans (van Berkum & Eardly, 2002)

Family Hyphomicrobiaceae

Azorhizobium (Dreyfus *et al.*, 1988)

A. caulinodans (Dreyfus *et al.*, 1988)

Devosia

D. neptuniae (Rivas *et al.*, 2002)

Family Methylobacteriaceae

Methylobacterium

M. nodulans (Jourand *et al.*, 2004; Sy *et al.*, 2001) [8]

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[4] Species from this genus were isolated from root nodules, but have not been shown to be capable of nodulation.
[5] Bacteria that develop within leaf nodules of tropical ornamental plants, such as *Ardisia*.
[6] Sulfur-oxidising bacteria, nodulation unknown.
[7] Tumour forming bacterium.

Class β-Proteobacteria
Order Burkholderiales
Family Burkholderiaceae
Burkholderia (Moulin *et al.*, 2001)
B. caribensis (Chen *et al.*, 2003)
B. cepacia (Rasolomampianina *et al.*, 2005) [9]
B. tuberum (Moulin *et al.*, 2001; Vandamme *et al.*, 2002)
B. phymatum (Vandamme *et al.*, 2002)
Cupriavidus (Vandamme & Coenye, 2004)
C. taiwanensis (Chen *et al.*, 2001; Chen *et al.*, 2003)
Family Oxalobacteraceae
Herbaspirillum (Baldani *et al.*, 1996)
H. lusitanum [10] (Valverde *et al.*, 2003)

Unlike their host plants, rhizobia are a very diverse group of gram-negative bacteria. However, there is no obvious correlation between bacterial phylogeny and the host range of the species.

The cross-inoculation group concept was formerly used to define classes of rhizobial strains that were able to nodulate a particular group of host plants. However, many species of rhizobia have not been tested for their host range. Two main groups of host ranges can be distinguished, the narrow host range and the broad host range. Furthermore, some rhizobia are adapted to particular cultivars of host species (Hadri *et al.*, 1998).

Chickpea rhizobia have been described as very specific microsymbionts, which means that they only nodulate chickpea (Gaur & Sen, 1979). Chickpea has also been considered a non-promiscuous host (Broughton & Perret, 1999), although it can be nodulated by rhizobia from diverse phylogenetic groups.

The symbiosis between rhizobia and chickpea has not been extensively analysed and there are only a few studies addressing the genetic diversity of chickpea rhizobia (Aouani *et al.*, 2001; Cadahía *et al.*, 1986; Kuykendall *et al.*, 1993; Maâtallah *et al.*, 2002a; Maâtallah *et al.*, 2002b; Nour *et al.*, 1994b). Two species of rhizobia have been described to specifically nodulate chickpea, namely *Mesorhizobium ciceri* (Jarvis *et al.*, 1997; Nour *et al.*, 1994a) and *Mesorhizobium mediterraneum* (Jarvis *et al.*, 1997; Nour *et al.*, 1995). However, previous works on rhizobia nodulating chickpea have shown the strains to be both phenotypically and genotypically diverse (Nour *et al.*, 1994a; Nour *et al.*, 1995; Nour *et al.*, 1994b). *Sinorhizobium medicae*, which was originally isolated from *Medicago spp.* nodules (Rome *et al.*, 1996), has also been found to form nodules on chickpea, but this

[8] Facultative methylotroph.
[9] Strain STM1424.
[10] This species has been isolated from root nodules, but has not been shown to be able to nodulate.

symbiosis is ineffective (Aouani *et al.*, 2001). More recently, strains of *M. tianshanense* isolated in Spanish soils have also been described to nodulate chickpea (Rivas *et al.*, *In Press.*).

1.5.1. Molecular methods for phylogeny estimation

Before the emergence of modern molecular methods, the main criteria used in the assignment of rhizobia into different species were host range (based on the cross-inoculation group concept) together with growth rate, morphology and metabolic tests.

Nowadays, the identification of rhizobia is often based on a polyphasic approach using several different techniques to describe new genera and species.

The Subcommittee on *Rhizobium* and *Agrobacterium* of the International Committee on Systematic Bacteriology of the International Union of the Microbiological Societies has recommended a minimal set of standard criteria for identifying new genera and species of nodule bacteria (Graham *et al.*, 1991; Stackebrandt *et al.*, 2002). These criteria include cultural, biochemical and molecular data. The most common approaches are 16S rDNA Restriction Fragment Length Polymorphism (RFLP) (Laguerre *et al.*, 1994) and sequencing (Willems & Collins, 1993; Yanagi & Yamasato, 1993; Young *et al.*, 1991; Young, 1996; Young & Haukka, 1996); DNA-DNA hybridisation (Willems *et al.*, 2001b; Willems *et al.*, 2001c), Fatty Acids Methyl Esters (FAME) analysis (Jarvis *et al.*, 1996; So *et al.*, 1994) and Multilocus Enzyme Electrophoresis (MLEE) (Strain *et al.*, 1995).

DNA-DNA hybridisation has been required for the description of a new species (Kämpfer *et al.*, 2003; Stackebrandt *et al.*, 2002). New species descriptions based upon other genomic techniques are encouraged, provided that congruence between the method used and DNA-DNA reassociation is demonstrated, within the taxa under study (Stackebrandt *et al.*, 2002). However, the DNA-DNA hybridisation method is time-consuming and has low reproducibility. Furthermore, in rhizobia, DNA-DNA hybridisation results may be difficult to interpret due to the large accessory genome (plasmids, megaplasmids and symbiosis islands) of these bacteria. Therefore, DNA-DNA hybridisation values should in a near future no longer be an absolute requirement for the description of a new species (J. Peter W. Young, personal communication).

More recently, a new approach, which combines DNA-DNA hybridisation with DNA microarrays, was described for the identification of bacteria (Cho & Tiedje, 2001). Bacterial total DNA is randomly cut into 1 kb pieces and representative fragments are spotted on microarrays and hybridised to type genomes. This method has the advantage that a database of hybridisation profiles can be established.

16S rRNA gene sequencing has been fundamental for the identification of species and its full sequence is required for the description of a new species. However, occasionally recombination events between species have caused some incoherencies between phylogenies based on different parts

of the gene (Young & Haukka, 1996). Transfer of parts of the 16S rDNA sequences between species has been suggested and there is indeed evidence for its recombination (Hashimoto *et al.*, 2003; van Berkum *et al.*, 2003). Thus, the 16S rDNA sequence should be only one of the many characters used for bacterial taxonomy, instead of the predominant one (van Berkum *et al.*, 2003).

Recently, the Ad Hoc Committee for the re-evaluation of the species definition in bacteriology has recommended sequence analysis of different housekeeping protein-coding genes in order to define the taxon species and to differentiate between closely related species. The number of genes to be analysed will depend on the level of genetic diversity present within a given taxon and should be evaluated on the basis of the robustness of clusters obtained with different phylogenetic methods (Stackebrandt *et al.*, 2002).

The most recent guidelines from the Subcommittee on the Taxonomy of *Agrobacterium* and *Rhizobium* (of the International Committee on Systematics of Prokaryotes) recommend the analysis of several gene sequences from several strains, in agreement with the genomic core hypothesis (Daubin *et al.*, 2002). Sequence databases of housekeeping protein-coding genes, such as *dnaK* (70 kDa molecular chaperone) (Eardly *et al.*, 2005; Parker, 2004; Stepkowski *et al.*, 2003), *glnA* and *glnII* (glutamine synthetases I and II) (Turner & Young, 2000; Vinuesa *et al.*, 2005a), *atpD* (ATP synthase β) (Gaunt *et al.*, 2001; Vinuesa *et al.*, 2005a) and *recA* (DNA recombinase A) (Gaunt *et al.*, 2001; Vinuesa *et al.*, 2005a), and *gltA* (citrate synthase) (Hernandez-Lucas *et al.*, 2004), should be extended to most rhizobial species, in order to constitute a basic framework in which to place representatives of a new species (J. Peter W. Young, personal communication). Furthermore, the 16S-23S Internal Transcribed Spacer (ITS), which includes the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes, and is known to be more useful as a marker for intraspecific diversity (Willems *et al.*, 2001a; Willems *et al.*, 2003a), has recently been presented as a phylogeny marker for rhizobia (Kwon *et al.*, 2005). Additionally, its sequence database has increased in recent years (Tan *et al.*, 2001a; van Berkum & Fuhrmann, 2000; Vinuesa *et al.*, 2005c).

Although the 16S rDNA phylogeny has become an universal paradigm, it is quite obvious that the true bacterial evolutionary history can only be inferred from the comparative phylogeny of several gene sequences. Several comprehensive phylogenetic studies of rhizobia have reported congruence between 16S rDNA based phylogeny and gene trees of other housekeeping genes, such as *glnA* (Turner & Young, 2000), *atpD* and *recA* (Gaunt *et al.*, 2001), *dnaK* (Stepkowski *et al.*, 2003) and *gltA* (Hernandez-Lucas *et al.*, 2004).

With the purpose of determining the most accurate species tree possible, it is desirable to include information from several different housekeeping genes phylogenies. However, gene trees and species trees need not necessarily always agree, for they merely reflect different aspects of the same phylogenetic process.

Symbiosis loci are part of the accessory genome and may have an evolutionary history independent of other loci. Phylogenetic comparisons with core genes reveal lateral gene transfer of symbiosis

genes among rhizobia species (Haukka *et al.*, 1998; Laguerre *et al.*, 2001; Moulin *et al.*, 2001; Suominen *et al.*, 2001; Vinuesa *et al.*, 2005a; 2005c). These are therefore unsuitable characters for defining species, but can be used for biovar descriptions (Amarger *et al.*, 1997; Laguerre *et al.*, 2001; Silva *et al.*, 2003; Vinuesa *et al.*, 2005c; Wang *et al.*, 1999b).

1.5.2. Molecular methods for diversity evaluation

The evaluation of rhizobial diversity is essential for the identification and selection of appropriate inoculant strains for a given crop.

The study of rhizobial populations' diversity can be achieved using both phenotypic and molecular approaches. Several methods have been used for strain differentiation, that evaluate rhizobia at various levels, namely DNA, RNA, phenotypic traits, protein expression and functions, fatty acids and chemotaxonomic markers. The great variety of available techniques has led to the proposal of a polyphasic approach of bacterial taxonomy that include results obtained with the different methods (Stackebrandt *et al.*, 2002).

Some of the most commonly used differentiation approaches include phenotypic methods like assimilation, biochemical and enzymatic tests (de Lajudie *et al.*, 1994), intrinsic antibiotic resistance (IAR) patterns (Abaidoo *et al.*, 2002), along with molecular methods, such as Multilocus Enzyme Electrophoresis (MLEE) (Strain *et al.*, 1995), SDS-PAGE analysis of total cell proteins (de Lajudie *et al.*, 1994; Dupuy *et al.*, 1994) and DNA based methods.

DNA fingerprinting techniques have been widely used to study biodiversity of rhizobial populations (Demezas, 1998). Restriction fragment length polymorphism (RFLP) of total genomic DNA (Glynn *et al.*, 1985) was certainly one of the first approaches with resolution up to the species level (Laguerre *et al.*, 1992). DNA restriction profiles with rare-cutting endonucleases provide less complex patterns, but the generated fragments are too large and need to be separated by Pulsed Field Gel Electrophoresis (PFGE) (Haukka & Lindström, 1994).

Plasmid profile analysis through an in situ lysis method (Eckhardt, 1978) is also a commonly used DNA fingerprinting technique, which has a discriminating power at the same level as total DNA restriction profiles (Laguerre *et al.*, 1992). This approach assumes a particular importance in rhizobia, whose genome is composed, in most cases, by several plasmids and megaplasmids.

RNA fingerprinting by arbitrarily primed (RAP) - PCR can be used for example to detect differentially expressed genes in cells that have been subjected to different environmental conditions (McClelland *et al.*, 1995). RNA fingerprinting techniques also include the Low Molecular Weight RNA (LMW RNA) method that allows the separation of ribosomal and transfer RNAs (Velázquez *et al.*, 1998).

Due to the improvement in the technology of PCR in the last twenty years, numerous PCR-based techniques have been developed for evaluation of rhizobial diversity and strain differentiation.

Some of these methods consist in the amplification of repeated regions in the genome, like REP (Repetitive Extragenic Palindromic) - PCR (de Bruijn, 1992; Judd *et al.*, 1993), ERIC (Enterobacterial Repetive Intergeneric Consensus) - PCR (de Bruijn, 1992; Niemann *et al.*, 1999) and BOX (Box element) - PCR (Vinuesa *et al.*, 1998), that can be used to distinguish among closely related strains of rhizobia. The obtained patterns are specific and reproducible and can be used in bacterial taxonomy at the strain level (Nick *et al.*, 1999b).

Another type of PCR-based techniques consist in the random amplification of genome fragments and this kind of approach has been used to differentiate among rhizobial strains (Kishinevsky *et al.*, 1996). Random Amplified Polymorphic DNA (RAPD) (Dooley *et al.*, 1993; Harrison *et al.*, 1992; Richardson *et al.*, 1995; Selenska-Pobell *et al.*, 1996; van Rossum *et al.*, 1995) is a PCR-based approach that uses one random primer of about 10 nucleotides combined with a low annealing temperature to generate strain specific profiles. However, this technique has been proved to have some reproducibility constraints (Selenska-Pobell *et al.*, 1995).

TP (Two Primers) - RAPD is another technique in which two primers, homologous to highly conserved regions of the 16S rDNA, are used (Rivas *et al.*, 2001). The distinctiveness of this method is that these two primers are used in very high concentrations and with a relatively high annealing temperature (approximately 50°C). The obtained profiles seem subspecies characteristic and not strain dependent. Originally, the TP-RAPD profiles were obtained only for the 16S rDNA. However, primers for the techniques of 5S-TP-RAPD and 23S-TP-RAPD have recently been designed (Rivas González, 2003) and similar results have been observed.

1.6. The host: chickpea

Chickpea is the third most important legume in the world. It is cultivated for its seeds, which are an important source of protein.

Chickpea is a dicotyledonous annual herbaceous plant, which belongs to the tribe Cicereae genus *Cicer* (Saxena & Singh, 1987).

Chickpea (*Cicer arietinum* L.) is indigenous to South Caucasus and North Persia (Saxena & Singh, 1987). It has been cultivated in Portugal since ancient times, but the time of its introduction into the Iberian Peninsula (Portugal and Spain) is not known (Gordillo, 1991). None of the chickpea wild relatives (*C. bijugum*, *C. echinospermum* and *C. reticulatum*) are found in Portugal (Talavera *et al.*, 1999).

Chickpea is an important pulse crop, according to the International Legume Database Information Service (ILDIS) (http://www.ildis.org/), the third most widely cultivated legume in the world, and the

first in the Mediterranean region (Gordillo, 1991). Nevertheless, studies addressing its inoculation response and rhizobial strain availability and competition are limited (Somasegaran *et al.*, 1988). The two main cultivar groups are known as "Desi" and "Kabuli" types: ICRISAT (International Crops Research Institute for the Semi-Arid Tropics)-India holds "Desi"-type germplasm collections and ICARDA (International Center for Agricultural Research in the Dry Areas)-Syria holds "Kabuli"-type germplasm collections.

The ICARDA focuses most of its efforts on "Kabuli"-type chickpea in the Mediterranean region, where the crop is generally grown in rotation with cereals. The ability of chickpea rhizobia to fix atmospheric nitrogen lessens chickpea's dependence on soil nitrogen and reinforces its role in the cropping systems of the region. Practices that increase symbiotic effectiveness will minimise the quantity of soil nitrogen utilised by the crop (Beck, 1992). The introduction of cold tolerant, ascochyta blight (*Ascochyta rabiei*) resistant lines for winter sowing into new, drier production areas has been accompanied by nodulation deficiency in several areas of the Mediterranean region (Beck, 1992). In these new production areas soils are less likely to contain adequate populations of specific rhizobia than traditional chickpea areas, and crops may show significant yield increases when seeds are inoculated with selected rhizobial strains. Indications are that highly specific rhizobial requirements of chickpea (Gaur & Sen, 1979) may also extend to strain-cultivar specificity for symbiotic effectiveness (Arsac & Cleyet-Marel, 1986; Rai & Prasad, 1986; Somasegaran *et al.*, 1988). The potential for increased nitrogen fixation in symbiosis with native rhizobial populations have developed in co-adaptation with local land-races (Beck, 1992).

1.7. Chickpea rhizobia used in the present study

The strains studied in this work were isolated in different locations from South Portugal (Alentejo region) in the course of several research projects from the Soil Microbiology Laboratory of ICAM, University of Évora.

In the Mediterranean region, grain legumes, and chickpea in particular, are important alternative crops to cereals, since they are a major source of protein both for human nutrition and animal feeding. In Portugal, chickpea is traditionally a spring crop destined to human nutrition. It is cultivated in almost the whole country, with some exceptions, but the main producing area is the Alentejo region (Beja, Portalegre and Évora). However, the traditional spring varieties, adapted to the dry land conditions, show very low production yields. Nevertheless, since the late 20th century the chickpea cultivated area has been reduced, due to the low yields obtained, among other factors. Improved chickpea cultivars made possible its use of as a winter crop with higher yield potential compared to the traditional spring varieties.

The rhizobia obtained within the scope of this work were isolated using chickpea trap plants grown in pots containing different soils: Beja, Elvas-Casas Velhas (Elvas), Elvas-Estação Nacional de Melhoramento de Plantas (Elvas-ENMP), Évora and Setúbal. An improved chickpea winter cultivar, named Chk 3226, recently developed in the ENMP, in Elvas (Duarte Maçãs, 2003; Tavares de Sousa & Duarte, 1990) was used.

1.8. Aims of the study

The present work focused on rhizobia able to nodulate chickpea, for which few studies are available, contrary to other microsymbionts, such as bean, pea or soybean rhizobia. The phenotypic and molecular characterisation of native chickpea rhizobia were addressed in order to increase our knowledge on this symbiosis model and to evaluate the soil natural populations in Portugal.

The introduction in agriculture of an inoculant strain effective in atmospheric nitrogen fixation and, simultaneously, adapted to host plant and to edapho-climatic conditions can be an important contribution to enhance chickpea productivity in the Mediterranean region. However, until the present work, a single study had been published on genetic diversity and symbiotic effectiveness of rhizobia isolated in Portugal.

Studies on diversity of chickpea rhizobia are important to elucidate the phylogeny and evolution of rhizobia. However, it is necessary to include a sufficient number of isolates in order to be able to draw conclusions at the population level. In the present thesis, four different indigenous chickpea rhizobia populations from South Portugal, namely Beja, Elvas, ENMP and Évora, as well as an isolate from Setúbal, were investigated, with the long-term purpose of understanding the symbiotic relation with the host plant and contribute to the development of efficient inoculants.

Forty-one isolates, ten from each population along with an isolate from Setúbal, were used to perform a diversity evaluation of natural populations (aims 1 to 4). Twenty-one isolates, five from each population together with the isolate from Setúbal, were selected for further analysis and a more detailed phylogenetic study (aims 5 to 7).

The main aim of this thesis was to study the genetic diversity and symbiotic effectiveness of rhizobia able to nodulate chickpea, to further elucidate rhizobial phylogeny and evolution and their role in the rhizobium-legume symbiosis.

The specific aims of this study were:

(1) to estimate the native soil populations of rhizobia able to nodulate chickpea;

(2) to determine the genus and species of chickpea rhizobia isolates by 16S rDNA sequence analysis;

(3) to evaluate the diversity of natural rhizobia populations by both phenotypic and molecular approaches, including auxanographic tests and symbiotic effectiveness, and DAPD analysis, SDS-PAGE total protein patterns and plasmid profiles;

(4) to evaluate the suitableness of DAPD analysis, a new PCR based method, in determining genetic relationships among isolates;

(5) to determine the symbiotic effectiveness of native chickpea rhizobia isolates;

(6) to investigate the chromosomal diversity and phylogeny of chickpea rhizobia by a multilocus phylogenetic approach using several housekeeping loci, besides 16S rDNA, such as *rec*A, *atp*D and *gln*A genes, as well as the ITS region;

(7) to study the genomic location, copy number and phylogenies of the symbiosis genes, *nif*H and *nod*C, of chickpea rhizobia and their correlation with the chromosomal background, plasmid content and symbiotic effectiveness, so as to contribute to the elucidation of the molecular bases of symbiotic effectiveness and host specificity.

2. MATERIALS AND METHODS

2.1. Bacterial strains

The bacterial strains used in the present study were isolated in the scope of the EU (European Community) project AIR3 CT92-0029, and the national projects PRAXIS/P/AGR/11129/1998 and POCTI/BME/44140/2002, both from Fundação para a Ciência e a Tecnologia (FCT). All strains are part of the collection maintained in the Soil Microbiology Laboratory-ICAM, University of Évora.

Rhizobia reference strains and type species were kindly provided in the course of this work by different people to whom acknowledges are due.

2.1.1. Reference and type strains

Rhizobia reference and type strains (Table 6) from different origins were used in this work for comparative purposes.

Table 6. Rhizobial reference strains and type species used in the present study.

SPECIES	STRAIN	ORIGINAL HOST PLANT	ORIGIN	REFERENCE
A. caulinodans	ORS 571^T	*Sesbania rostrata*	Senegal	(Dreyfus *et al.*, 1988)
B. japonicum	USDA 6^T	*Glycine max*	Japan	(Jordan, 1982)
B. japonicum	USDA 110	*Glycine max*	USA	(Kaneko *et al.*, 2002a)
M. amorphae	ACCC 19665^T	*Amorpha fruticosa*	China	(Wang *et al.*, 1999a)
M. chacoense	LMG 19008^T	*Prosopis alba*	Argentina	(Velázquez *et al.*, 2001)
M. ciceri	UPM-Ca7^T	*Cicer arietinum*	Spain	(Nour *et al.*, 1994a)
M. huakuii	CCBAU 2609^T	*Astragalus sinicus*	China	(Chen *et al.*, 1991)
M. loti	NZP 2213^T	*Lotus corniculatus*	New Zealand	(Jarvis *et al.*, 1982)
M. loti	R7A	*Lotus corniculatus*	New Zealand	(Kaneko *et al.*, 2000a)
M. mediterraneum	UPM-Ca36^T	*Cicer arietinum*	Spain	(Nour *et al.*, 1995)
M. plurifarium	ORS 1032^T	*Acacia senegal*	Senegal	(de Lajudie *et al.*, 1998b)
M. septentrionale	HAMBI 2582^T	*Astragalus adsurgens*	China	(Gao *et al.*, 2004)
M. temperatum	HAMBI 2583^T	*Astragalus adsurgens*	China	(Gao *et al.*, 2004)
M. tianshanense	A-1BS^T	*Glycyrrhiza pallidiflora*	China	(Chen *et al.*, 1995)
Rhizobium sp.	NGR 234	*Lablab purpureus* (broad host range)	Papua New Guinea	(Freiberg *et al.*, 1997; Streit *et al.*, 2004)
R. etli	CFN 42^T	*Phaseolus vulgaris*	Mexico	(Segovia *et al.*, 1993)
R. leguminosarum	USDA 2370^T	*Pisum sativum*	USA	(Frank, 1879)
R. leguminosarum bv. *viciae*	3841*	*Pisum sativum*	-	(Glenn *et al.*, 1980; Johnston & Beringer, 1975)
S. medicae	A-321^T	*Medicago* spp.	France	(Rome *et al.*, 1996)
S. meliloti	USDA 1002^T	*Medicago sativa*	USA	(Dangeard, 1926)
S. meliloti	1021	*Medicago sativa*	Australia	(Galibert *et al.*, 2001)

2.1.2. Portuguese native rhizobia

Soil samples were collected into a deep of 15 to 20 cm from five different regions in South Portugal: Beja, Elvas, Elvas-ENMP, Évora and Setúbal. The first four collection sites were agricultural soils located in experimental farms where leguminous plants, not necessarily chickpea, have been grown in a recent past, despite these crops are at present mostly absent from the rotations commonly used (Carvalho & co-workers, 1996).

Soils were analysed in the Chemistry-Agriculture Laboratory, from the University of Évora. The following soil parameters were analysed: type (Zobler, 1986), texture (Zobler, 1986), pH, organic

* *R. leguminosarum* bv. *viciae* strain 3841 is a spontaneous streptomycin resistant mutant of strain 300.

matter (Rauterderg & Kremskuf, 1951), nitrates (NO_3) (1980), potassium oxide (K_2O) and phosphates (P_2O_5) (available K and P) (Knudsen, 1975; Riehm, 1958).

2.1.2.1. Estimation of the natural populations size

The size of the natural chickpea rhizobia populations was estimated according to the Brockwell's Most Probable Number (MPN) plant infection method (Brockwell, 1963), as described before (Vincent, 1970). A tenfold dilution series with three replicates was used for each soil for the enumeration of the most probable number of rhizobia per gram of soil. Plastic pots with approximately 11 cm diameter were disinfected in a commercial sodium hypoclorite solution (approximately 5% active chlor) for 72 hours. They were afterwards washed with distilled water, filled with vermiculite and sand in equal portions, and sterilised by autoclaving at 121°C for two hours. Certified chickpea commercial seeds from a recently developed winter cultivar (Chk 3226) were used (Duarte Maçãs, 2003). Seeds were previously treated with a 14% calcium hypochlorite solution (w/v) (5% available chlor) and rinsed with sterile distilled water. Seeds were pre-germinated in 0.75 % water agar (w/v) for 48 hours at 28°C, before being sown in the pots at 1 cm profundity. After sowing, seeds were inoculated with the soil dilutions. Pots were irrigated every other day, or whenever necessary, with a nitrogen-free nutrient solution (Broughton & Dilworth, 1971).

The growth chamber was programmed for 65% humidity and a photoperiod of 16 hours:

Day Cycle:	22°C for 60 min
	24°C for 840 min
	20C for 60 min
Night cycle:	20°C for 60 min
	18°C for 360 min
	22°C for 60 min

After five weeks, plants were harvested and nodulation in each replicate was scored as positive or negative.

2.1.2.2. Isolation of native rhizobia

Indigenous rhizobia isolates used in this study were obtained from nodulated chickpea used as trap plants grown in pots (Somasegaran & Hoben, 1994; Vincent, 1970), using soil from the different origins, as described by Somasegaran and Hoben (1994).

Setúbal isolates used in this study were originally from the work by Rodrigues (2002).

Ceramic pots with approximately 22 cm diameter were disinfected and sterilised as described in 3.1.2.1. for plastic pots.

Seeds were pre-germinated and five seeds per pot were sown as described in 3.1.2.1. Plants were irrigated every other day, or whenever necessary, with sterile distilled water.

The growth chamber was programmed as described in 3.1.2.1.

After ten weeks, the resulting nodules were harvested from the roots, rinsed with 96% ethanol for 30 seconds, surface sterilised by immersion for 2 to 3 minutes in 3% hydrogen peroxide, and washed six times with sterile distilled water (Somasegaran & Hoben, 1994; Vincent, 1970). The nodule was was pierced with a pin, which was afterwards streaked onto a Yeast Mannitol Agar (YMA) (1% mannitol, 0.05% yeast extract, 0.02% $MgSO_4$, 0.06% KH_2PO_4, 0.01% NaCl, pH 6.8-7.2) (Vincent, 1970) plate supplemented with Congo red (0.025 mg/ml) and cycloheximide (0.02 mg/ml) and incubated at 28°C for 3 to 5 days. The obtained isolates were purified by at least three single colony passages. One isolate was recovered from each nodule.

Isolates were validated as rhizobia by plant re-inoculation under axenic conditions, to ensure that each isolate alone is responsible for the development of root nodules.

2.1.3. Maintenance of rhizobia isolates and strains

All isolates and strains were routinely stored at 4°C on Yeast Mannitol Agar (YMA) (2.9 mM K_2HPO_4, 0.8 mM $MgSO_4$, 1.7 mM NaCl, 1% mannitol, 0.05% yeast extract, 1.5% agar, pH 7.0-7.2) (Vincent, 1970) or Tryptone Yeast (TY) (0.5% tryptone, 0.3% yeast extract, 7 mM $CaCl_2$, pH 6.8-7.2) (Beringer, 1974) agar slopes and plates.

The original collection of isolates and strains was maintained at –80°C in liquid medium containing 20% glycerol.

Rhizobia were routinely grown on TY plates and incubated at 28°C for 3 to 5 days or in TY liquid medium and incubated at 28°C for 16 to 48 hours with shaking (approximately 150 rpm).

2.2. Phenotypic methods

2.2.1. Morphology, Gram staining and growth characteristics

The growth, morphology and colony pigmentation was observed in YMA (Vincent, 1970). All isolates were observed for their shape under an optic microscope with an amplification of 1000 times.

Gram staining (Doetsch, 1981) was also performed in order to ensure that all isolates were Gram negative.

YMA (Vincent, 1970) supplemented with bromothymol blue (Hirsch & Skinner, 1992) was used to check for of acid or alkali production.

2.2.2. Auxanographic tests

The auxanographic analysis was performed with API20NE galleries (bioMérieux, France) for the identification of Gram-negative rods, according to the manufacturer's instructions, except that the strips were incubated at 28°C for 5 days (de Lajudie *et al.*, 1994). TheAPI20NE system is a standardised method combining 8 conventional tests and 12 assimilation tests for the identification of non-fastidious (pathogenic) Gram-negative rods not belonging to the *Enterobacteriaceae*. This microbiological test strip includes in total 21 enzymatic and assimilation tests that are summarised in Table 7. Inocula were obtained from 72 hours TY plates cultures and readings were recorded at 24, 48 and 120 hours.

The software NTSYSpc (version 2.02h; Applied Biostatistics, Inc) was used for analysis of API20NE results. For cluster analysis the similarity matrix Dice coefficient (Dice, 1945) and Unweighted Pair Group Mean Averages (UPGMA) (Sneath & Sokal, 1973) were used.

Table 7. API20NE tests.

TEST	REACTIONS/ENZYMES
NO_3	reduction of nitrates to nitrites reduction of nitrates to nitrogen
TRP	indole production
GLU	acidification
ADH	arginine dihydrolase
URE	urease
ESC	hydrolysis(β-glucosidase)
GEL	hydrolysis(protease)
PNG	β-galactosidase
GLU	glucose assimilation
ARA	arabinose assimilation
MNE	mannose assimilation
MAN	mannitol assimilation
NAG	N-acetyl-glucosamine assimilation
MAL	maltose assimilation
GNT	gluconate assimilation
CAP	caprate assimilation
ADI	adipate assimilation
MLT	malate assimilation
CIT	citrate assimilation
PAC	phenyl-acetate assimilation
OX	cytochrome oxidase

2.2.3. Symbiotic effectiveness

Seeds were pre-germinated, before being transferred to the pots and sown as described in section 3.1.2. Each seed was inoculated with 1 ml of a turbid rhizobia suspension, prepared in water from a fresh YMA culture.

Inoculated chickpea plants were grown in the growth chamber in plastic pots (11 cm diameter) filled with sterile vermiculite. Three replicates were used for each treatment.

A nitrogen-free nutrient solution (Broughton & Dilworth, 1971) was applied to irrigate the pots every other day, or whenever necessary,.

Uninoculated nitrogen-free plants served as negative controls, whereas uninoculated nitrogen-supplemented plants (140 ppm nitrogen as KNO_3) were used as positive controls.

The growth chamber was programmed as described in 3.1.2.

After eight weeks, plant shoot dry weight (SDW), root dry weight (RDW), nodules dry weight (NDW) and number of nodules (NN) were measured. SDW, RDW and NDW were determined from material dried to constant weight at 70°C for 48 hours (Gibson, 1987).

Symbiotic effectiveness (SE) was determined as the ratio of SDW inoculated plants – SDW non-inoculated control plants / SDW non-inoculated N-supplemented control plants – SDW non-inoculated control plants, according to Gibson (1987).

Data represent average of three replicas. Statistical analysis was done using SPSS 13.0 Software (SPSS Chicago, IL, USA). One-way analyses of variance (ANOVA) were carried out for each parameter. Duncan's multiple range test (Duncan, 1955) was used to distinguish between treatments and compare means, whenever the ANOVA was significant (P=0.05). Correlations between continuous variables were determined using the Pearson product-moment correlation coefficient.

2.3. Molecular methods

All material was sterilised either by autoclaving or in the oven. Bidestilled filtered water (Millipore, U.S.) was used for all applications.

2.3.1. DNA isolation

The DNA isolated as described in 3.3.1.1 was used only for RFLP of total DNA for Southern hybrisation. The DNA obtained using the protocol described in 3.3.1.2 was used for PCR amplifications and for determination of the GC content.

2.3.1.1. Total DNA isolation

Total DNA of isolates was obtained using the GenomicPrep™ Cells and Tissue DNA Isolation Kit (GE Healthcare, formerly Amersham Biosciences, U.K.) using the manufacturer's instructions.

Isolates were grown in 5 ml TY at 28°C with shaking (150 rpm). 4 ml of this overnight culture was added to a 2 ml microtube. Cells were pelleted by centrifugation at 16 000 x g for 3 minutes at 4°C. Cells were gently ressuspended in 600 µl of cell lysis solution. After about 10 minutes of lysis at 80°C, samples were cooled to room temperature, treated with RNase A solution at 37°C for one hour and then cooled to room temperature again, before treatment with protein precipitation solution. DNA was precipitated with isopropanol and ressuspended in 50 µl of water.

DNA concentration was determined at 260 nm using a UV/Vis spectrophotometer DU 530 Life Sciences (Beckman) and by agarose gel electrophoresis. The ratio 260/280 nm was used to evaluate DNA quality.

2.3.1.2. Rapid DNA extraction

Total DNA was extracted as described previously (Rivas *et al.*, 2001). Rhizobial isolates were inoculated in TY medium. Cells were recovered by centrifugation at 9500 x g for four minutes. The pellet was washed with 200 µl 0.1% Sarkosyl and spun down again to remove the detergent. The DNA was extracted with 100 µl 0.05 M NaOH by heating at 100°C for four min. The reaction was stopped on ice for a few seconds. 900 µl of water were added followed by gentle shaking. Finally, the suspension was centrifuged at 1500 x g for three min, to purge cell remains. 700 µl of the suspension were transferred to a new microtube, which was kept at -20°C.

2.3.2. GC Content

Total DNA was extracted from each isolate using the method described in section 3.3.1.2. The GC content of DNA was measured using the thermal denaturation method of (Marmur & Doty, 1961), according to the following equation:

$$\% \text{ G+C} = \text{K (Tm} - 69.3) \times 2.44$$

The DNA was diluted in SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) and heated in a water bath. Optical density was measured at 260 nm for temperatures above 50°C, in order to determine the melting temperature (Tm).

The constant K was determined using *E. coli* K12, whose GC content is known (Marmur & Doty, 1962). In our case, K was 1.

2.3.3. Restriction Fragment Lenghth Polymorphism (RFLP) of total DNA

2.3.3.1. Electrophoresis

Total DNA was digested with restriction endonucleases *Bam*HI (Invitrogen) and *Eco*RI (Invitrogen), according to the manufacturer's instructions. The digestion mixture was combined with 5 µl of loading buffer (0.25% Orange G, 30% glycerol) (Sambrook & Russell, 2001) and electrophoresed on 0.8% (w/v) agarose (Invitrogen) gel with Tris-acetate buffer (TAE) (40 mM Tris, 40 mM acetic acid, 1 mM EDTA, pH 8.3) (Sambrook & Russell, 2001) at 80 V for 5 hours. 1 Kb DNA Plus (Invitrogen) was used as a molecular weight marker. Gels were stained with ethidium bromide (QBiogene) (0.5 µg/ml) and photographed under UV light using a Kodak DC 120 camera with the 1D Kodak Digital Science software (version 2.0.3, Eastman Kodak Company).

2.3.3.2. Southern hybridisation

Total DNA RFLP gels were capilllary transferred overnight to a nylon membrane (Roche Applied Science, Germany) by Southern blot (Sambrook & Russell, 2001) using 10X SSC (1.5 M NaCl, 0.15 M trisodium citrate, pH 7.0).

Hybridisations were performed overnight at 37°C and 42°C with Dig Easy Hyb hybridisation buffer (Roche Applied Science, Germany) using *nif*H and *nod*C, of the type strain of *M. mediterraneum* UPM-Ca36^T or of isolate 29-Beja, as probes. The recommended hybridisation temperature range for the Dig Easy Hyb buffer is between 37°C and 42°C, according to the manufacturer's instructions. Approximately 1 µg purified PCR product was used for random primed labelling. Probe labelling was performed with digixigenin-11-dUTP (Dig-High Prime Labelling and Detection Starter Kit II, Roche Applied Science, Germany), as recommended by the manufacturer. After hybrisation, membranes were washed twice at room temperature for five minutes in Low Stringency Buffer (2X SSC, 0.1% SDS, pH 7.0) and once at 60 °C to 68°C for 10 to 15 min in High Stringency Buffer A (0.5X SSC, 0.1% SDS, pH 7.0) or B (0.1X SSC, 0.1% SDS, pH 7.0). The following incubations were performed at room temperature with shaking. Membranes were equilibrated in Washing Buffer (0.1 M maleic acid, 0.15 NaCl, pH 7.5, 0.3% (v/v) Tween 20) for 5 min. Afterwards, they were incubated for 30 min in Blocking Solution (Roche Applied Science, Germany) and for an additional 30 min period in Antibody Solution (Anti-Digoxigenin-AP [75 mU/ml] in Blocking Solution). They were then washed twice for 15 min in Washing Buffer and subsequently equilibrated in Detection Buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 5 min. Hybridisation signals were detected with CSPD using the chemiluminescent method (Dig-High Prime Labelling and Detection Starter Kit II, Roche Applied

Science, Germany), as recommended by the manufacturer. The damp membranes were finally sealed in folders and exposed to X-Ray film (Kodak) for 2 hours to overnight.

2.3.4. Direct Amplified Polymorphic DNA (DAPD) analysis

DAPD analysis has been developed in our Lab. It is a PCR-based fingerprint method that uses a high annealing temperature (Niemann *et al.*, 1997) together with a single non-arbitrary directed primer of 20 nucleotides with homology to the *nif* promoter consensus sequence, RPO1 (Richardson *et al.*, 1995).

RPO1: 5' AAT TTT CAA GCG TCG TGC CA 3'

A 25 µl PCR reaction was prepared with 10 µl of diluted cell suspension (Harrison *et al.*, 1992), 1 U *Taq* polymerase (MBI Fermentas), 3.0 mM $MgCl_2$ (MBI Fermentas), 0.2 mM each dNTP (Invitrogen) and 15 pmol of primer RPO1 (Invitrogen). The amplification program was: 3 min at 95°C, 45 cycles of 1 min at 95°C, 1 min at 60°C, 2 min at 72°C, and finally 3 min at 72°C. The amplification was done in a termocycler "Personal Cycler" (Biometra) with hot lid. Amplified products were separated on 1% (w/v) agarose gels running in 0.5 X Tris-borate buffer (TBE) (Tris 90 mM, boric acid 90 mM, EDTA 2 mM, pH 8.3) (Sambrook & Russell, 2001). 1Kb DNA Plus (Invitrogen) was used as a molecular weight marker. Gels were stained and photographed as described in 3.3.3.1.

DAPD patterns were analysed using the software BioNumerics (version 2.0; Applied Maths). For cluster analysis the similarity matrix Dice coefficient (Dice, 1945) and Unweighted Pair Group Mean Averages (UPGMA) (Sneath & Sokal, 1973) were used.

2.3.5. SDS-PAGE of total proteins

The treatment of cells for protein fingerprinting by SDS-PAGE (Laemmli, 1970) was adapted from Tan and co-workers (1997). Isolates were grown at 28°C for 48 hours in TY medium. Cells were collected by centrifugation at 16,000 × g for 3 minutes. The pellet was washed and suspended in 0.5 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The concentration of protein was adjusted by measuring the optical density at 280 nm with a spectrophotometer CE 1021 (Cecil). The samples were incubated at 100°C for 10 minutes with sample buffer (62.5 mM Tris-HCl, 2% (w/v) SDS, 5% (v/v) mercaptoethanol, 10% (v/v) glycerol, 0.001% (w/v) bromophenol blue) (Laemmli, 1970).

Stacking gel (4% (v/v) acrylamide, 0.04% (v/v) bis-acrylamide, 0.125 M Tris-HCl, pH 6.8, 0.1 % SDS (w/v), 0.1% (v/v) ammonium persulfate, 0.1% (v/v) TEMED) and resolving gel (12.5% (v/v) acrylamide, 0.1% (v/v) bis-acrylamide, 0.375 M Tris-HCl, pH 8.8, 0.1 % SDS (w/v), 0.1% (v/v) ammonium persulfate, 0.1% (v/v) TEMED) and electrophoresis buffer (24.8 Tris-HCl, 192 mM glycine, 0.1% (w/v), pH 8.3) were prepared in the meanwhile. Samples were subsequently subjected to discontinuous slab gel electrophoresis (Laemmli, 1970) at 150 V for approximately four hours. SigmaMarker™ High Range (Sigma-Aldrich) and Dalton Mark® VII-L Standard Mixture (Sigma-Aldrich) were used as molecular weight markers. The gels were stained in a Coomassie® blue staining solution (40% (v/v) methanol, 7% (v/v) acetic acid, 0.025% (w/v) Coomassie blue R-250) overnight and destained with a destaining solution (7% (v/v) acetic acid, 5% (v/v) methanol). Protein patterns gels were scanned and computer images were explored with 1D Kodak Digital Science software (version 2.0.3, Eastman Kodak Company).

Protein profiles were analysed with BioNumerics (version 2.0; Applied Maths), as described in 3.3.4.

2.3.6. Plasmid profiles

Due to the very high molecular weight of plasmids, their analysis involved the direct lysis of bacterial cells on an agarose gel prior to electrophoresis. The procedure was adapted from (Haukka *et al.*, 1998; Hynes *et al.*, 1985) and uses an in-well lysis method to determine the plasmid profiles by horizontal agarose gel electrophoresis. Isolate 29-Beja, whose plamids molecular weights were determined using *Rhizobium leguminosarum* bv. *viciae* strain T83K3 as standard, was used in all agarose gels as a molecular weight marker.

2.3.6.1. Cell treatment

The isolates were grown in 5 ml TY medium for six hours at 28°C with shaking (150 rpm). After this initial growth, cells were cultured overnight in half-concentrated TY liquid medium in the same conditions. This less concentrated TY liquid medium was used to prevent excessive polysaccharide production (Haukka, 1997). The cell cultures (1 to 1.5 ml) were then spun down at 3000 x g for 10 minutes and then washed 50 µl with 0.2% (w/v) N-laurylsarcosine (Sigma). The pellet was ressuspended in 50 µl lysis solution (25 mM Tris-HCl, pH 8.3, 10% sucrose, 10 µg/ml RNase (Pharmacia), 100 µg/ml lysozyme) (Harrison *et al.*, 1989; Hynes *et al.*, 1985) and 4 µl sample buffer

(0.25% (w/v) bromophenol blue, 0.25% (w/v) xylen cyanol FF blue, 30% (v/v) glycerol) were added (Sambrook & Russell, 2001). The samples were then immediately loaded into the gel.

2.3.6.2. Electrophoresis

The analysis of plasmid profiles was performed using a two-comb system, which allows the in situ lysis of the bacterial cells in the agarose gel itself (Eckhardt, 1978).

A 0.9% agarose was prepared with TBE buffer. The first comb was filled with a solution containing 2% SDS and 0.4% agarose. In the cell lysis step it was essential that the lysis solution was added to one sample at a time, which was then immediately loaded into the gel before proceeding to the next sample. The electrophoresis with an initial run at low voltage (35 V for two hours) in order to permit the migration of the SDS in the direction of the wells containing the cells, so that lysis can occur and plasmids may be separated. Next, the voltage was switched to 100 V, and the gel was run overnight (16 to 18 hours) at 4ºC.

After electrophoresis, the gel was stained with ethidium bromide (1.5 µg/ml) and documented as described in 3.3.3.1.

2.3.6.3. Southern hybridisation

Plasmids were subsequently transferred to a nylon membrane (Roche Applied Science, Germany) by Southern blot (Sambrook & Russell, 2001) using 20X SSC (3.0 M NaCl, 0.3 M trisodium citrate, pH 7.0).

Southern hybridisation was performed as described in 3.3.3.2.

2.4. Gene sequence analysis

2.4.1. PCR amplification and sequencing

All amplifications were done in a termocycler "Personal Cycler" (Biometra) with hot lid.

Amplified products were run on 1% (w/v) agarose gels with 0.5X TBE buffer. 1 Kb DNA Plus (Invitrogen) was used as a molecular weight marker. Agarose gels were stained and photographed as described in 3.3.3.1.

PCR products were purified using ExoSAP-It (Amersham Biosciences), CONCERT™ Rapid PCR Purification System (Gibco BRL) or GFX PCR DNA and Gel Band Purification Kit (Amersham

Biosciences) according to the manufacturer's instructions. Alternatively, PCR products were run on an agarose gel, the desired band excised and purified using GFX PCR DNA Gel Band Purification Kit (Amersham Biosciences).

Sequences obtained from native isolates were compared with those of rhizobial type strains available in the GenBank database or determined within the present study.

2.4.1.1. 16S rRNA gene RFLP and sequencing

The primers used for 16S rDNA amplification and sequencing are listed in Table 8. The 16S rRNA gene was amplified by PCR using primers Y1 (Young *et al.*, 1991) (positions 20-43) and Y3 (positions 1482-1507), which amplify a 1440 bp fragment. The positions indicated correspond to the 16S rDNA gene sequence of *Escherichia coli* (V00348: region 1518-2959).

Table 8. Primer sequences for 16S rDNA amplification and sequencing. **Y**-C/T; **S**-C/G; **R**-A/G.

Primer	Sequence
Y1	5' - TGG CTC AGA ACG AAC GCT GGC GGC - 3'
Y3	5' - CCC ACT GCT GCC TCC CGT AGG AGT -3'
IntF	5' - GCT YAA CST GGG AAC TGC - 3'
IntR	5' - TTT ACR GCG TGG ACT ACC - 3'

A 50 µl PCR reaction was prepared with a "tip" of bacterial culture from a TY plate, 1 U Taq polymerase (Invitrogen), 1.25 mM $MgCl_2$ (Invitrogen), 0.2 mM each dNTP (Invitrogen) and 16 pmol each primer (Gibco-BRL). The amplification programme was as follows: 3 min at 95°C, and 25 cycles of 1 min at 94°C, 1 min at 62°C, 2 min at 72°C, and finally 3 min at 72°C.

For 16S rDNA RFLP, PCR products were digested with six endonucleases namely: *Alu*I, *Hha*I, *Hin*fI, *Hpa*II, *Rsa*I and *Taq*I, according to supplier's instructions.

Digested PCR products were run on 2.5% (w/v) agarose gels with 0.5X TBE buffer.

Twenty-one representative strains from all the obtained RFLP genotypes were chosen for sequencing.

Sequencing reactions were done using ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit with Amplitaq® DNA polymerase, FS (Perkin Elmer). Sequences were generated with Perkin Elmer's ABI PRISM™ 377 DNA Automatic Sequencer. An estimated amount of 100 ng of DNA was used for each reaction along with 1.6 pmoles of primer and 4 µl of ready reaction mix in a 10 µl final volume. Primers IntF and IntR were used as internal primers for double-stranded sequencing.

2.4.1.2. 16S-23S intergenic spacer (ITS)

In the present study, the ITS sequence between 16S and 23S was used to distinguish among closely related isolates within *Mesorhizobium*.

The intergenic spacer (800-1000 bp) between the 16S and 23S rRNA genes was amplified, as described previously (Laguerre *et al.*, 1996), using primers FGPS1490 (Navarro *et al.*, 1992) and FGPS132' (Ponsonnet & Nesme, 1994), and sequenced directly. These primers amplify one or more fragments of variable length, depending on the isolate or strain analysed. The positions indicated correspond to the numbering of the 16S-23S intergenic spacer sequence of *Escherichia coli* (V00348: region 2960-3499).

FGPS1490:	5' TGC GGC TGG ATC ACC TCC TT	3' (positions 1521-1541)
FGPS132':	5' CCG GGT TTC CCC ATT CGG	3' (positions 115-133)

A 50 µl PCR reaction was prepared with 10 µl DNA, 1 U Fideli*Taq*™ polymerase (usb), 1X buffer (usb), containing 1.5 mM $MgCl_2$, 5% DMSO (Duchefa), 0.2 mM each dNTP (Invitrogen) and 25 pmol each primer (Thermo Electron). The amplification programme was as follows: 2 min at 95°C, and 35 cycles of 30 sec at 95°C, 30 sec at 68°C, 2 min at 68°C, and finally 5 min at 68°C.

Sequencing reactions were performed in Macrogen (Seoul, Korea).

2.4.1.3. Protein coding genes

2.4.1.3.1. *atp*D (ATP synthase β) gene

The primers used for amplification and sequencing of the *atp*D gene are listed in Table 9.

Table 9. Primer sequences for amplification and sequencing of the *atp*D gene.

Primer	Sequence	Reference
***atp*D-F**	5' - ATC GGC GAG CCG GTC GAC GA - 3'	(Gaunt *et al.*, 2001)
***atp*D-R**	5' - GCC GAC ACT TCC GA CCN GCC TG - 3'	(Gaunt *et al.*, 2001)
***atp*D-3**	5' – TGA TAG CCC ACG GCC GAC- 3'	This study

The first pair of *atp*D primers (Gaunt *et al.*, 2001) amplify a 477 bp fragment between positions 294 and 771 in the *Rhodobacter capsulatus* sequence (X99599). Primer *atp*D-3 was used to replace primer *atp*D-R for some isolates together with *atp*D-F to amplify a fragment of approximately 450 bp.

A 50 µl PCR reaction was prepared with 10 µl DNA, 1 U Fideli*Taq*™ polymerase (usb), 1X buffer (usb), containing 1.5 mM $MgCl_2$, 5% DMSO (Duchefa), 0.2 mM each dNTP (Invitrogen) and 25 pmol

each primer (Thermo.Electron). The amplification programme was as follows: 2 min at 95°C, and 35 cycles of 30 sec at 95°C, 30 sec at 60°C, 2 min at 68°C, and finally 5 min at 68°C.

2.4.1.3.2. *rec*A (DNA recombinase A) gene

*rec*A primers (Gaunt *et al.*, 2001) amplify a 441 bp fragment between positions 63 and 504 in the *R. leguminosarum* sequence (X59956).

*rec*A-F:	5'	ATC GAG CGG TCG TTC GGC AAG GG	3'
*rec*A-R:	5'	TTG CGC AGC GCC TGG CTC AT	3'

A 50 µl PCR reaction was prepared with 3 µl DNA, 2 U *Taq* polymerase (Fermentas), 1X buffer (Fermentas), 1.5 mM $MgCl_2$ (Fermentas), 0.004% BSA (Promega), 0.2 mM each dNTP (Invitrogen) and 25 pmol each primer (Biomers). The amplification programme was as follows: 5 min at 95°C and 30 cycles of 45 sec at 94°C, 60 sec at 55°C, 1 min at 72°C.

2.4.1.3.3. *gln*A (glutamine synthetase I) gene

Amplification and sequencing of the *gln*A gene was only performed for a subgroup of isolates, which were considered to be interesting, because they grouped separately from all known species.

*gln*A primers (Turner & Young, 2000) amplify a 1085 bp fragment between positions 58 and 1143 in the *Sinorhizobium meliloti* sequence (U50385).

GSI-3:	5'	GAY CTG CGY TTY ACC GAC C	3'
GSI-2:	5'	GTC GAG ACC GGC CAT CAG CA	3'

A 50 µl PCR reaction was prepared with 5 µl DNA, 1 U Fideli*Taq*™ polymerase (usb), 1X buffer (usb), containing 1.5 mM $MgCl_2$, 0.2 mM each dNTP (Invitrogen) and 15 pmol each primer. The amplification programme was as follows: 2 min at 94°C, and 30 cycles of 45 sec at 94°C, 90 sec at 55°C, 2 min at 68°C, and finally 7 min at 68°C.

Sequencing reactions were performed in Macrogen (Seoul, Korea).

2.4.1.5. Symbiosis genes

The primers used for amplification of symbiosis genes are listed in Table 10. *nif*H primers (Eardly *et al.*, 1992) amplify a 601 bp fragment between positions 256 and 856 in the *S. meliloti* sequence (M55229). *nod*C primers were designed from conserved regions of the gene in different bacteria (Rivas González, 2003), and amplify a fragment of approximately 800 bp.

Table 10. Primer sequences of symbiosis genes.

Primer	Sequence
***nif*H-1**	5' – AAG TGC GTG GAG TCC GGT GG – 3'
***nif*H-2**	5' – GTT CGG CAA GCA TCT GCT CG - 3'
***nod*CF**	5' – CGA (CT)CG (AG)AG (AG)TT CCA (CT)TT C - 3'
***nod*CR**	5' – CT(CT) AAT GTA CAC A(AG)(GC) GC - 3'

2.4.1.5.1. *nif*H gene

A 50 µl PCR reaction was prepared with 3 µl DNA, 1 U *Taq* polymerase (Ferementas), 1X buffer (Fermentas), 3 mM $MgCl_2$ (Fermentas), 0.2 mM each dNTP (Invitrogen) and 16 pmol each primer (Operon). The amplification programme was as follows: 2 min at 93°C, and 35 cycles of 45 sec at 93°C, 45 sec at 62°C, 2 min at 72°C, and finally 5 min at 72°C.

2.4.1.5.2. *nod*C gene

A 50 µl PCR reaction was prepared with 10 µl DNA, 1 U *Taq* polymerase (Fermentas), 1X buffer (Fermentas), 5 mM $MgCl_2$ (Fermentas), 0.004% BSA (Promega), 0.2 mM each dNTP (Invitrogen) and 50 pmol each primer (MWG). The amplification programme was as follows: 9 min at 95°C, and 35 cycles of 60 sec at 94°C, 60 sec at 50°C, 2 min at 72°C, and finally 7 min at 72°C.

Sequencing reactions were performed by STABVida (Oeiras, Portugal) or Macrogen (Seoul, Korea).

2.4.2. Phylogenetic analysis

Phylogenetic analysis generally comprises four fundamental steps: sequence multiple alignment, determination of the substitution model, tree reconstruction and evaluation (Brinkman & Leipe, 2001).

2.4.2.1. Multiple alignment and substitution model

The most important parameters in an alignment are those that determine the placement of insertions and deletions (indels) in alignment of length variable sequences. This will depend on all elements of the evolutionary model, including nucleotide transition/transversion rates. Unless phylogenetic relations are known beforehand, there is no way of determine if an alignment is appropriate for phylogenetic analysis, but it is always advisable to inspect the computer generated alignment. The most extreme way to treat indels is to remove from the analysis all sites that include gaps. A gap-free alignment requires no consideration of gap interpretation specific to a given tree-building method, but has the disadvantage of discarding phylogenetic signal contained in indels (Brinkman & Leipe, 2001).

The substitution model influences both alignment and the tree building, therefore a recursive approach is needed (Brinkman & Leipe, 2001).

2.4.2.2. Tree reconstruction and evaluation

Distance methods use the amount of dissimilarity between two aligned sequences to draw trees. The great advantage of these methods is that they can use the same models of sequence evolution than maximum likelihood, but are much less computationally intensive. Their disadvantage is that the actual character data is discarded. The most commonly applied methods are described as follows.

- Unweighted Pair Group Method with Arithmetic Mean (UPGMA). It is a clustering or phenetic algorithm, which joins tree branches based on greatest similarity among pairs and average of joined pairs.
- Neighbour-Joining (NJ) (Saitou & Nei, 1987). It is related to traditional cluster analysis, but allows for unequal rates of molecular change among branches. At each step of the analysis, a transformed distance matrix is constructed that has the net effect of adjusting branch lengths between pairs of nodes on the basis of mean divergence from all other nodes. It produces only one tree. This method is fast, but seems less efficient when the number of taxa increases and the branches are short.

- Fitch-Margoliash (FM). It seeks to maximise the fit of the observed pairwise distances to a tree by minimising the squared deviation of all possible observed distances relative to all possible path lengths in the tree (Felsenstein, 1997).
- Minimum Evolution (ME). This method searches for the shortest tree consistent with the path lengths measured by minimising the squared deviation of observed to tree-based distances.

NJ is the fastest procedure and generally yields the actual ME tree or a tree very close to it (Rzhetsky & Nei, 1992).

Character-based methods use character data in all steps of the analysis, which allows assessment of the reliability of each base position in an alignment on the basis of all other base positions.

- Maximum Parsimony (MP). It is based on the criterion that the best explanation of the data is the simplest one. MP analyses generally yield numerous trees with the same score, but only groupings present in the strict consensus of all trees are considered to be supported by the data.
- Maximum Likelihood (ML). This method searches for the evolutionary model and the tree that has the highest likelihood of producing the observed data. In fact, ML is derived for each base position in alignment. ML is computationally very intense.

Tests of tree support from resampling of observed data include:

- Bootstrap (Felsenstein, 1985). Non-parametric bootstrapping works with distance, parsimony and likelihood. The result is a number associated with a particular branch in the tree, which gives the proportion of bootstrap replicates that support the monophyly of the clade. It has been considered to be a measure of accuracy, a biologically relevant parameter, which gives the probability that the true phylogeny has been recovered (Felsenstein & Kishino, 1993).
- Jackknife. The jackknifing resamples the original data set by dropping one or more alignment positions in each replicate. Consequently, each replicate is smaller than the original data set and cannot contain duplicated data points.

2.4.2.3. Phylogenetic approach in the present study

In the present work, sequences were analysed and aligned using ClustalW (Thompson *et al.*, 1994) as implemented in BioEdit Sequence Alignment Editor (version 7.0.5) (Hall, 1999). Molecular phylogeny was reconstructed with Molecular Evolutionary Genetics Analysis (MEGA3) software (version 3.1) (Kumar *et al.*, 2004) using the Neighbour-Joining (Saitou & Nei, 1987) and the Minimum Evolution methods with bootstrap (Felsenstein, 1985) support (1000 replicates) based on a distance matrix with the distance correction calculated by Kimura's two-parameter model (Kimura, 1980). Gaps or indels were treated using the complete deletion option in MEGA3 (Kumar *et al.*, 2004). Since *Azorhizobium* and *Bradyrhizobium* are both equally distant from the fast-growing rhizobia (Sawada *et al.*, 2003), trees were rooted using the *Azorhizobium-Bradyrhizobium* "clade" as

the outgroup, with the exception of the *rec*A tree, which was rooted using only the *Bradyrhizobium* branch. Maximum likelihood phylogenies were also constructed using the DNAml program included in the PHYLIP package (version 3.66, July 2006) (Felsenstein, 2006).

The partition homogeneity test or incongruence length difference (ILD) test (Cunningham, 1997) was applied to check whether trees for the different genes were sufficiently similar to be appropriate to combine the data. The test was calculated using PAUP*4.0 (Swofford, 2003). Combined phylogenetic trees were constructed from the concatenated sequences of the three protein coding genes (*atp*D, *gln*A and *rec*A).

Statistical analyses were done using SPSS 13.0 Software (SPSS Chicago, IL, USA). Relationships between categorical variables were determined using the chi-square test of association. Results are presented as the test statistic (χ^2), degrees of freedom (df), and probability of equal or greater deviation (P). Since our samples did not satisfy Cochran's criteria (some categories were represented by only one isolate, and more than 20% of the categories were represented by less than five isolates), the exact value of *P*, the critical probability, was computed rather than the asymptotic *P* value, which is an approximation reserved for large samples (Louvrier *et al.*, 1996). Relationships between a continuous variable and an unordered categorical variable were tested using analyses of variance (ANOVA).

3. IDENTIFICATION OF CHICKPEA RHIZOBIA ISOLATES

This chapter is based on the following manuscripts:

Laranjo, M., Rodrigues R., Alho L. and Oliveira, S. 2001. Rhizobia of chickpea from southern Portugal: symbiotic efficiency and genetic diversity. *J. Appl. Microbiol.* 90: 662-667.

Laranjo, M., Branco, C., Soares, R., Alho, L., Carvalho, M. and Oliveira, S. 2002. Comparison of chickpea rhizobia isolates from diverse Portuguese natural populations based on symbiotic effectiveness and DNA fingerprint. *J. Appl. Microbiol.* 92: 1043-1050.

Laranjo, M., Machado, J., Young, J. P.W. and Oliveira, S. 2004. High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. *FEMS Microbiol. Ecol.* 48: 101-107.

Alexandre, A., Laranjo, M. and Oliveira, S. 2006. Natural populations of chickpea rhizobia evaluated by antibiotic resistance profiles and molecular methods. Microb. Ecol. 51: 128-136.

3.1. Introduction

Chickpea is an alternative crop to cereals in the dry land conditions of south Portugal, namely in the Alentejo region. Nevertheless, chickpea rhizobia have been poorly studied worldwide and no study has been published on Portuguese strains.

Only two species have been described to effectively nodulate chickpea, namely *M. ciceri* (Nour *et al.*, 1994a) and *M. mediterraneum* (Nour *et al.*, 1995).

Identification methods that often lead to phylogeny inference include 16S rDNA sequencing (Stackebrandt *et al.*, 2002), and 16S rDNA RFLP analysis (Laguerre *et al.*, 1994).

The analysis of the 16S rRNA gene is a standard method established for phylogenetic (Young *et al.*, 1991) and taxonomic studies . Thus, full (or almost full) sequence is absolutely necessary for the description of a new species of bacteria. Less than 97% similarity between the 16S rDNA sequences of two isolates usually means that they belong to different species (Stackebrandt & Goebel, 1994). However, the sequence identity between several species, namely of *Mesorhizobium*, is actually much higher. While, the lowest sequence similarity between two *Mesorhizobium* species is 97.6% between *M. ciceri* and *M. huakuii*, the highest sequence similarity is 99.7% between *M. amorphae* and *M. septentrionale*, and between *M. mediterraneum* and *M. temperatum*. The sequence identity between the two chickpea microsymbionts, *M. ciceri* and *M. mediterraneum*, is 98.5%.

Chickpea rhizobia populations were analysed to characterise native isolates obtained from four sites with different environmental conditions, in order to explore new rhizobium-chickpea associations. Therefore, rhizobia able to nodulate chickpea were isolated and their species affiliation was determined by 16S rDNA sequence analysis.

3.2. Results

3.2.1. Rhizobia isolates

Isolation of native Portuguese chickpea rhizobia was accomplished using trap plants in pots with soil from different geographical origins in South Portugal, namely Beja, Elvas, Elvas-ENMP, Évora and Setúbal. The soils from the first four locations belong to the region known as Alentejo and are agricultural soils. The Elvas-ENMP soil is the only one where chickpea is usually cultivated.

In Figure 2 the collection sites are marked with a red spot. Elvas corresponds to two collection sites: Elvas-CV and Elvas-ENMP.

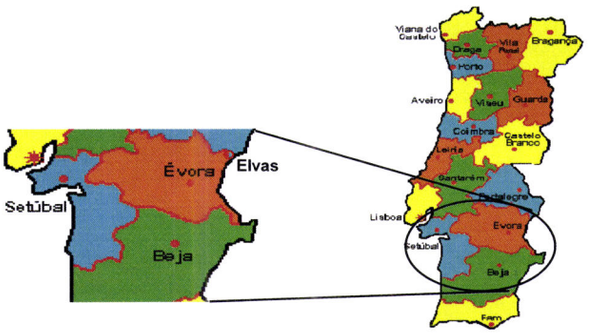

Figure 2. Map of Portugal showing the sites where soil samples were collected.

3.2.1.1 Soil characteristics

Some physical and chemical analyses were performed to the different soils, as described in the following table. The size of natural rhizobia population of the different soils was also estimated.

Table 11 shows some basic soil characteristics, including the soil type as determined by (Zobler, 1986).

Table 11. Physical and chemical properties of the soils used in the present study. n.d.-not detectable; n.dt.-not determined.

Origin	Type	Texture	pH	Organic Matter (%)	P_2O_5 ppm	NO_3 ppm	K_2O ppm	Ca meq/100g	Mg meq/100g	Na meq/100g	Rhizobia (nfu/g soil
Beja	Vertisol	Medium	7.9	1.2	43.4	6.0	180.9	n.dt.	n.dt.	n.dt.	$6.00x10^4$
Elvas	Luvisol	Medium	5.8	1.7	25	25.5	275.5	n.dt.	n.dt.	n.dt.	$1.05 x10^2$
			6.2	n.dt.	48	63.5	143	5.89	1.56	0.06	$1.50x10^3$
Elvas-ENMP	Luvisol	Medium	7.9	n.dt.	529	12.0	137	11.59	1.35	0.06	$4.27x10^6$
Évora	Luvisol	Gross	5.8	0.8	58	5.3	75.5	n.dt.	n.dt.	n.dt.	n.d.
Setúbal	-	Medium	8.1	n.dt.	631	17.5	350	n.dt.	n.dt.	n.dt.	n.dt.

For three sites (Beja, Elvas and Évora) two different soil parameters values are shown, which correspond to samples that were collected in different times at approximately the same locations. In these cases, the two soil samples were analysed and the results are shown in Table 11.

The size of the rhizobia population was found to be positively correlated ($P<0.01$) with soil pH, and nitrates and phosphates.

The soil from Évora is the most acidic soil, and it is the only one with a different texture, namely gross. Interestingly, it is the soil with the fewest rhizobia. All other soils are either moderately acidic or moderately alkaline with a medium texture.

The largest native population found in the Elvas-ENMP soil could be due to the intensive use of chickpea in crop rotation (cereals x grain legumes) (I. Duarte, personal communication). A possible explanation to the large native population found also in the Beja soil could be the traditional use of legumes, in particular chickpea, in the rotation (chickpea x wheat x barley), although this rotation has been abandoned in the last thirty years. However, the population survived even without the presence of the specific host, probably due to the favourable soil conditions of this soil (high clay content and high pH). The very low population present in the Évora soil, which was not detected by the low sensitivity MPN method, is probably a result of the lack of grain legumes in the rotation (sunflower x oat x follow) (Carvalho & co-workers, 1996).

An appropriate soil for the cultivation of chickpea should have a medium texture, be well drained and have a pH between 6 and 8 (Saxena & Singh, 1987). Acidic soils are prone to *Fusarium* spp. bursts and alkaline soils are usually calcareous, and chickpea seeds should not absorb calcium, since it difficults the cooking process (Gordillo, 1991; Saxena & Singh, 1987). However, a moderately acidity seems to favour the symbiosis between chickpea and rhizobia (Howieson *et al.*, 2000). Furthermore, the presence of low amounts of available nitrogen in the soil favours nodulation of chickpea by rhizobia. Nevertheless, if the concentration of nitrates increases, nodulation will be inhibited (Saxena & Singh, 1987; Zahran, 1999).

The larger native population, rather than individual rhizobia, could contribute to the higher chickpea yields obtained in Elvas-ENMP (Carvalho & co-workers, 1996; Pereira *et al.*, 1992).

3.2.1.2. Portuguese native isolates

In practical terms it is not always easy to distinguish between a new rhizobium and a non-symbiotic soil bacterium. Contaminants can originate from the surface of a nodule, or even from inside, where they could have entered together with a rhizobium, which later died during cultivation or agar Petri dish. For determining which isolates are true rhizobia, we used re-inoculation in the original host plant, and excluded from our analysis those isolates which did not seem to be able form nodules on their own, under axenical conditions.

A large amount of strains was isolated within the scope of this work (about 400), of which only about one hundred were confirmed to be responsible for the formation of root nodules by re-inoculation of host plant. From these, an initial screening of genetic diversity by means of plasmid profile analysis was performed. This background information was very helpful in choosing our study group material.

Figure 3 is a partial view of the growth chamber, where chickpea plants were sown in large pots filled with the different soils, for trapping rhizobia.

Figure 3. Chickpea trap plants growing in the growth chamber.

The present study includes 41 isolates of Portuguese chickpea rhizobia from the four different Portuguese soils (ten from each location), Beja, Elvas, Elvas-ENMP and Évora, as well as one isolate from Setúbal. Table 12 enumerates the isolates used in this work. Among these, 21 isolates were chosen for identification by 16S rDNA sequence (this chapter), symbiotic effectiveness evaluation (chapter 4) and gene sequence analysis (chapters 5 and 6).

Table 12. Rhizobia used in the present study.

ORIGIN	ISOLATES	ORIGIN	ISOLATES	ORIGIN	ISOLATES	ORIGIN	ISOLATES
Beja	4a.	Elvas	75	ENMP	EE-2	Évora	87
Beja	6b.	Elvas	77	ENMP	EE-3	Évora	89a.
Beja	7a.	Elvas	78	ENMP	EE-7	Évora	90
Beja	9	Elvas	79	ENMP	EE-9	Évora	92
Beja	21	Elvas	83	ENMP	EE-11	Évora	93
Beja	27	Elvas	85	ENMP	EE-12	Évora	94
Beja	29	Elvas	CV-1	ENMP	EE-13	Évora	96
Beja	31	Elvas	CV-11	ENMP	EE-14	Évora	98
Beja	54b.	Elvas	CV-16	ENMP	EE-20	Évora	101
Beja	64b.	Elvas	CV-18	ENMP	EE-29	Évora	102
						Setúbal	ST-2

In Figure 4 a detail of a root and its nodules, containing isolate ST-2-Setúbal, is shown. The nodules are large, have the typical "Y" ('epsilon') shape and exhibit a red colour, which suggests the presence of the leghemoglobin pigment. Reds nodules are usually active in nitrogen-fixation, because

leghemoglobin binds free oxygen, so that it will not hamper the activity of nitrogenase (Appleby, 1984).

Figure 4. Chickpea roots exhibiting nodules.

3.2.2. Identification of native rhizobia

The 16S rRNA gene was chosen for identification of native rhizobia. The gene was first amplified for all 41 isolates (Table 12) and a RFLP analysis was performed, in order to choose a subgroup of isolates for sequencing of the 16S rDNA.

21 isolates representative of all origins and all RFLP genotypes were chosen for further analysis by sequencing.

3.2.2.1. 16S rDNA RFLP genotypes

PCR products were restricted with six endonucleases: *Alu*I, *Hha*I, *Hin*fI, *Hpa*II, *Rsa*I and *Taq*I.

Each endonuclease generated three to six profiles. The number of fragments obtained varied between three and six. The enzymes *Hin*fI and *Hpa*II were the most discriminating ones. Five different RFLP genotypes were identified. The 16S rDNA genotypes obtained for the 41 isolates were compared with those of type strains from known species. All endonucleases except *Rsa*I were necessary to resolve both isolates and reference strains. Nevertheless, a minimum of two enzymes, *Hha*I and *Hpa*II, was sufficient to separate the native isolates. This combination had the same discriminating power as the combination of the six enzymes. Table 13 shows the RFLP genotype for all 41 isolates.

Table 13. 16S rDNA RFLP genotypes. * denotes isolates chosen for 16S rDNA sequencing.

ISOLATES	16S RESTRICTION PATTERNS						16S RFLP TYPE	
	*Alu*I	*Hha*I	*Hin*fI	*Hpa*II	*Rsa*I	*Taq*I		
4a.-Beja	a	a	a	c	a	a	C	
6b.-Beja	a	a	a	c	a	a	C	*
7a.-Beja	a	b	b	b	a	a	D	*
9-Beja	a	a	a	c	a	a	C	
21-Beja	a	a	a	c	a	a	C	
27-Beja	a	b	b	b	a	a	D	*
29-Beja	a	a	a	c	a	a	C	*
31-Beja	a	a	a	c	a	a	C	
54b.-Beja	a	b	b	b	a	a	D	
64b.-Beja	a	a	c	a	a	a	A	*
75-Elvas	a	b	a	b	a	a	D	*
77-Elvas	a	b	a	b	a	a	D	
78-Elvas	a	a	c	a	a	a	A	*
79-Elvas	a	a	c	a	a	a	A	
83-Elvas	a	a	c	a	a	a	A	
85-Elvas	a	a	c	a	a	a	A	*
CV-1-Elvas	a	a	c	a	a	a	A	
CV-11-Elvas	a	a	c	a	a	a	A	
CV-16-Elvas	a	b	a	b	a	a	D	*
CV-18-Elvas	a	a	c	a	a	a	A	*
EE.2-ENMP	a	a	a	c	a	a	C	*
EE-3-ENMP	a	a	a	c	a	a	C	
EE-7-ENMP	a	b	b	b	a	a	D	*
EE-9-ENMP	a	b	b	b	a	a	B	
EE-11-ENMP	a	a	a	c	a	a	C	
EE-12-ENMP	a	b	b	b	a	a	D	*
EE-13-ENMP	a	b	b	b	a	a	D	
EE-14-ENMP	a	a	a	c	a	a	C	*
EE-20-ENMP	a	b	b	b	a	a	D	
EE-29-ENMP	a	a	a	c	a	a	C	*
87-Évora	a	a	c	a	a	a	A	
89a.-Évora	a	a	c	a	a	a	A	
90-Évora	a	a	c	a	a	a	A	*
92-Évora	a	a	c	a	a	a	A	
93-Évora	a	b	b	c	a	a	B	*
94-Évora	a	a	c	a	a	a	A	*
96-Évora	a	a	c	a	a	a	A	
98-Évora	a	a	c	a	a	a	A	*
101-Évora	a	a	c	a	a	a	A	
102-Évora	a	a	c	a	a	a	A	*
ST-2-Setúbal	a	b	b	c	a	a	B	*

Figure 5 shows the dendrogram based on the analysis of the 16S rDNA RFLP from the 41 isolates.

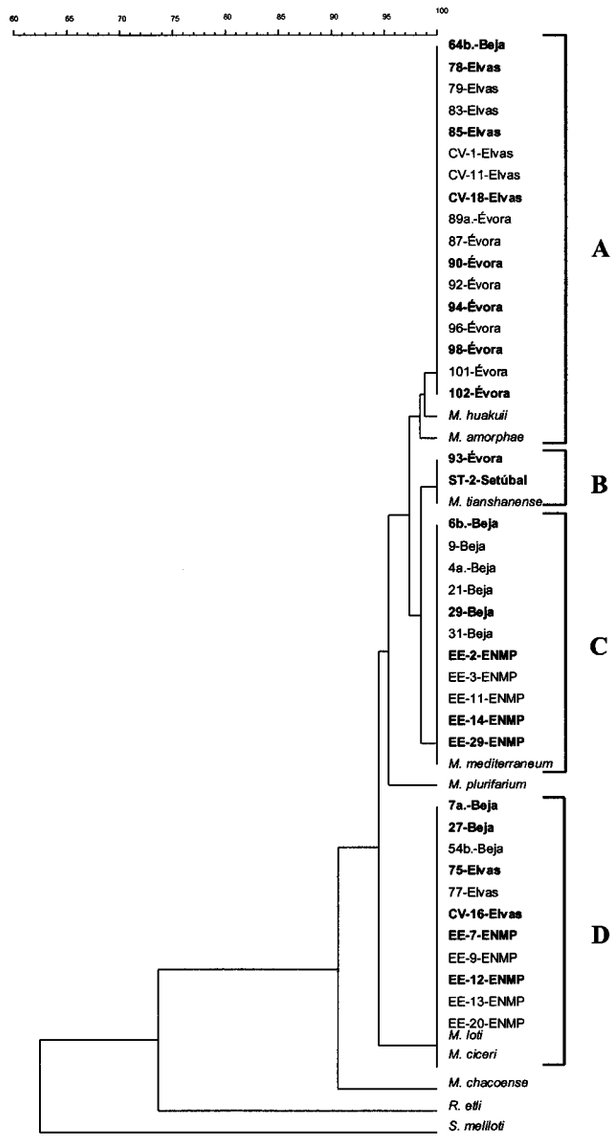

Figure 5.UPGMA Dendrogram based on 16S rDNA RFLP analysis from the 41 isolates and type strains.

All isolates cluster with *Mesorhizobium* species type strains and four main clusters can be identified (Figure 5). Cluster A contains 17 isolates from Beja, Elvas, and Évora and the type strains of *M. amorphae* and *M. huakuii*, although the isolates form a separate branch. Cluster B encloses isolates 93-Évora and ST-2-Setúbal plus the type strain of *M. tianshanense*. Cluster C includes 11

isolates from Beja and Elvas-ENMP and also the type strain of *M mediterraneum*. Cluster D has 11 isolates from Beja, Elvas and Elvas-ENMP and the type strains of *M. ciceri* and *M. loti*.

16S rDNA RFLP suggests that all isolates belong to the genus *Mesorhizobium*. However, clustering of isolates reveals the existence of species able to nodulate chickpea, other than *M. ciceri* and *M. mediterraneum*, the known microsymbionts.

Although not all isolates from the same geographic origin are in the same cluster, there is a relationship between isolates clustering and origin (χ^2=51.908, df=12, P<0.001). For example, isolates from Elvas-ENMP are found only in clusters C and D, and all isolates from Évora, except one, are in cluster A.

Based on the 16S-rDNA RFLP genotype analysis, a subgroup of 21 isolates (marked in Table 13), containing representatives from all origins and all RFLP genotypes, was chosen for sequencing of the 16S rRNA gene.

3.2.2.2 16S rDNA phylogeny

GenBank accession numbers for the 16S rDNA sequences of the 21 isolates determined in this study are AY225381 to AY225401.

GenBank accession numbers for the 16S rDNA sequences of the type strains used for phylogenetic analysis are shown in Table 14.

Table 14. GenBank accession number or RhizoBase record of 16S rDNA sequences from rhizobia type and reference strains used for phylogenetic analysis. Sequence in bold was determined in this study. *Sequence data produced by the *R. leguminosarum* bv. *viciae* Sequencing Group at the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/rl/).

SPECIES	STRAIN	ACCESSION NUMBER
Azorhizobium caulinodans	ORS 571^T	X67221
Bradyrhizobium japonicum	USDA 6^T	U69638
B. japonicum	USDA 110	rrn16S
Mesorhizobium amorphae	ACCC 19665^T	AF041442
M. chacoense	LMG 19008^T	AJ278249
M. ciceri	UPM-Ca7^T	**DQ444456**
M. huakuii	CCBAU 2609^T	D127979
M. loti	NZP 2213^T	X67229
M. loti	R7A	U50166
M. mediterraneum	UPM-Ca36^T	L38825
M. plurifarium	ORS 1032^T	Y14158
M. septentrionale	HAMBI 2582^T	AF508207
M. temperatum	HAMBI 2583^T	AF508208
M. tianshanense	A-1BS^T	AF041447
Rhizobium sp.	NGR234	AY260147
R. etli	CFN 42^T	U28916
R. leguminosarum	USDA 2370^T	U28386
R. leguminosarum bv. *viciae*	3841	16S rRNA 1
Sinorhizobium medicae	A-321^T	L39882
S. meliloti	USDA 1002^T	X67222
S. meliloti	1021	rRNA-16S_1

Phylogenetic analysis of the 21 isolates based on 16S rDNA sequence is shown in Figure 6.

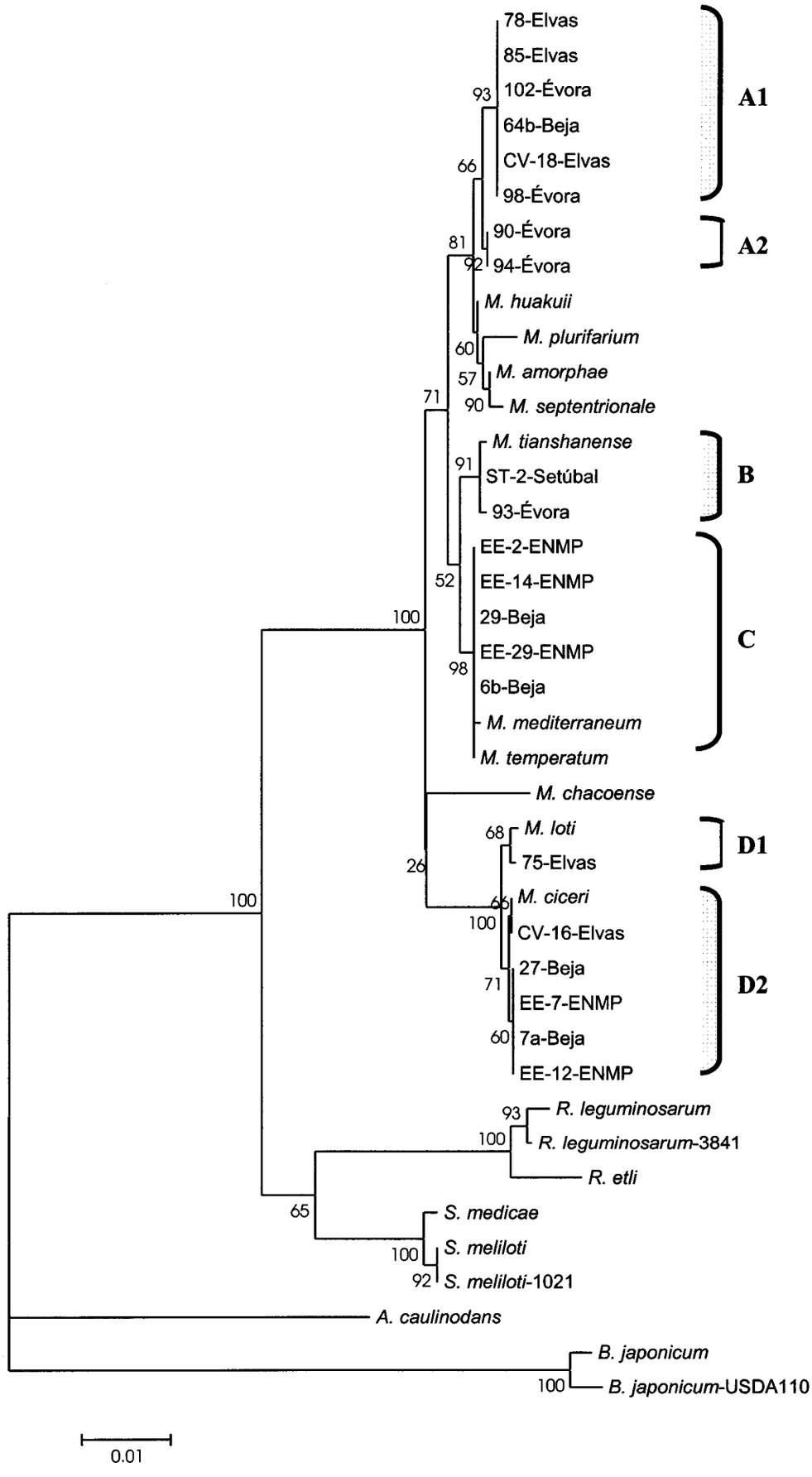
78-Elvas
85-Elvas
93
102-Évora
64b-Beja
66
CV-18-Elvas
98-Évora
A1
81
90-Évora
92
94-Évora
A2
M. huakuii
60
M. plurifarium
57
M. amorphae
71
90
M. septentrionale
M. tianshanense
91
ST-2-Setúbal
93-Évora
B
52
EE-2-ENMP
EE-14-ENMP
100
29-Beja
EE-29-ENMP
98
6b-Beja
M. mediterraneum
M. temperatum
C
M. chacoense
68
M. loti
75-Elvas
D1
26
100
M. ciceri
66
100
CV-16-Elvas
27-Beja
71
EE-7-ENMP
60
7a-Beja
EE-12-ENMP
D2
93
R. leguminosarum
100
R. leguminosarum-3841
R. etli
65
S. medicae
100
S. meliloti
92
S. meliloti-1021
A. caulinodans
B. japonicum
100
B. japonicum-USDA110
0.01

Figure 6.(on opposite page) Neighbour-Joining phylogeny based on 16S rDNA sequences from the 21 isolates (alignment length 1361 bp). Percentage bootstrap support is indicated on internal branches (1000 replicates); scale bar indicates 0.01 substitutions per site.

According to the 16S rDNA based molecular phylogeny (Figure 6), all isolates were assigned to the genus *Mesorhizobium*, since they form a cluster together with the type strains of all ten species of the genus, which received 100% bootstrap support. Among Portuguese isolates, six species groups may be identified. Group **A1** contains six isolates from Beja, Elvas and Évora that may be a putative new species. The sequence similarity within group **A1** is of 100%. Group **A2** is composed by two Évora isolates that may constitute another putative new species. The two group **A2** isolates are 100% identical in sequence. Sequence identity between isolates from the two groups is 99.7%. The sequence identity of the 16S-rDNA sequences of isolates from group **A1** to the type strains of *M. amorphae*, *M. huakuii*, *M. plurifarium* and *M. septentrionale*, the closest known species, ranged from 99.2% to 99.5%, and the sequence identity of isolates from group **A2** to the same four type strains, ranged from 99.3% to 99.6%. Since isolates in groups **A1** and **A2** form independent branches apart from the closest type strains, they could probably be assigned to new species, but further studies are required. Group **B** includes *M. tianshanense* and isolates ST-2-Setúbal (99.9%) and 93-Évora (99.8%). Group **C** contains five isolates from Beja and Elvas-ENMP, closely related to *M. mediterraneum* (99.9%) and *M. temperatum* (100%). Group **D1** has isolate 75-Elvas and the type strain of *M. loti* (99.8%). Group **D2** comprises five isolates from Beja, Elvas and Elvas-ENMP closely related to *M. ciceri* (99.7–99.8%).

The *Mesorhizobium* type strains and isolates form a highly sustained branch (100% bootstraps), which supports their monophyletic origin (Gaunt *et al.*, 2001). The two groups representing new species (**A1** and **A2**) are very close. The groups of *M. tianshanense* (**B**) and *M. mediterraneum* (**C**) cluster together. More distantly related to the latter four groups, are the groups of *M. loti* (**D1**) and *M. ciceri* (**D2**), which are very similar to each other in their sequences.

As expected, there is coherence between the 16S rDNA sequence (Figure 6) and the 16S rDNA RFLP (Figure 5) analyses: groups **A1** and **A2**, the two putative new species, are comprised by cluster A; group **B** corresponds to cluster B; group **C** matches cluster C; groups **D1** and **D2** are enclosed in cluster D.

3.3. Discussion

Clustering of chickpea rhizobia isolates with *M. ciceri* and *M. mediterraneum* was expected, but representatives of the other species, namely isolates closely related to *M. loti* and *M. tianshanense*,

have not been reported before as chickpea symbionts. This was unpredicted, since the known host plants of these rhizobia, *Lotus corniculatus* and *Glycyrrhiza pallidiflora*, respectively, are unrelated to chickpea. However, strains related to *M. amorphae* and *M. tianshanense*, able to nodulate chickpea, have been recently isolated from Spanish soils (Rivas *et al.*, *In Press.;* Rivas *et al.*, *Submitted.*), although their first described hosts are *Amorpha fruticosa* and *Glycyrrhiza pallidiflora*, respectively.

The existence of one or two new species of *Mesorhizobium* able to nodulate chickpea is suggested by 16S rDNA sequence analysis.

Phylogenetic analysis of the 16S rDNA gene revealed identical topologies with neighbour-joining (Figure 6), minimum evolution (data not shown) and maximum likelihood methods (data not shown).

It is interesting that a considerable number of distinct *Mesorhizobium* species, able to nodulate chickpea, were isolated from soils in a small region of only about 100 km2 (Beja, Elvas and Évora) (Figure 2). In these soils, no inoculant strains have been used (I. Duarte, personal communication). It is possible that the limited chickpea crop in this region (I. Duarte, personal communication), the absence of chickpea wild relatives (Talavera *et al.*, 1999) and the host-specificity of chickpea rhizobia (Gaur & Sen, 1979) might contribute to the high rhizobial diversity found in such a small region. This hypothesis is also supported by other studies that reported a decrease in rhizobia diversity associated with the presence of the host (Coutinho *et al.*, 1999).

The 16S rDNA RFLP based clustering of isolates (Figure 5) is generally correlated with their geographical origin. However, different chromosomal genotypes could be detected at a single site, as well as, an identical genotype was detected in very different geographical locations. The Évora population seems to be the most homogeneous, since most isolates are grouped within cluster A. The Elvas-ENMP isolates are found in clusters C and D. These results are not in agreement with other studies on chickpea rhizobia (Nour *et al.*, 1994b), which reported an absence of correlation between species and origin of isolates. Nevertheless Jarabo-Lorenzo and co-workers (2003) have observed similar results for bradyrhizobia.

Regarding the 16S rDNA species groups (Figure 6), significant differences were found between different origins (χ^2=32.480, df=20, P=0.042), which means that there is an association between 16S species groups and origin of individual isolates. Furthermore, a correlation is also found between 16S rDNA groups and origin, when a larger number of isolates is analysed (Alexandre *et al.*, *Manuscript in preparation.*).

Contrary to the original idea of other authors (Cadahía *et al.*, 1986; Gaur & Sen, 1979), previous studies (Maâtallah *et al.*, 2002a; Maâtallah *et al.*, 2002b; Nour *et al.*, 1994b) had already suggested that chickpea rhizobia strains are indeed an heterogeneous group, based on phenotypic characteristics, such as tolerance to pH, salt, temperature and antibiotics and carbon substrate assimilation tests and on 16S rDNA RFLP analysis.

Studies on other hosts, such as bean or soybean have also reported an unexpected diversity of strains in the same region. An extraordinarily high diversity of *S. fredii* strains able to nodulate

soybean were found in a particular region of China (Camacho *et al.*, 2002). *Phaseolus vulgaris* is nodulated by at least five rhizobial species in a Spanish soil (Herrera-Cervera *et al.*, 1999) and by *R. gallicum* and *R. giardinii* in France (Amarger *et al.*, 1997).

Moreover, in New Zealand, very diverse *Mesorhizobium* strains as well as *R. leguminosarum* were found to nodulate a range of native woody legumes (Weir *et al.*, 2004).

The present 16S rDNA RFLP analysis revealed to be a useful method for grouping new isolates, indicating their putative species and estimating genetic relationships between groups, as described previously by other authors (Laguerre *et al.*, 1994; Wang *et al.*, 1999a; Wang *et al.*, 1999b; Wang *et al.*, 1998). Indeed, the variations detected between 16S rDNA RFLP within the genus *Mesorhizobium* seem sufficient to allow species identification. The 16S rDNA RFLP analysis provides enough information for prediction of phylogenetic assignment of new isolates.

Chickpea nodulating rhizobia isolated from soils of a small region in the south of Portugal are described to show high phylogenetic diversity. Evidence is provided for one or even two putative new species of *Mesorhizobium* that are able to nodulate chickpea, as supported with 16S rDNA sequence data.

Further studies are needed in order to assess strain diversity and to confirm phylogenetic assignment of isolates within the genus *Mesorhizobium* and, in particular, of the proposed new species.

Until now, chickpea has been considered to be specific in its symbiotic relationships with rhizobia (Broughton & Perret, 1999). The finding that it can be nodulated by several species of mesorhizobia, raises the question of whether chickpea is in fact a restrictive or promiscuous host for nodulation by rhizobia Therefore, it is necessary to investigate genes involved in symbiosis, particularly nodulation genes, to further elucidate this matter.

4. DIVERSITY OF CHICKPEA RHIZOBIA POPULATIONS

This chapter is based on the following manuscripts:

Laranjo, M., Rodrigues R., Alho L. and Oliveira, S. 2001. Rhizobia of chickpea from southern Portugal: symbiotic efficiency and genetic diversity. *J. Appl. Microbiol.* 90: 662-667.

Laranjo, M., Branco, C., Soares, R., Alho, L., Carvalho, M. and Oliveira, S. 2002. Comparison of chickpea rhizobia isolates from diverse Portuguese natural populations based on symbiotic effectiveness and DNA fingerprint. *J. Appl. Microbiol.* 92: 1043-1050.

Laranjo, M., Machado, J., Young, J. P.W. and Oliveira, S. 2004. High diversity of chickpea *Mesorhizobium* species isolated in a Portuguese agricultural region. *FEMS Microbiol. Ecol.* 48: 101-107.

Alexandre, A., Laranjo, M. and Oliveira, S. 2006. Natural populations of chickpea rhizobia evaluated by antibiotic resistance profiles and molecular methods. Microb. Ecol. 51: 128-136.

4.1. Introduction

Large bacterial soil populations are hard to analyse. It is necessary to employ fast and cheap methods in an initial screening to evaluate the diversity within and between different populations.

In the pursuit of appropriate inoculant strains to use in association with host legumes, it is necessary to study the diversity of natural rhizobia populations.

Several methods have been used in rhizobia to evaluated their populations biodiversity, including phenotypic methods, such as assimilation, biochemical and enzymatic tests (de Lajudie *et al.*, 1994), IAR patterns (Abaidoo *et al.*, 2002), and mainly molecular methods. Some of the most commonly used are MLEE (Strain *et al.*, 1995), SDS-PAGE analysis of total cell proteins (de Lajudie *et al.*, 1994; Dupuy *et al.*, 1994), plasmid profile analysis (Eckhardt, 1978; Haukka *et al.*, 1998; Hynes *et al.*, 1985), RFLP of total genomic DNA (Glynn *et al.*, 1985; Laguerre *et al.*, 1992), PFGE (Haukka & Lindström, 1994), LMW RNA (Velázquez *et al.*, 1998), REP-PCR (de Bruijn, 1992; Judd *et al.*, 1993), ERIC-PCR (de Bruijn, 1992; Niemann *et al.*, 1999) and BOX-PCR (Vinuesa *et al.*, 1998) and RAPD (Dooley *et al.*, 1993; Harrison *et al.*, 1992; Richardson *et al.*, 1995; Selenska-Pobell *et al.*, 1996; van Rossum *et al.*, 1995).

In order to investigate the diversity of Portuguese chickpea rhizobia populations, 41 isolates, together with 10 reference strains, were used for phenotypic and molecular characterisation by a polyphasic approach including auxanographic tests, symbiotic effectiveness, DAPD analysis, SDS-PAGE total protein patterns and plasmid profiles.

The suitableness of the new method DAPD, after Direct Amplified Polymorphic DNA, for determining genetic relationships among isolates, was also evaluated. This new method was named by analogy to RAPD (Random Amplified Polymorphic DNA), the main differences being the use of a direct instead of a random primer, and also the high annealing temperature.

SE was determined to compare of the ability of isolates to nodulate and fix nitrogen in association with chickpea plants. This is fundamental to investigate symbiosis genes contributing to SE. Furthermore, the relationship between SE and species affiliation of native isolates was evaluated.

4.2. Results

4.2.1. Phenotypic characteristics

4.2.1.1. Morphology, Gram stain and growth characteristics

All 41isolates showed intermediate growth rates between fast- and slow-growing rhizobial strains. They are aerobic, non-spore-forming flagellated rods (data not shown).

All isolates were confirmed to be Gram-negative and not to absorb Congo red (data not shown).

According to the pH test in YMA supplemented with bromothymol blue all isolates are acid producers, except for isolate 75-Elvas, which causes no change in the medium's pH (data not shown).

These characteristics were expected, since all known effective chickpea microsymbionts belong to the genus *Mesorhizobium*, which was created to accommodate rhizobia with distinctive phenotypic characteristics that include an intermediate growth rate and acid production (Jarvis *et al.*, 1997).

4.2.1.2. Auxanographic tests

Although the API20NE strips (bioMérieux, France) have not been conceived for the identification of rhizobia, these tests were performed with the purpose of analysing strain diversity and ensuring that all 41 isolates were different. The obtained results were consistent and reproducible.

Figure 7 shows some API20NE results obtained with three isolates.

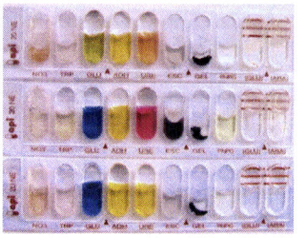

Figure 7. Selection of API20NE strips of isolates ST-2-Setúbal, 77-Elvas and EE-29-ENMP.

Table 15 shows the results obtained with the 41 isolates.

Table 15. (on opposite page) API 20NE results of the 41 isolates. NO_3-nitrates; TRP-tryptophan; GLU-glucose acidification; ADH-arginine; URE-urea; ESC-esculin; GEL-gelatin; PNG-β-galactosidase; GLU-glucose assimilation; ARA-arabinose; MNE-mannose; MAN-mannitol; NAG-N-acetyl-glucosamine; MAL-maltose; GNT-gluconate; CAP-caprate; ADI-adipate; MLT-malate; CIT-citrate; PAC-phenyl-acetate; OX-cytochrome oxidase.

Table 14. (see legend on the opposite page).

ISOLATES	NO_3	TRP	GLU	ADH	URE	ESC	GEL	PNG	GLU	ARA	MNE	MAN	NAG	MAL	GNT	CAP	ADI	MLT	CIT	PAC	OX
4a.-Beja	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6b.-Beja	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
7a.-Beja	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	-	-	+	-	-	-
9-Beja	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21-Beja	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
27-Beja	-	-	-	-	-	-	-	-	+	+	+	+	-	+	-	-	-	-	-	-	-
29-Beja	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31-Beja	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
54b.-Beja	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
64b.-Beja	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
75-Elvas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
77-Elvas	-	-	+	-	+	+	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-
78-Elvas	-	-	-	-	+	+	-	+	+	+	-	+	+	-	-	-	-	-	-	-	+
79-Elvas	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	+	-	+
83-Elvas	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
85-Elvas	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
CV-1-Elvas	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+
CV-11-Elvas	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-
CV-16-Elvas	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
CV-18-Elvas	-	-	-	-	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	+
EE-2-ENMP	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
EE-3-ENMP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
EE-7-ENMP	-	-	-	-	+	-	-	-	+	+	+	+	+	+	+	-	-	+	-	-	-
EE-9-ENMP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EE-11-ENMP	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EE-12-ENMP	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
EE-13-ENMP	-	-	+	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-
EE-14-ENMP	-	-	+	-	+	+	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-
EE-20-ENMP	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-
EE-29-ENMP	-	-	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
87-Évora	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	+	-	-	+
89a.-Évora	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-
90-Évora	-	-	-	-	+	+	-	+	+	+	+	+	+	+	-	-	-	-	-	-	-
92-Évora	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
93-Évora	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+
94-Évora	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
96-Évora	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	+
98-Évora	-	-	-	-	+	+	-	+	+	+	+	-	+	+	-	-	-	-	-	-	+
101-Évora	-	-	-	-	+	-	-	+	+	+	+	+	+	+	-	-	-	+	-	-	-
102-Évora	-	-	-	-	-	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
ST-2-Setúbal	-	-	-	-	+	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-

Taken together, the results obtained with the API 20NE galleries revealed strain heterogeneity at the biochemical level among mesorhizobia able to nodulate chickpea.

All isolates were negative for tryptophan, arginine, caprate, adipate and phenyl-acetate.

Six tests (nitrates, glucose, esculin, gelatin, gluconate and citrate) showed little differentiation ability between isolates, and the remaining ten tests showed a higher discriminating power.

The auxanographic tests yielded isolate-specific profiles. The dendrogram based on these profiles (data not shown) shows two main groups, which are not consistent with 16S rDNA RFLP dendrogram (Figure 5) nor with the 16S rDNA phylogeny (Figure 6).

The lack of coherence between 16S rDNA based phylogeny and API 20NE tests was expected.

Most enzymes involved in the metabolic activities searched for in these tests, are not encoded by housekeeping genes. Some of these genes are not present in the rhizobia sequenced genomes (e.g. arginine dihydrolase), and others are located either in the chromosome (e. g. β-glucosidase), in its symbiosis island or even in plasmids (e. g. cytochrome oxidase). Besides, even if genes exist, they may not be functional or might not be expressed when cells are grown in rich laboratory media.

Thus, one major reason for the discrepancy observed between 16S rDNA based phylogeny and auxanographic tests might be the loss and gain of plasmids, leading to inconsistencies in metabolic traits of strains, since the presence or absence of most of these enzymes is independent from the core genes chromosomal background, which tends to reflect bacterial phylogeny.

Due to the absence of correspondence with 16S rDNA based phylogeny, the API 20NE profiles may not be a useful technique in rhizobia taxonomy. It might, however, be useful for strain differentiation by means of their metabolic profiles, since the patterns are consistent.

There is no correlation between API based clustering and origin, which was expected, since exchange of genetic material is frequent among and between populations, above all when genes are plasmid encoded.

4.2.1.3. Symbiotic effectiveness

The SE was determined for the subgroup of 21 isolates, for which the 16S rDNA had been previously sequenced (chapter 3).

Figure 8 is a partial view of a SE trial in the growth chamber.

Figure 8.SE trial with chickpea inoculated plants in the growth chamber.

Table 16 summarises the shoot dry weight (SDW), root dry weight (RDW), number of nodules, nodules dry weight (NDW) and symbiotic effectiveness (SE) of the 21 isolates.

Table 16. SDW, RDW, number of nodules, NDW and SE ± the standard deviation of the 21 isolates and control plants. Letters denote significantly different SE values according to the Duncan's multiple range test.

ISOLATES	SDW (g)	RDW (g)	NUMBER OF NODULES	NDW (g)	SE (%)
N-free control	0.451 ± 0.053	0.761 ± 0.076	-	-	-
N-fed control	2.706 ± 0.423	1.646 ± 0.118	-	-	-
6b.-Beja	2.168 ± 0.199	0.956 ± 0.096	51 ± 10	0.305 ± 0.027	76 ef
7a.-Beja	1.321 ± 0.622	0.604 ± 0.174	149 ± 67	0.157 ± 0.044	39 abcdef
27-Beja	1.386 ± 0.434	0.746 ± 0.309	115 ± 38	0.266 ± 0.047	41 abcdef
29-Beja	2.060 ± 0.190	0.817 ± 0.127	53 ± 19	0.304 ± 0.099	71 def
64b.-Beja	2.024 ± 0.201	0.857 ± 0.262	77 ± 26	0.284 ± 0.083	70 def
75-Elvas	1.248 ± 0.804	0.719 ± 0.187	136 ± 75	0.179 ± 0.067	35 abcde
78-Elvas	1.870 ± 0.461	0.755 ± 0.043	102 ± 39	0.216 ± 0.026	63 cdef
85-Elvas	1.811 ± 0.790	0.743 ± 0.417	65 ± 23	0.222 ± 0.084	60 cdef
CV-16-Elvas	1.393 ± 0.899	0.820 ± 0.304	99 ± 52	0.208 ± 0.110	42 abcdef
CV-18-Elvas	2.463 ± 0.189	0.993 ± 0.138	121 ± 7	0.285 ± 0.076	72 def
EE-2-ENMP	1.256 ± 0.977	0.549 ± 0.274	46 ± 17	0.241 ± 0.084	36 abcde
EE-7-ENMP	2.339 ± 0.074	0.971 ± 0.045	123 ± 22	0.324 ± 0.053	84 f
EE-12-ENMP	0.689 ± 0.373	0.712 ± 0.056	45 ± 44	0.077 ± 0.048	10 ab
EE-14-ENMP	1.176 ± 0.170	0.557 ± 0.095	56 ± 26	0.234 ± 0.052	32 abcde
EE-29-ENMP	0.937 ± 0.454	0.560 ± 0.144	44 ± 10	0.273 ± 0.075	21 abc
90-Évora	1.831 ± 0.309	0.960 ± 0.112	116 ± 21	0.266 ± 0.004	49 abcdef
93-Évora	1.067 ± 0.663	0.569 ± 0.092	79 ± 14	0.138 ± 0.029	27 abcd
94-Evora	1.207 ± 0.934	0.654 ± 0.138	134 ± 31	0.203 ± 0.058	33 abcde
98-Évora	2.076 ± 0.039	0.805 ± 0.074	88 ± 10	0.245 ± 0.017	72 def
102-Évora	1.678 ± 0.231	0.790 ± 0.081	93 ± 14	0.250 ± 0.101	54 bcdef
ST-2-Setúbal	0.550 ± 0.347	0.486 ± 0.267	83 ± 27	0.144 ± 0.092	4 a
UPM-Ca7^T	1.368 ± 0.453	0.769 ± 0.140	95 ± 4	0.170 ± 0.033	41 abcdef
UPM-Ca36^T	1.327 ± 0.554	0.583 ± 0.065	58 ± 26	0.319 ± 0.118	39 abcdef

The symbiotic effectiveness of the different isolates varied from 4 to 84% (Table 16). Nine isolates (6b.-Beja, 29-Beja, 64b.-Beja, 78-Elvas, 85-Elvas, CV-18-Elvas, EE-7-ENMP, 98-Évora and 102-Évora) showed a high SE (above 50%) and, among these, six isolates (6b.-Beja, 29-Beja, 64b.-Beja, CV-18-Elvas, EE-7-ENMP and 98-Évora) were found to establish very effective symbiosis (SE above 70%) with the host chickpea.

The SE values obtained with the two chickpea rhizobia type strains, *M. ciceri*-UPM-Ca7^T and *M. mediterraneum*-UPM-Ca36^T (Table 16), are similar to the mean of all isolates of the corresponding species group based on 16S rDNA phylogeny (Figure 6), which is 43% and 47%, respectively.

SE was found to be positively correlated with RDW ($r=0.771$, $P<0.01$) and NDW ($r=0.720$, $P<0.01$), among the 21 Portuguese isolates.

No correlation was depicted between SE and origin of individual isolates ($P>0.05$). However, Beja isolates show the highest mean SE (59%), and Elvas-ENMP isolates have the lowest mean SE (30%), despite including the isolate with the highest efficiency (EE-7-ENMP).

Although there is no significant correlation of SE with species, it is clear that the six members of the putative new species, corresponding to group **A1** in the 16S rDNA based phylogeny (Figure 6), are far more efficient than isolates from the other species groups. Isolates from the putative new species show a 65% average SE, and not only is this the only mean SE above 50%, but also each of the six isolates from this group show a SE above 50%.

4.2.2. Molecular features

4.2.2.1. DAPD profiles

DAPD analysis was used for strain differentiation and to evaluate the genetic diversity of the different populations.

DAPD fingerprinting is a recently described PCR-based method, developed in the Soil Microbiology Laboratory-ICAM, which uses a single non-arbitrary direct primer (RPO1) with homology with the *nif*HDK promoter consensus sequence (Richardson *et al.*, 1995).

All 41 isolates together with reference and type strains were analysed under standardised conditions. Figure 9 and Figure 10 show the DAPD profiles of 40 isolates from Beja, Elvas, Elvas-ENMP and Évora.

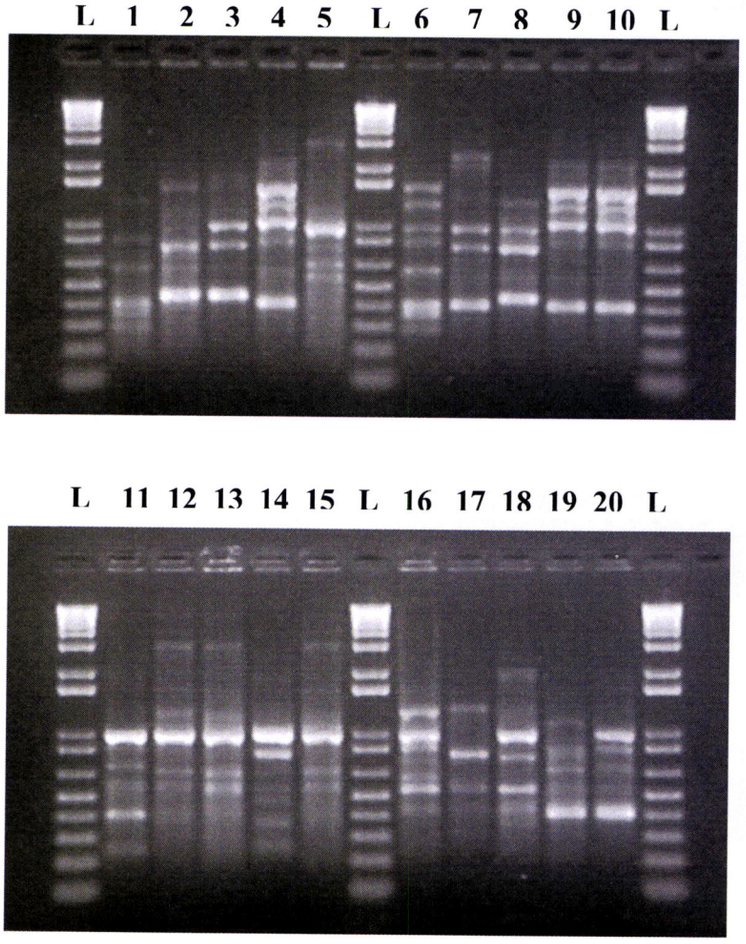

Figure 9.DAPD profiles of the isolates from the populations of Beja and Elvas-ENMP. L-1 Kb DNA Plus Ladder (Invitrogen), lane 1-6b.-Beja, lane 2-7a.-Beja, lane 3-27-Beja, lane 4-29-Beja, lane 5-64b.-Beja, lane 6-EE-2-ENMP, lane 7-EE-7-ENMP, lane 8-EE-12-ENMP, lane 9-EE-14-ENMP, lane 10-EE-29-ENMP, lane 11-4a.-Beja, lane 12-9-Beja, lane 13-21-Beja, lane 14-31-Beja, lane 15-54b.-Beja, lane 16-EE-3-ENMP, lane 17-EE-9-ENMP, lane 18-EE-11-ENMP, lane 19-EE-13-ENMP, lane 20-EE-20-ENMP.

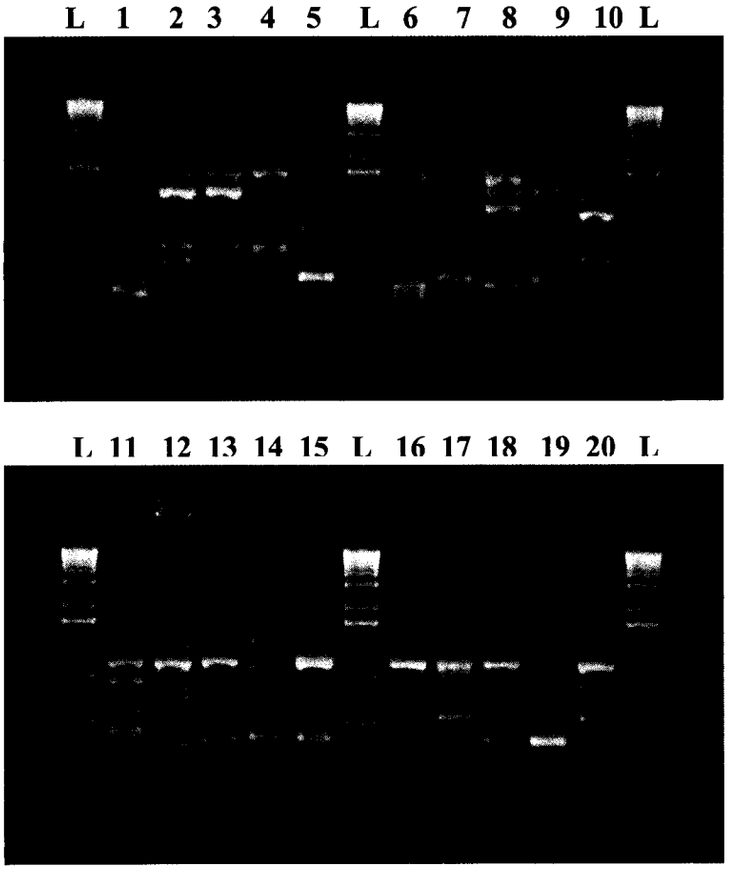

Figure 10. DAPD profiles of the isolates from the populations of Elvas and Évora. L-1 Kb DNA Plus Ladder (Invitrogen), lane 1-75-Elvas, lane 2-78-Elvas, lane 3-85-Elvas, lane 4-CV-16-Elvas, lane 5-CV-18-Elvas, lane 6-90-Évora, lane 7-93-Évora, lane 8-94-Évora, lane 9-98-Évora, lane 10-102-Évora, lane 11-77-Elvas, lane 12-79-Elvas, lane 13-83-Elvas, lane 14-CV-1-Elvas, lane 15-CV-11-Elvas, lane 16-87-Évora, lane 17-89a.-Évora, lane 18-92-Évora, lane 19-96-Évora, lane 20-101-Évora.

Most amplification products were between 250 bp and 3 kb in length and the number of fragments obtained varied between two and ten.

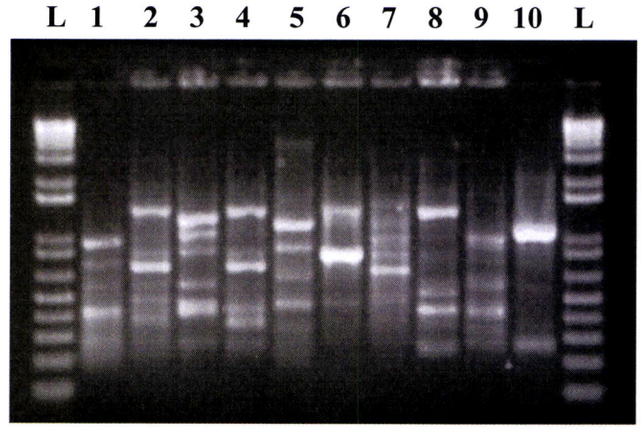

Figure 11. DAPD profiles of the isolate from Setúbal and the rhizobia reference and type strains. L-1 Kb DNA Plus Ladder (Invitrogen), lane 1-*S. meliloti*-1021, lane 2-*R. etli*-CFN 42^T, lane 3-ST-2-Setúbal, lane 4-*M. amorphae*-ACCC 19665^T, lane 5-*M. chacoense*-LMG 19008^T, lane 6-*M. ciceri*-UPM-Ca7^T, lane 7-*M. huakuii*-CCBAU 2609^T, lane 8-*M. mediterraneum*-UPM-Ca36^T, lane 9-*M. plurifarium*-ORS 1032^T, lane 10-*M. tianshanense*-A-1BS^T, L-1 Kb DNA Plus Ladder (Invitrogen).

Most isolates exhibited unique and distinct profiles. However, some isolates showed similar patterns: 29-Beja, EE-14-ENMP and EE-29-ENMP; EE-2-ENMP and EE-3-ENMP; EE-9-ENMP and EE-13-ENMP; 77-Elvas and 89a.-Évora; 64b.-Beja, 78-Elvas, 83-Elvas, 85-Elvas and CV-18-Elvas. This had also been reported before for RAPD analysis of rhizobia (Harrison *et al.*, 1992; Richardson *et al.*, 1995).

DAPD patterns were usually reproducible. The reproducibility of our DAPD profiles, in contrast to RAPD analysis (Selenska-Pobell *et al.*, 1995), probably relies on the use of stringent amplification conditions, as recommended by Stackebrandt and co-workers (Stackebrandt *et al.*, 2002) for validated methods of DNA profiling. The DAPD amplification profiles were less susceptible to random errors and less dependent on the cultural state of the bacteria, when compared to arbitrarily amplified DNA techniques (Kay *et al.*, 1994; Schierwater, 1995; Selenska-Pobell *et al.*, 1995).

The reproducibility of DAPD PCR is probably due to the use of a sequence specific 20-nucleotide primer and a high annealing temperature. The *nif*-directed RPO1 primer includes a promoter consensus sequence highly conserved and reiterated in rhizobial genomes (Schofield & Watson, 1985). Analysis of the genome database for *Mesorhizobium loti* (http://www.kazusa.or.jp/rhizobase/search.html) revealed more than 60 regions with similarity to the RPO1 primer sequence.

DAPD patterns obtained with two different annealing temperatures, 55ºC and 60ºC, were similar (data not shown), contrary to results from other studies concerning the effect of annealing temperature on RAPD patterns (Richardson *et al.*, 1995).

The dendrogram based on DAPD analysis (Figure 12) shows that the clustering of isolates is in good agreement with the clustering revealed by 16S rDNA RFLP dendrogram (Figure 5), as the same four clusters are obtained. Only isolates 75-Elvas and 77-Elvas group within cluster A, far apart from its 16S rDNA RFLP cluster (D) (Figure 12).

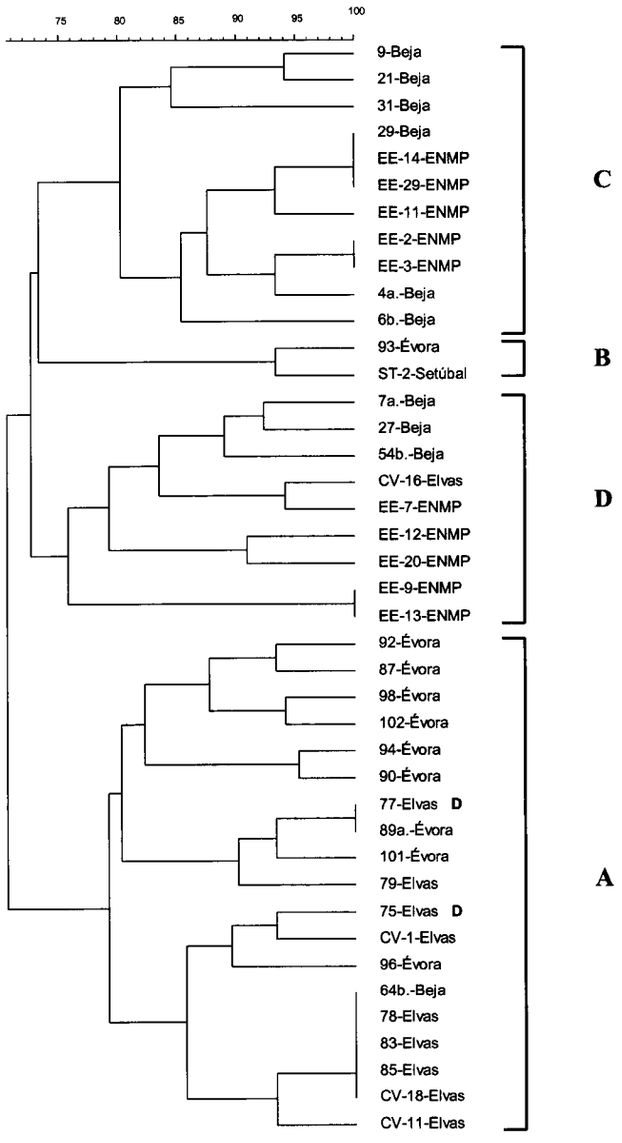

Figure 12. UPGMA dendrogram based on the analysis of DAPD profiles from the 41 isolates. Letters A, B, C and D denote 16S rDNA RFLP clusters (Figure 5).

Isolate clustering based on DAPD profiles of the 21 isolates (Figure 13) correlates with the 16S rDNA based phylogeny (Figure 6). For instance, the *M. mediterraneum* (**C**) and *M. tianshanense* (**B**) groups, as well as groups **D1** (*M. loti*) and **D2** (*M. ciceri*), are mostly conserved. The grouping of all isolates from groups **A1** and **A2** separately from any type strain within the genus *Mesorhizobium* is confirmed, supporting their assignment to one or two new species. Since the DAPD dendrogram generally reflects 16S rDNA-based phylogeny, it seems that this DNA profiling method may be a useful tool to fingerprint rhizobial genomes and to predict the phylogenetic position of isolates.

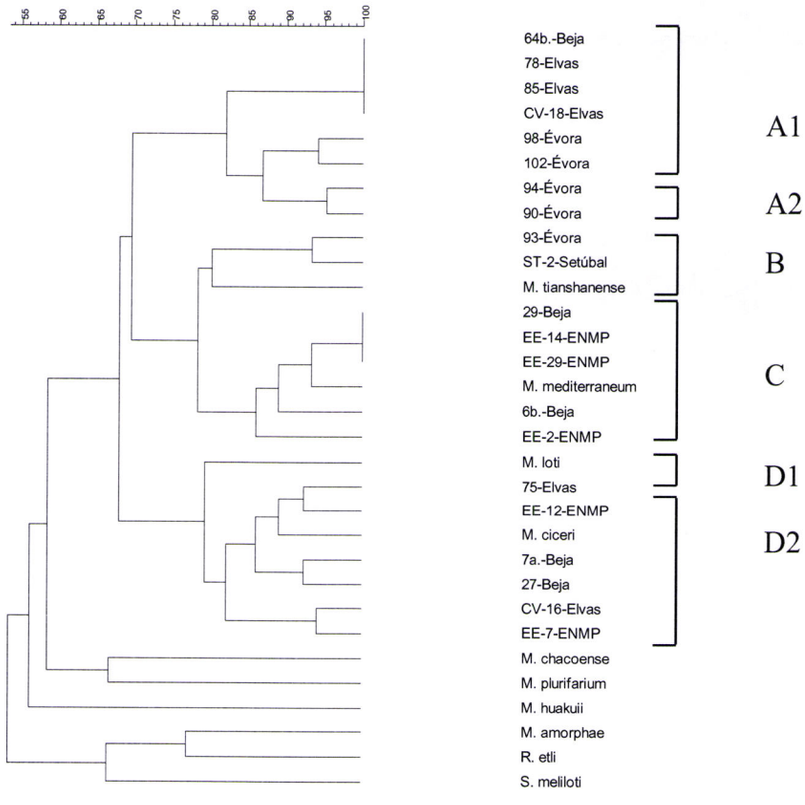

Figure 13. UPGMA dendrogram based on the analysis of DAPD profiles from the 21 isolates together with the type and reference strains. Letters A1, A2, B, C, D1 and D2 denote 16S rDNA species groups (Figure 6).

Furthermore, some species groups are clustered together in the same way as in the 16S rDNA based phylogeny (Figure 6) , which emphasise their phylogenetic proximity: the two new species groups (**A1** and **A2**); the *M. tianshanense* (**B**) and *M. mediterraneum* (**C**) groups; and the groups of *M. loti* (**D1**) and *M. ciceri* (**D2**).

4.2.2.2. Total protein profiles

All isolates were fingerprinted for their protein profiles. These were reproducible, and many isolates were observed to share common patterns.

Figure 14 to Figure 19 show the total protein profiles of the 40 isolates from Beja, Elvas, Elvas-ENMP and Évora.

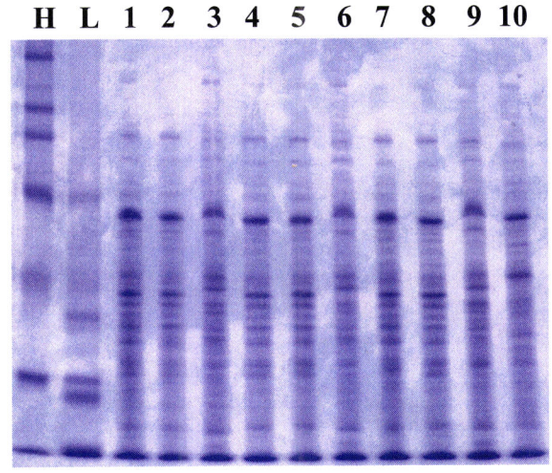

Figure 14. SDS-PAGE total protein profiles of the isolates of the Beja population. H-High Molecular Weight Marker (Sigma), L-Low Molecular Weight Marker (Sigma); lane 1-4a.-Beja, lane 2-6b.-Beja, lane 3-7a.-Beja, lane 4-9-Beja, lane 5-21-Beja, lane 6-27-Beja, lane 7-29-Beja, lane 8-31-Beja, lane 9-54b.-Beja, lane 10-64b.-Beja.

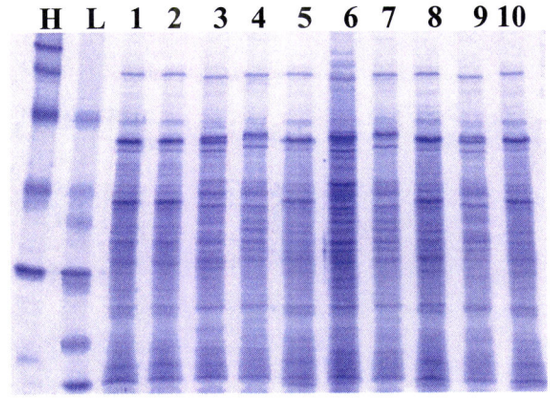

Figure 15. SDS-PAGE total protein profiles of the isolates of the Elvas-ENMP population. H-High Molecular Weight Marker (Sigma), L-Low Molecular Weight Marker (Sigma); lane 1-EE-2-ENMP, lane 2-EE-3-ENMP, lane 3-EE-7-ENMP, lane 4-EE-9-ENMP, lane 5-EE-11-ENMP, lane 6-EE-12-ENMP, lane 7-EE-13-ENMP, lane 8-EE-14-ENMP, lane 9-EE-20-ENMP, lane 10-EE-29-ENMP.

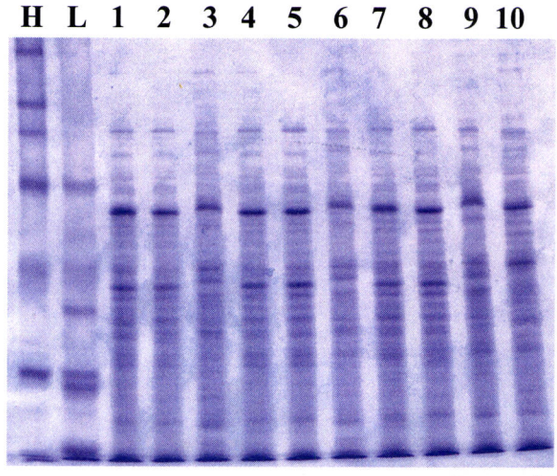

Figure 16. SDS-PAGE total protein profiles of the isolates of the Elvas population. H-High Molecular Weight Marker (Sigma), L-Low Molecular Weight Marker (Sigma); lane 1-75-Elvas, lane 2-77-Elvas, lane 3-78-Elvas, lane 4-79-Elvas, lane 5-83-Elvas, lane 6-85-Elvas, lane CV-1-Elvas, lane 8-CV-11-Elvas, lane 9-CV-16-Elvas, lane 10-CV-18-Elvas.

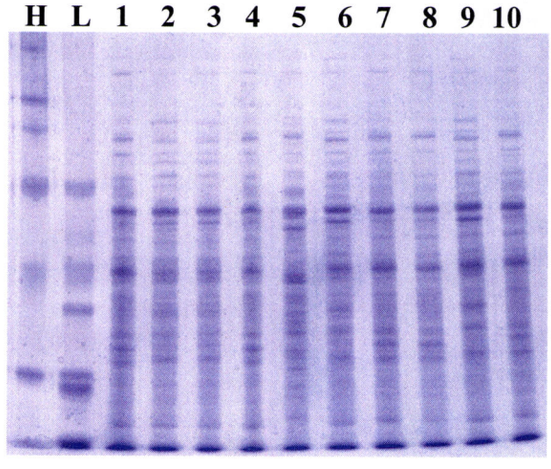

Figure 17. SDS-PAGE total protein profiles of the isolates of the Évora population. H-High Molecular Weight Marker (Sigma), L-Low Molecular Weight Marker (Sigma); lane 1-87-Évora, lane 2-89a.-Évora, lane 3-90-Évora, lane 4-92-Évora, lane 5-93-Évora, lane 6-94-Évora, lane 96-Évora, lane 8-98-Évora, lane 9-101-Évora, lane 10-102-Évora.

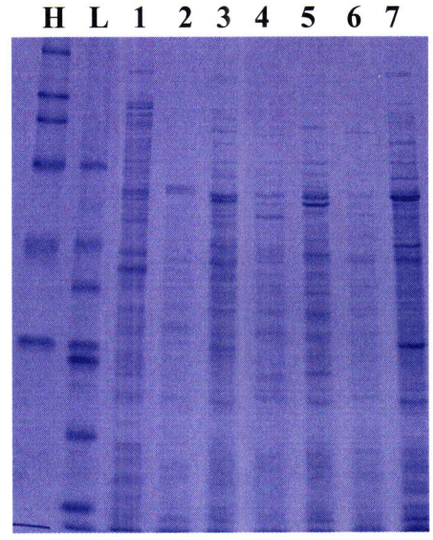

Figure 18. SDS-PAGE total protein profiles of *Mesorhizobium* type strains. H-High Molecular Weight Marker (Sigma), L-Low Molecular Weight Marker (Sigma); lane 1-*M. amorphae* ACCC 19665^T, lane 2-*M. chacoense* LMG 19008^T, lane 3-*M. ciceri*-UPM-Ca7^T, lane 4-*M. huakuii*-CCBAU 2609^T, lane 5-*M. mediterraneum*-UPM-Ca36^T, lane 6-*M. plurifarium*-ORS 1032^T, lane 7-*M. tianshanense*-A-1BS^T.

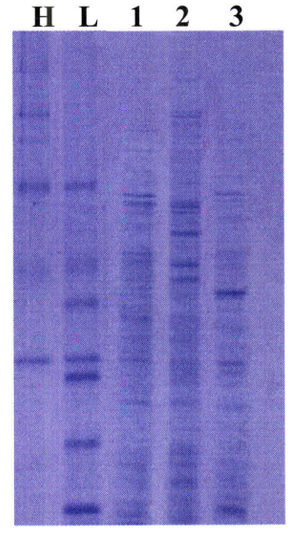

Figure 19. SDS-PAGE total protein profiles of the isolate from Setúbal and two rhizobia reference and type strains. H-High Molecular Weight Marker (Sigma), L-Low Molecular Weight Marker (Sigma); lane 1-ST-2-Setúbal, lane 2-*R. etli* CFN 42^T, lane 3-*S. meliloti*-1021.

Clustering of isolates based on total protein profiles (Figure 20) globally agrees with the 16S rDNA RFLP dendrogram, as shown in Figure 5. Nevertheless, isolates 78-Elvas and 85-Elvas showed SDS–PAGE patterns identical to CV-16-Elvas, and are included in cluster D, instead of cluster A from 16S

rDNA RFLP dendrogram. Similarly, isolates 79-Elvas, 83-Elvas, CV-1-Elvas, and CV-11-Elvas showed protein profiles identical to isolates from group D instead of group A.

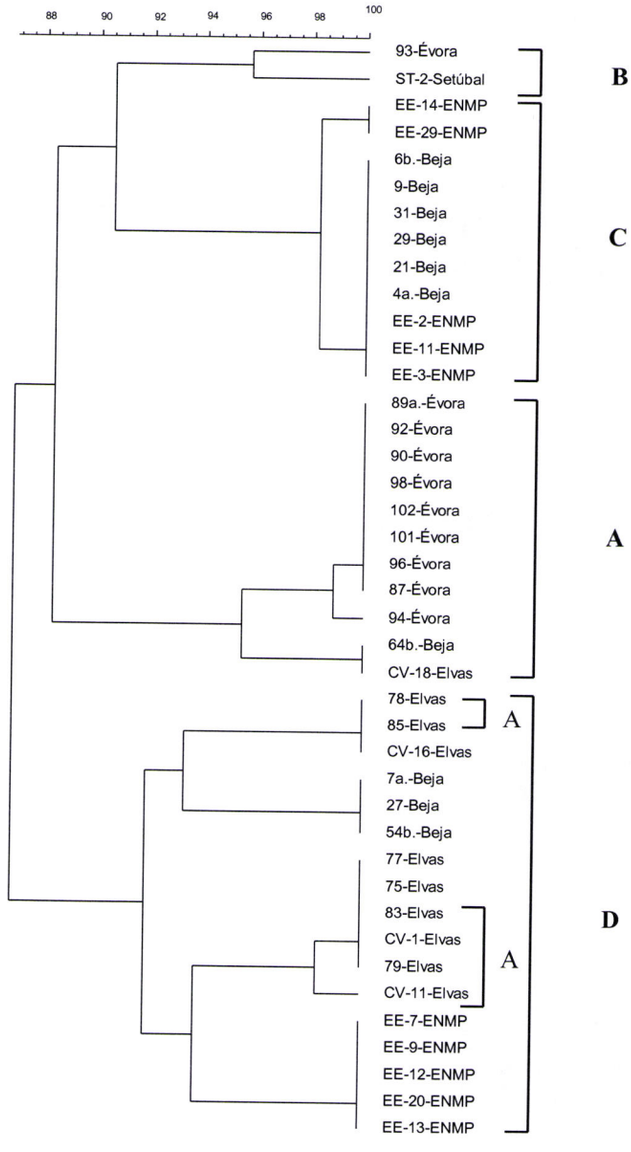

Figure 20. UPGMA dendrogram obtained from the analysis of total protein profiles of the 41 isolates. Letters A, B, C and D denote 16S rDNA RFLP clusters (Figure 5).

There is a correlation between SDS-PAGE total protein profiles and origin of isolates (χ^2=143.500, df=44, P<0.001). For example, all Évora isolates are in the same cluster, with the exception of isolate 93-Évora (Figure 20).

Protein fingerprinting revealed clustering of the 21 isolates (Figure 21) generally according to 16S rDNA sequence phylogeny (Figure 6).

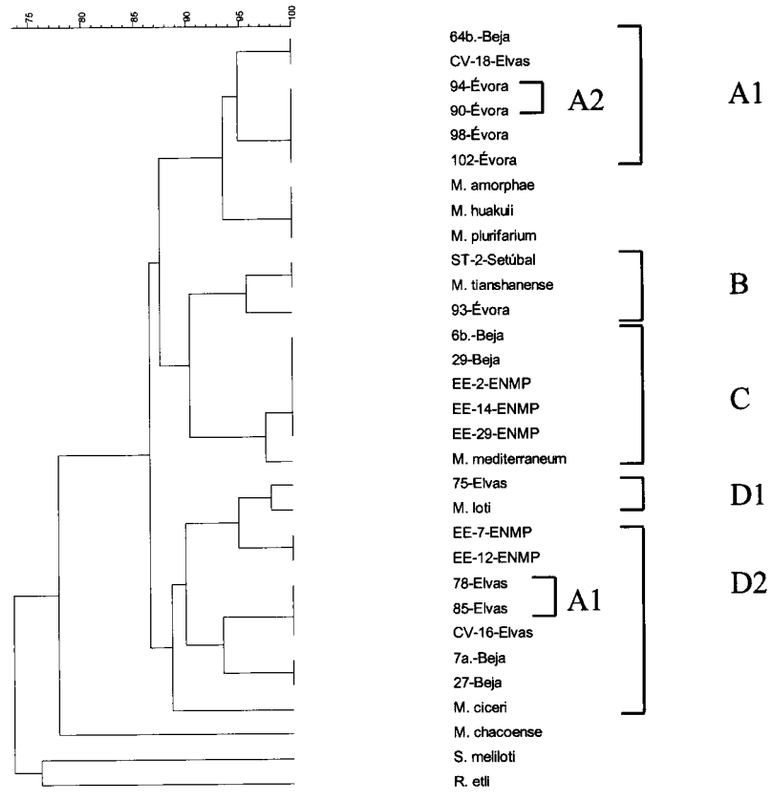

Figure 21. UPGMA dendrogram obtained from the analysis of total protein profiles of the 21 isolates together with the type strains. Letters A1, A2, B, C, D1 and D2 denote 16S rDNA species groups (Figure 6).

Isolates with identical SDS–PAGE protein patterns generally belong to the same 16S-rDNA cluster with one exception: isolates 78-Elvas and 85-Elvas (group **A1**) had similar whole-cell protein patterns to CV-16-Elvas (group **D2**). We could tentatively suggest that these isolates represent recombinants. In general, though, SDS–PAGE analysis of total proteins is a useful method to identify very close genetic relationships and has been used for differentiation and identification of new species (de

Lajudie *et al.*, 1994; Nick *et al.*, 1999a; Velázquez *et al.*, 2001). In our case, SDS–PAGE total protein fingerprinting supports the new putative species suggested by 16S rDNA sequence and DAPD analysis, because groups **A1** and **A2** isolates (except for 78-Elvas and 85-Elvas) are grouped together and apart from the closest type strains.

4.2.2.3. Plasmid profiles

Plasmid profiles were determined by horizontal agarose gel electrophoresis using an in-well lysis method. Examples of plasmid profile gels are shown in Figure 22.

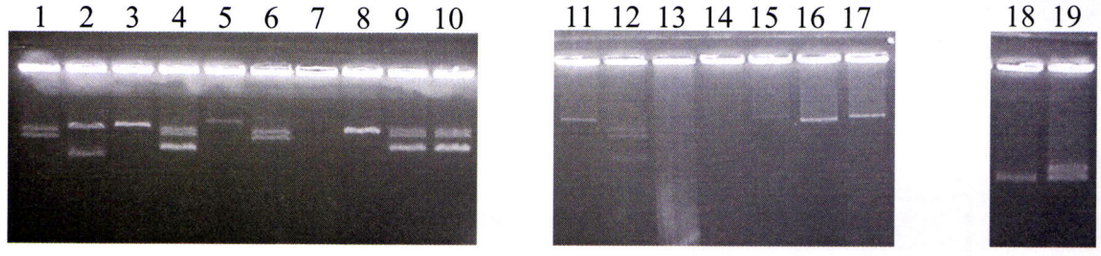

Figure 22. Typical plasmid profiles of isolates from the four populations (Beja, ENMP, Elvas and Évora). Lane 1-6b.-Beja, lane 2-7a.-Beja, lane 3-27-Beja, lane 4-29-Beja, lane 5-64b.-Beja, lane 6-EE-2-ENMP, lane 7.EE-7-ENMP, lane 8-EE-12-ENMP, lane 9-EE-14-ENMP, lane 10-EE-29-ENMP; lane 11-UPM-Ca7^T (*M. ciceri*) (~370 kb) (Cadahía *et al.*, 1986), lane 12- UPM-Ca36^T (*M. mediterraneum*) (245, 195 and 75 kb) (Cadahía *et al.*, 1986), lane 13-75-Elvas, lane 14-78-Elvas, lane 15-85-Elvas, lane 16-CV-16-Elvas, lane 17-CV-18-Elvas; lane 18-94-Évora, lane 19-101-Évora.

Plasmid profiles were determined for all 41 isolates and the obtained profiles were consistent and reproducible. However, for some isolates plasmids were never detected. This apparent lack of plasmids could mean that either the isolates do not carry plasmids or that the technique used was not able to detect very high molecular weight plasmids. The absence of discernible plasmids has already been described for chickpea nodulating rhizobia (Cadahía *et al.*, 1986)

Plasmid number of isolates ranged from zero to six (Table 17). Plasmids with a molecular weight ranging from approximately 15 kb to higher than 500 kb were detected (Table 17). In most isolates, one or two large plasmids were detected.

Although similar plasmid profile features were detected among isolates of the same geographical area, no association was found between plasmid number and isolates origin (P=0.127). Among Elvas isolates, the variation was low and most of them showed a single plasmid. Among Beja isolates, the

variation was high as isolates with one to six plasmids were detected. In most Évora isolates, no plasmids or a single plasmid were detected.

No association was found between plasmid number and species group (P=0.278). Groups **A1** and **A2**, the putative new species, contain isolates with zero or one plasmid. The *M. tianshanense* group (**B**) only includes isolates with no plasmids. The *M. mediterraneum* group (**C**) encloses isolates with two to six plasmids. Isolate 75-Elvas from group **D1**, shows one plasmid. Group **D2** comprises isolates close to *M. ciceri* with none to three plasmids.

Table 17. Plasmid number and approximate molecular weights of the 41 isolates. n.d.-not detectable; n.dt.-not determined.

ISOLATES	PLASMID NUMBER	PLASMID MOLECULAR WEIGHT (Kbp)
4a.-Beja	3	150 / 200 / 230
6b.-Beja	2	190 / 240
7a.-Beja	3	160 / 250
9-Beja	3	70 / 220 / 310
21-Beja	3	170 / 210 / 310
27-Beja	1	310
29-Beja	6	15 / 20 / 140 / 145 / 210 / 260
31-Beja	2	200 / 300
54b.-Beja	1	215
64b.-Beja	1	400
75-Elvas	1	210
77-Elvas	1	400
78-Elvas	1	400
79-Elvas	1	400
83-Elvas	1	400
85-Elvas	1	400
CV-1-Elvas	n.d.	-
CV-11-Elvas	n.d.	-
CV-16-Elvas	1	320
CV-18-Elvas	1	400
EE-2-ENMP	2	190 / 240
EE-3-ENMP	3	n.dt.
EE-7-ENMP	n.d.	-
EE-9-ENMP	1	n.dt.
EE-11-ENMP	4	n.dt.
EE-12-ENMP	2	270
EE-13-ENMP	1	n.dt.
EE-14-ENMP	4	140 / 145 / 210 / 260
EE-20-ENMP	1	n.dt.
EE-29-ENMP	4	140 / 145 / 210 / 260
87-Évora	1	> 500
89a.-Évora	4	> 500
90-Évora	1	90
92-Évora	2	> 500
93-Évora	n.d.	-
94-Évora	1	200
96-Évora	2	> 500
98-Évora	2	> 500
101-Évora	2	280
102-Évora	n.d.	-
ST-2-Setúbal	n.d.	-

4.3. Discussion

The study of rhizobial diversity has been achieved using several phenotypic and molecular approaches. Some of the most commonly used differentiation methods, which have allowed the establishment of genetic relationships, are random amplified polymorphic DNA (RAPD) (Harrison *et al.*, 1992; Richardson *et al.*, 1995), total protein profiles (de Lajudie *et al.*, 1994; Nick *et al.*, 1999a; Velázquez *et al.*, 2001) and assimilation and biochemical tests (de Lajudie *et al.*, 1994). Studies on soybean and bean rhizobia have revealed incongruence between clustering based on phenotypic and molecular data (Abaidoo *et al.*, 2002; Vásquez-Arroyo *et al.*, 1998).

Dendrograms generated with DAPD and total protein profiles are consistent with the 16S rDNA sequence analysis (Figure 6). The same six species groups were identified, with a few exceptions mentioned in section 4.2. In particular, the putative new species group is supported.

The present study suggests that DAPD analysis can be used for rhizobial strain differentiation and as a first approach in rhizobial phylogeny studies. It is a global fingerprinting technique as the sequence of the RPO1 primer is reiterated in rhizobial genomes, including plasmids and chromosomes. Indeed, the analysis of *Mesorhizobium loti* strain MAFF 303099 genome revealed more than 60 regions with homology to RPO1 primer sequence. Thus, DAPD fingerprinting is shown as a reliable and fast method for assessing biodiversity and genetic relatedness among rhizobia.

The distribution of isolates within each 16S rDNA cluster (Figure 5) can be analysed in terms of SE. Thus, cluster A contains symbiotically effective isolates; cluster B encloses ineffective isolates; cluster C includes isolates showing low SE; and cluster D isolates show high SE.

However, no significant correlation was detected between SE and species, contrary to the findings of Abaidoo *et al.* (2000), regarding the symbiosis *Bradyrhizobium*-soybean. Still, it is clear that members of the putative new species corresponding to group **A1**, as described in 3.2.2.2, are far more efficient than isolates from the other species groups.

Although no correlation was found between SE and soil pH, isolates from the Beja soil, the most alkaline one (pH=7.9), show the highest mean SE (59%). Isolates originally from alkaline soils have been reported to be more effective (Ibekwe *et al.*, 1997).

Despite plasmid number was not found to be correlated with both isolates origin and species group, some isolates from the same species group or the same origin seem to have common plasmids taking into account their identical molecular weight. For example, isolates 29-Beja, EE-14-ENMP and EE-29-ENMP, from the *M. mediterraneum* group (**C**), show four identical plasmids; and isolates 85-Elvas and CV-18-Elvas, from the new species group **A1**, have one identical plasmid. It is likely that at least some of the plasmids are transmissible and have moved between strains of different chromosomal background. Plasmids in rhizobia are commonly mobile, and in *Mesorhizobium* there is also direct evidence for transfer of large chromosomal islands (Sullivan & Ronson, 1998), so that the variation in mobile genes, such as those responsible for nodulation, may not be concordant with that in

chromosomal genes such as rDNA (Qian & Parker, 2002). Thus, isolates with similar host-range genes may be taxonomically diverse. Indeed, the *Lotus* nodulating strain MAFF 303099, for which the complete genome sequence has been determined (Kaneko *et al.*, 2000a), has nodulation genes very similar to those of the type strain of *M. loti*, but its chromosomal genes place it much closer to *M. huakuii* (Turner *et al.*, 2002).

In the present study, chickpea rhizobia diversity was assessed using total protein and plasmid profiles, and auxanographic tests, as well as the recently developed DAPD analysis.

The obtained results indicate the existence of different levels of heterogeneity within natural chickpea rhizobia populations, confirming previous studies on chickpea rhizobia from Spain and Morocco (Maâtallah *et al.*, 2002b; Nour *et al.*, 1994b).

Biochemical tests showed a high heterogeneity among rhizobia isolates able to nodulate chickpea refuting the original idea that chickpea-infective strains should be a very homogeneous group (Cadahía *et al.*, 1986; Gaur & Sen, 1979).

The results obtained with DAPD analysis and whole-cell protein fingerprinting analysis provide further evidence of higher genetic diversity within the population of Beja, since Beja isolates were found to be dispersed through clusters A, C and D. This had already been suggested by the 16S-rDNA RFLP and sequence analysis (chapter 3). The obtained plasmid profiles also suggest genetic differences among rhizobial populations of the different soil sites.

The distinct levels of genetic diversity of the indigenous populations may be correlated with the soil pH (Table 11), which is more acidic in Évora and more alkaline in Beja.

The genetic diversity of *Rhizobium* populations was described as low in acid soils (such as Évora) and high among isolates from more neutral soils (such as Elvas-ENMP or Beja) (Harrison *et al.*, 1989). Plasmid profiles of Elvas-ENMP and Beja isolates suggest a higher genetic variation and/or a higher frequency of plasmid transfer phenomena among isolates. This could be explained by the finding that the native rhizobial population is much larger in the Elvas-ENMP and Beja soils.

On the contrary, Anyango and co-workers (1995) had reported a negative correlation between soil pH and rhizobial diversity. Besides, Thurman *et al.* (1985) found no relationship between genetic diversity and soil pH.

Chickpea nodulating rhizobia isolated from soils of a small region in the south of Portugal show high phylogenetic diversity. At least a putative new species of *Mesorhizobium,* able to nodulate chickpea, suggested by 16S rDNA sequence data (chapter 3) is supported by DAPD patterns, and total protein profiles. However, more studies, such as sequencing of other housekeeping loci, are necessary to confirm this hypothesis.

5. PHYLOGENY OF CHICKPEA RHIZOBIA BASED ON MULTILOCUS SEQUENCE ANALYSIS: A NEW SPECIES OF *Mesorhizobium*

This chapter is based on the following manuscript:

Laranjo, M., Alexandre, A., Alho, L., Young, J. P. W. and Oliveira, S. A new species of *Mesorhizobium* supported by a multilocus sequence approach. *Manuscript in preparation.*

5.1. Introduction

Ribosomal RNA is the more widely accepted molecule to infer bacterial phylogeny, however there have been several reports indicating that it has suffered lateral gene transfer and is also subject to recombination (Coenye & Vandamme, 2003; Eardly *et al.*, 2005; Hashimoto *et al.*, 2003; Haukka *et al.*, 1996; Parker, 2001; Parker *et al.*, 2002; Ueda *et al.*, 1999; van Berkum *et al.*, 2003). Therefore, it is desirable to base prokaryotic phylogeny on a core of housekeeping genes (Daubin *et al.*, 2002).

It is necessary to analyse genes other than 16S rDNA in order to confirm and clarify the relationships among the rhizobia suggested by the 16S rDNA phylogeny. It is wishful and necessary to find additional markers and extend the current rhizobial sequence database (Hernandez-Lucas et al., 2004). It is also important to assure that the different loci have evolved independently.

One convenient approach to infer species phylogeny is to combine sequence of several unlinked and neutrally evolving loci, which are shown in advance to yield congruent topologies (Gaunt *et al.*, 2001; Vinuesa *et al.*, 2005a). The need for a population genetic approach, including analysis of several housekeeping genes (Stackebrandt *et al.*, 2002) and an adequate number of genetically diverse isolates used (Barraclough & Nee, 2001) has been put forward.

The intergenic regions are known for their variability in size and sequence (Doignon-Bourcier *et al.*, 2000). Thus, the analysis of the Intergenic Transcribed Spacer (ITS) region between 16S and 23S rDNA is a useful method for studying diversity among bacterial populations at the intraspecific level (Laguerre *et al.*, 1996). The variability in ITS sequences among strains of the same species has been demonstrated in several rhizobial isolates (de Oliveira *et al.*, 1999; Laguerre *et al.*, 1996; van Berkum & Fuhrmann, 2000; Vinuesa *et al.*, 1998; Willems *et al.*, 2001a).

Recently, the high variability in the ITS region between the 16S and the 23S rRNA genes has been shown to be useful in the differentiation of rhizobial strains (van Berkum & Fuhrmann, 2000; Willems *et al.*, 2001a). In general, the high sequence variation of the ITS region allows discrimination between closely related strains or isolates (Kwon *et al.*, 2005).

The phylogenies of the protein coding loci *atp*D (ATP synthase β) and *rec*A (recombinase A) have been shown to support the 16S rDNA based phylogeny in rhizobia (Gaunt *et al.*, 2001).

Glutamine synthetases GSI and GSII have been used in evolutionary molecular clock studies, and while GSI is exclusive of bacteria, GSII has been found predominately in eukaryotes and only in a few bacteria, among which are rhizobia. In rhizobia, *gln*A (glutamine synthetase I) phylogeny is broadly congruent with 16S rDNA phylogeny, while *gln*II (glutamine synthetase II)is not (Turner & Young, 2000).

Despite the coherence between the DAPD and total protein profiles dendrograms and the 16S rDNA phylogeny, the phylogenetic position of the Portuguese chickpea rhizobia used in this study was supported only on 16S rDNA sequence analysis.

In order to seek corroboration of the 16S rDNA groups, and also to resolve relationships among them, sequence analysis of other loci was performed. These loci are widely spaced in the genome, as can be seen by their positions in the *M. loti* MAFF303099 chromosome sequence (given in brackets) and hence unlikely to be cotransferred in recombination events. Thus, they provide independent genealogies from which to infer a species tree (Nichols, 2001; Rosenberg, 2002) and allow distinguishing the effects of different forces and contingencies of evolution (Lan & Reeves, 2000; Wernegreen & Riley, 1999). The protein-coding core loci, *atp*D (3245449-3246882), *rec*A (24004-25101) and *gln*A (238290-239699), were selected to expand the database of sequences available for mesorhizobia and in particular for isolates able to nodulate chickpea and type strains of mesorhizobia. The intergenic spacer between 16S and 23S rRNA genes is of course tightly linked to the 16S rDNA and does not provide independent evidence. There are usually several copies in rhizobial genomes, and there is gene conversion between them that keeps them close in sequence. Nevertheless, it was chosen because it may show more variation than 16S rDNA and hence be more informative.

This work was performed with the purpose of inferring a high resolution species phylogeny within the *Mesorhizobium* genus and to estimate the congruence between classifications of rhizobia based on four housekeeping genes (16S rDNA, *atp*D, *rec*A, *gln*A) and the ITS region. The main aim of this multilocus sequence approach was to confirm the existence of unexpected rhizobia species and a new species, able to nodulate chickpea, as suggested by 16S rDNA sequences and different molecular approaches (DAPD and protein profiles).

5.2. Results

5.2.1. 16S-23S intergenic spacer (ITS) phylogeny

GenBank accession numbers for the ITS sequences determined in this study are DQ659517 to DQ659535.

Repeated attempts to amplify and sequence the ITS region of *M. temperatum* type strain were unsuccessful.

The ITS sequences obtained varied in length (between 750 and 1000 nucleotides, approximately), but they all contained the $tRNA^{Ile}$ and $tRNA^{Ala}$ genes. Variable regions were interspersed throughout the entire ITS sequence, except for the two tRNA sequences. Since this is a non-coding spacer region it shows much more variation and is more prone to indels (insertions and deletions), which provide per se further phylogenetic evidence. Although the tree building method used does not take into account positions with gaps, in general, closely related isolates share the same indel regions.

The ITS based phylogeny, shown in Figure 23, generally supports the 16S rDNA groups (Figure 6). Groups **A1**-**D2** determined by 16S rDNA phylogeny are indicated for each isolate.

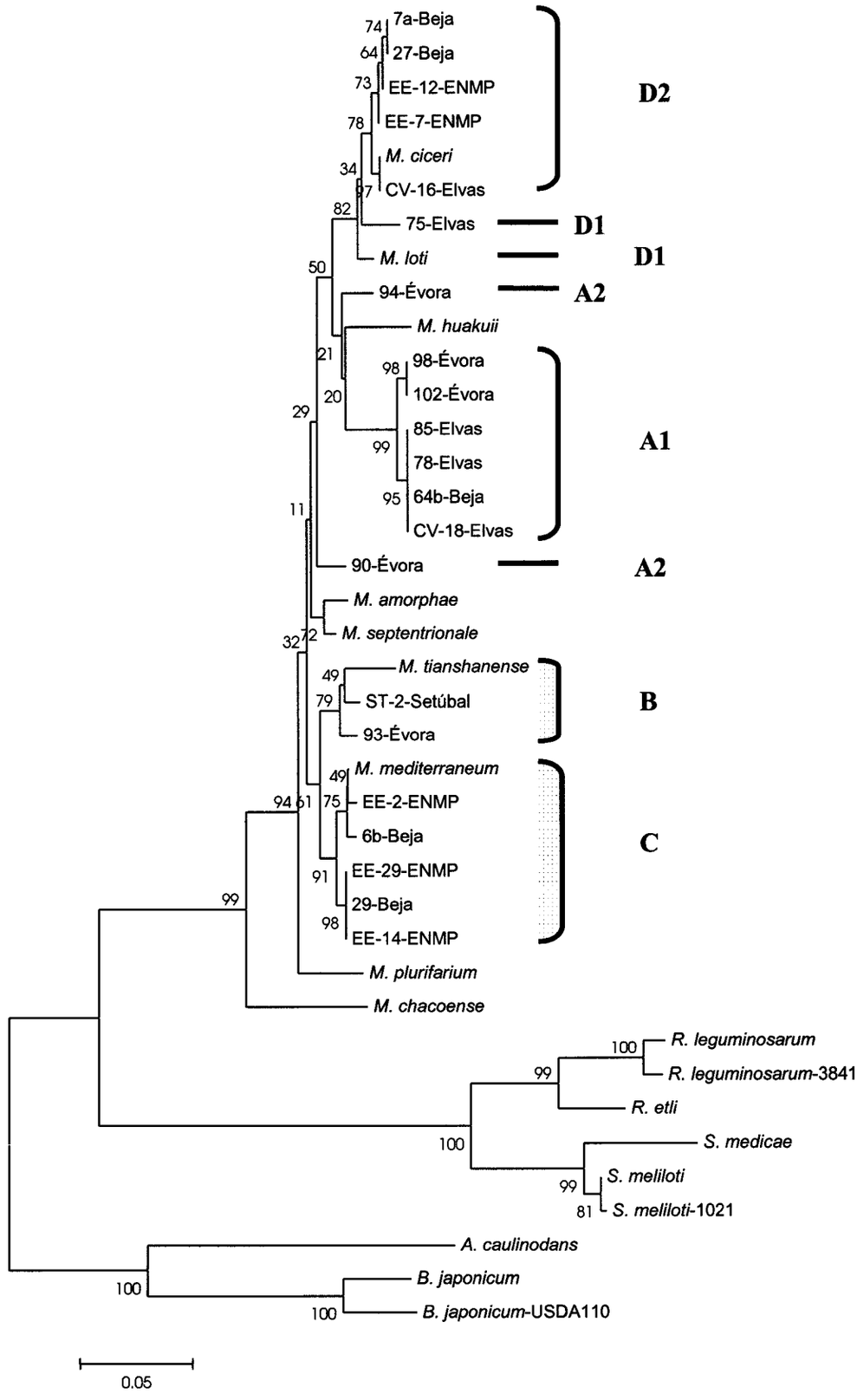

Figure 23. (on previous page) Neighbour-Joining phylogeny based on ITS sequences from the 21 isolates (alignment length 1686 bp). Percentage bootstrap is indicated on internal branches (1000 replicates); scale bar indicates 0.05 substitutions per site. Letters A1, A2, B, C, D1 and D2 denote 16S rDNA species groups (Figure 6).

Table 18 shows the accession numbers of the different loci sequences from the reference and type strains used in the phylogenetic analyses.

Table 18. GenBank accession numbers or RhizoBase records of the ITS, *atp*D, *rec*A and *gln*A sequences from rhizobia reference and type strains used in the phylogenetic analyses. Sequences in bold were determined in this study. *Sequence data produced by the *R. leguminosarum* bv. *viciae* Sequencing Group at the Sanger Institute (ftp://ftp.sanger.ac.uk/pub/pathogens/rl/). §Accession number of the complete genome sequence. n.dt.-not determined.

SPECIES	STRAIN	ACCESSION NUMBERS			
		ITS	*atp*D	*rec*A	*gln*A
Azorhizobium caulinodans	ORS 571^T	AF345253	AJ294389	AJ294363	Y10213
Bradyrhizobium japonicum	USDA 6^T	AF345255	AJ294388	AY591555	AF169576
Bradyrhizobium japonicum	USDA 110	NC 004463§	bll0440	bll5755	blr4949
Mesorhizobium amorphae	ACCC 19665^T	AF345257	AY493453	AY494816	**DQ659488**
M. chacoense	LMG 19008^T	**DQ659515**	AY493460	AY494825	**DQ659489**
M. ciceri	UPM-Ca7^T	AF345258	AJ294395	AJ294367	AF169565
M. huakuii	CCBAU 2609^T	AF345259	AJ294394	AJ294370	AF169562
M. loti	NZP 2213^T	AF345260	AJ294393	AJ294371	AF169563
M. mediterraneum	UPM-Ca36^T	AF345261	AJ294391	AJ294369	AF169564
M. plurifarium	ORS 1032^T	AF345263	AY688599	AY494824	**DQ659490**
M. septentrionale	HAMBI 2582^T	**DQ659516**	**DQ659498**	**DQ444304**	**DQ659491**
M. temperatum	HAMBI 2583^T	n.dt.	**DQ659499**	**DQ444305**	**DQ659492**
M. tianshanense	A-1BS^T	AF345264	AJ294392	AJ294368	AF169577
Rhizobium etli	CFN 42^T	AF541974	AJ294404	AJ294375	AF169567
R. leguminosarum	USDA 2370^T	AF345271	AJ294405	AJ294376	AF169566
R. leguminosarum bv. *viciae*	3841	AM236080§	RL4407	RL2637	RL2392
Sinorhizobium medicae	A-321^T	AF325824	AJ294401	AJ294381	AF169572
S. meliloti	USDA 1002^T	AF345286	AJ294400	AJ294382	AF169573
S. meliloti	1021	NC 003047§	SMc02501	SMc00760	SMc00948

ITS sequence identity among isolates from group **A1** is between 91.3% and 97.9 %. Group **A2** isolates which are separate in the ITS tree share 81% sequence similarity. The sequence identity between group **B** isolates ranged from 71.5% to 89.2%. Isolates from group **C** share between 69.3% and 99.8% sequence identity. Isolate 75-Elvas from group **D1** is separated from *M. loti* in the tree, although they share 73.4% sequence identity. The sequence similarity within group **D2** ranged from 86.9% to 99.2%.

The putative new species isolates from group **A1** are in the same ITS cluster and share the highest sequence similarity values.

The analysis of ITS sequences supports the integrity of the *Mesorhizobium* clade, but not its placement in the phylogeny regarding other genera.

The major differences of the ITS from the 16S rDNA phylogeny (Figure 6) are the taking apart of the two isolates from group **A2**, and the separate position of *M. loti* and isolate 75-Elvas, both from group **D1**.

In particular, the putative new species (group **A1**) is confirmed. The *M. tianshanense* (**B**), *M. mediterraneum* (**C**) and *M. ciceri* (**D2**) groups are supported. Besides, as in the 16S rDNA phylogeny, the groups of *M. loti* (**D1**) and *M. ciceri* (**D2**) and of *M. tianshanense* (**B**) and *M. mediterraneum* (**C**) are very close. However, the sequences of *M. loti* and 75-Elvas, both from group **D1**, do not cluster together. Furthermore, the sequences of isolates from group **A2**, 90-Évora and 94-Évora, are not very similar. The position of isolate 94-Évora remains close to the putative new species group **A1**, while isolate 90-Évora is more distantly related.

The ITS region is a good marker for assessing phylogenetic relationships among closely related strains, since it resolved isolates within each group clearer than 16S rDNA.

5.2.2. Protein coding gene based phylogenies

5.2.2.1. *atp*D phylogeny

PCR amplification of the *atp*D gene yielded low quantity products of approximately 450 bp.

Although the *atp*D gene might be useful for phylogenetic inferences in rhizobia, it is not an appropriate gene for sequence analysis due to many amplification problems, at least within the genus *Mesorhizobium*. The high sequence heterogeneity throughout most of the amplified fragment, made it difficult to use one pair of primers for all isolates.

GenBank accession numbers for the *atp*D sequences determined in this study are DQ659500 to DQ659514.

Repeated attempts to amplify and sequence the *atp*D gene of isolates 29-Beja, EE-12-ENMP, EE-14-ENMP, EE-29-ENMP, 94-Évora and ST-2-Setúbal were unsuccessful.

The *atp*D based phylogeny is shown in Figure 24.

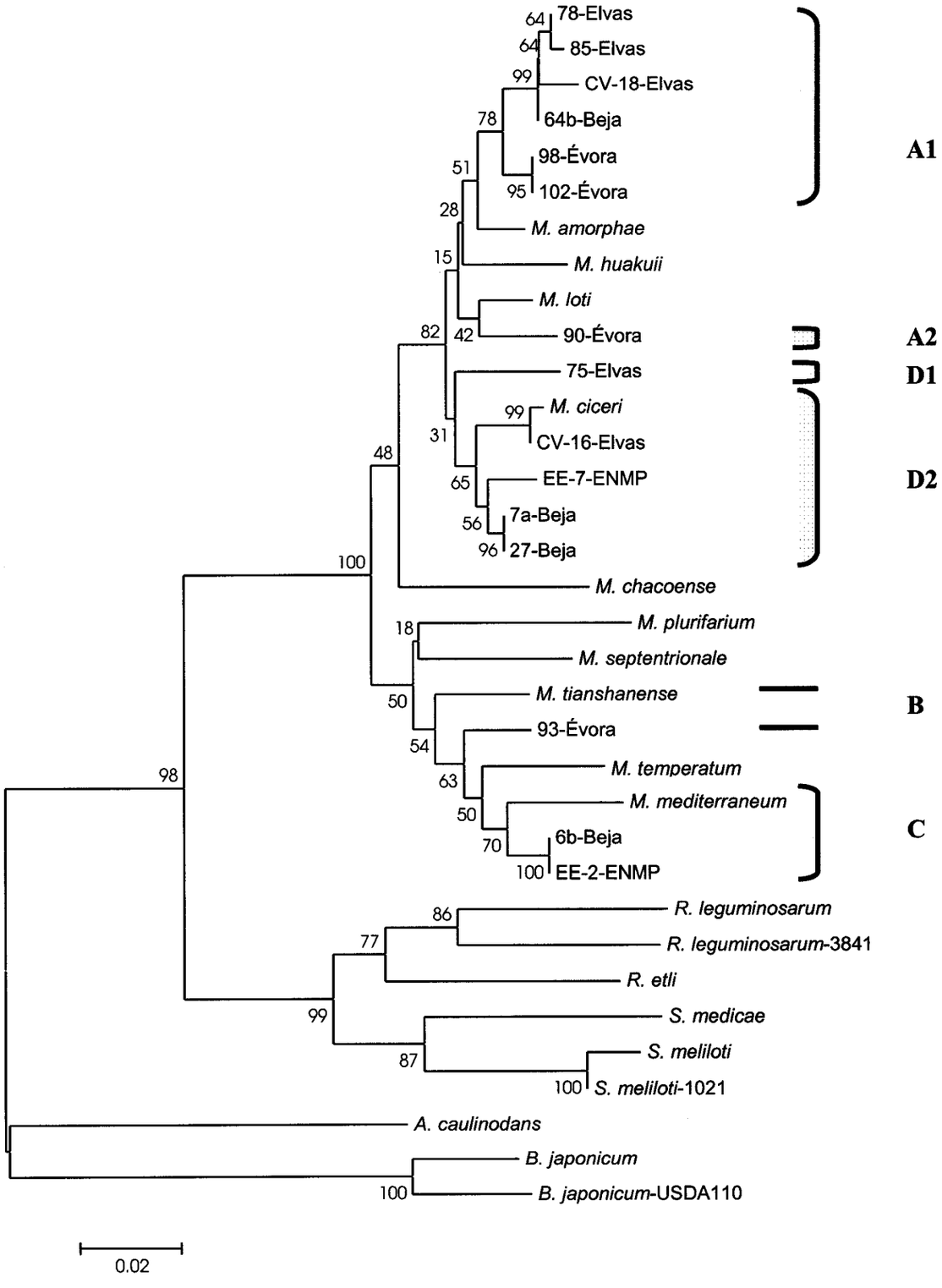

Figure 24. Neighbour-joining phylogeny based on *atp*D sequences of 15 isolates (alignment length 417 bp). Percentage bootstrap support is shown on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Letters A1, A2, B, C, D1 and D2 denote 16S rDNA species groups (Figure 6).

Relative to all the other sequences, *Azorhizobium caulinodans* and *Bradyrhizobium japonicum* have a 14 bp and 11 bp insertion, respectively, at position 279 in the alignment. These insertions are related in sequence and provide good support for the monophyly of these two species. To confirm this hypothesis a phylogenetic tree was constructed using sequences of more distantly related α-Proteobacteria, such as *Rhodobacter capsulatus* (X99599), which shows the ancestral state of the *atp*D gene sequence and suggests that there was an insertion in the branch leading to *Azorhizobium* and *Bradyrhizobium* (data not shown).

Despite incomplete, since it lacks six of the 21 isolates, the sequence analysis of the *atp*D gene supports the 16S rDNA groups (Figure 6) , with the exception of *M. loti*. The putative new species from group **A1** holds up. Isolate 90-Évora (group **A2**) is in the same clade as group **A1**. Groups **D1**, excluding *M. loti*, and **D2** (*M. ciceri*) are close together. The *M. tianshanense* (**B**) and *M. mediterraneum* (**C**) groups are also closely related to each other. Despite being in the same clade, isolate 93-Évora and *M. tianshanense* (group **B**), do not form a cluster, as in the 16S rDNA phylogeny (Figure 6).

5.2.2.2. *rec*A phylogeny

A fragment of approximately 450 bp of the *rec*A gene was obtained for all isolates.

GenBank accession numbers for the *rec*A sequences determined in this study are DQ441449 to DQ441469.

The *rec*A tree (Figure 25) has more resolution within groups than the 16S rDNA tree. *rec*A based phylogeny generally supports the 16S rDNA groups (Figure 6), with the exception of the position of the *M. loti* type strain. Furthermore, the *rec*A gene sequences strongly support the existence of two putative new species, proposed by the 16S rDNA gene sequence analysis. In the *rec*A phylogeny groups **A1** and **A2** are clearly different from each other and are not in the same clade. Again, groups **B** and **C**, as well as groups **D1** and **D2** are clustered together. *M. loti* is distant from the *M. ciceri* group (**D2**), as well as from isolate 75-Elvas, and clusters with group **A1**, the putative new species.

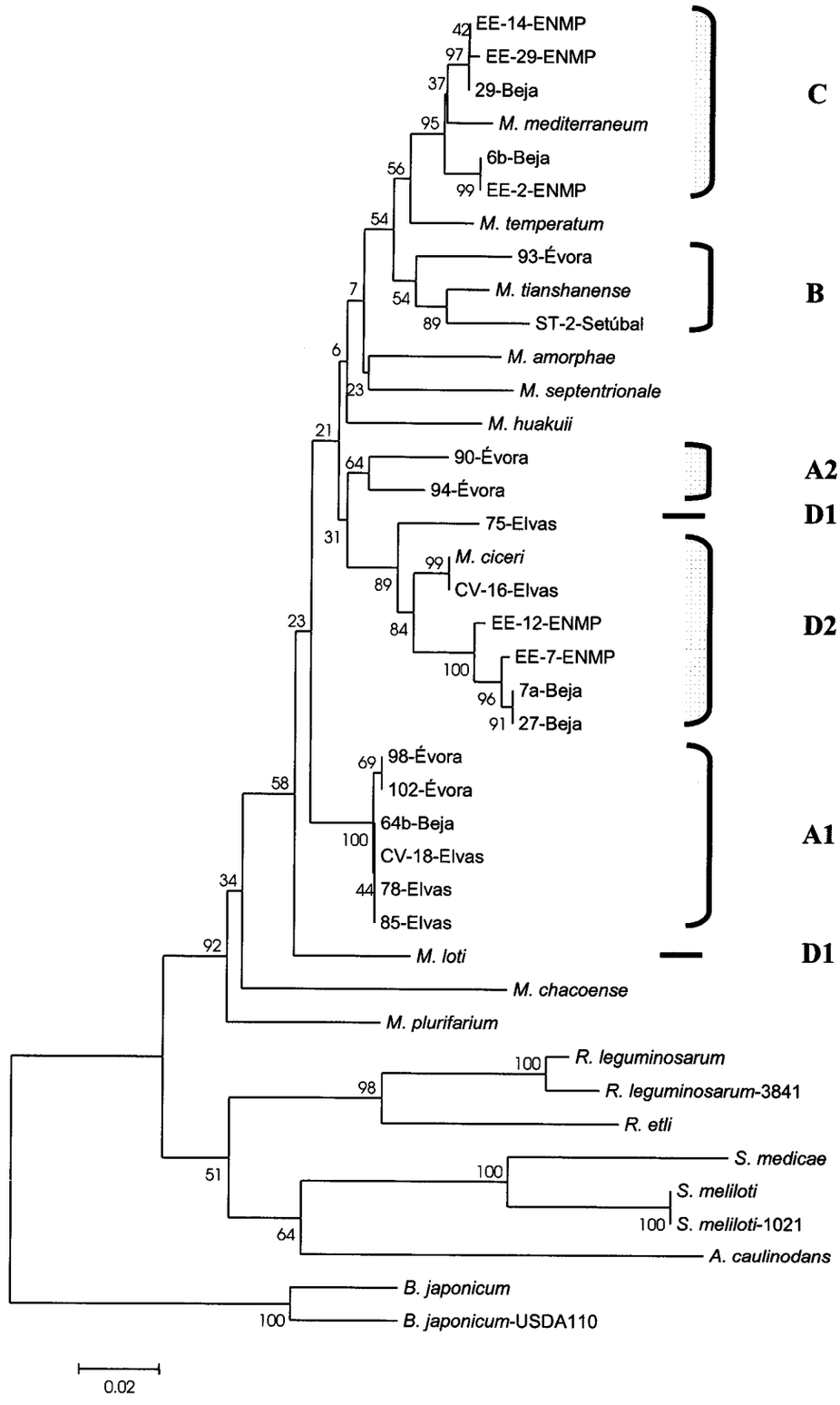

Figure 25. Neighbour-Joining phylogeny based on *rec*A sequences from the 21 isolates (alignment length 430 bp). Percentage bootstrap support is shown on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Letters A1, A2, B, C, D1 and D2 denote 16S rDNA species groups (Figure 6).

*rec*A sequence similarity among isolates from group **A1** is above 99%. Isolates 90-Évora and 94-Évora from group **A2** show almost 97% sequence identity. The *M. tianshanense* group **(B)** above 95% sequence similarity. The *M. ciceri* group **(D2)** presents above 96% sequence similarity.

Again, similarly to the ITS based analysis, *rec*A sequences from isolates from group **A1** show the highest similarity values among them.

5.2.2.3. *gln*A phylogeny

In order to confirm groups **A1** and **A2** as new species, a third protein coding gene was sequenced for the eight isolates belonging to these two groups.

GenBank accession numbers for the *gln*A sequences determined in this study are DQ659493 to DQ659497, DQ675602, DQ826574 and DQ826575.

The *gln*A based phylogeny (Figure 26) is congruent with the 16S rDNA phylogeny (Figure 6), as far as the type strains of known species and genera are concerned. It undoubtly supports the putative new species (group **A1**), although its position is different towards type species sequences compared to the positions in the 16S rDNA phylogeny. In the *gln*A phylogeny the group **A1** new species clusters with *M. ciceri* and not with *M. huakuii*, as in the 16S rDNA phylogeny.

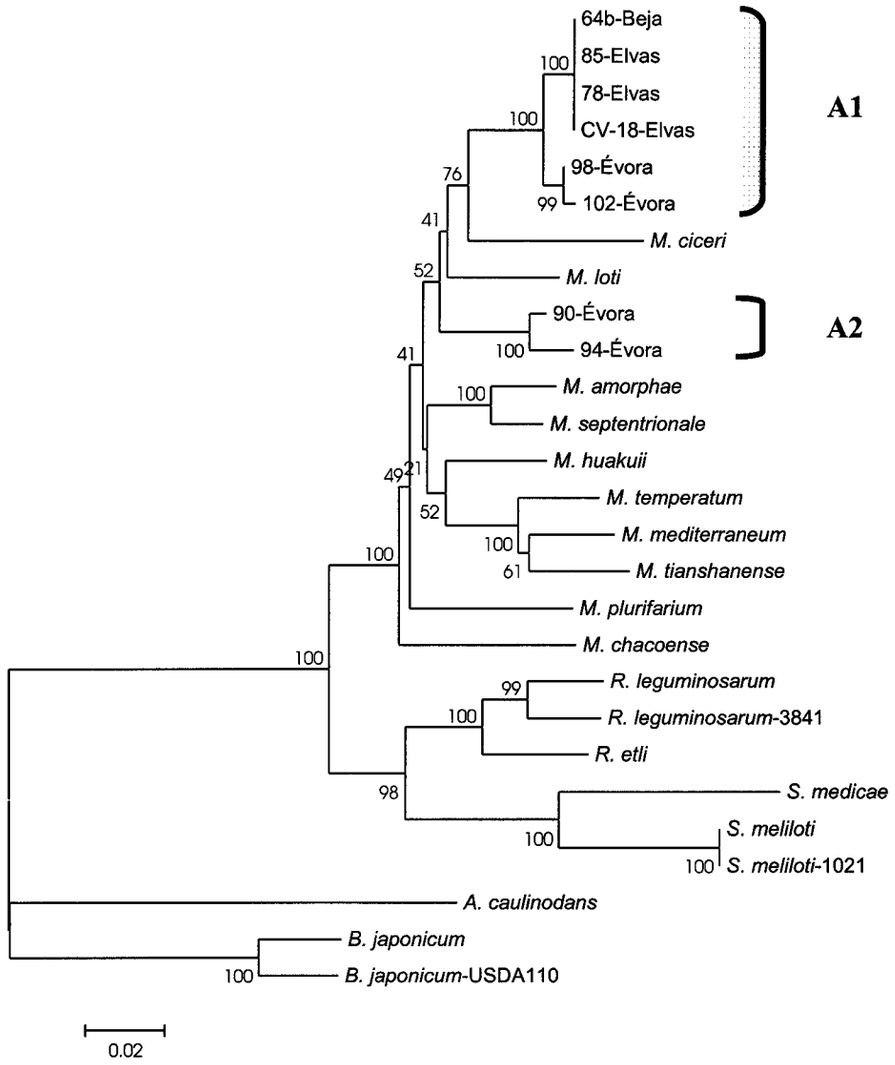

Figure 26. Neighbour-Joining phylogeny based on *gln*A sequences from the eight isolates of the 16S rDNA groups **A1** and **A2**, the two putative new species (alignment length 942 bp). Percentage bootstrap support is shown on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Letters A1 and A2 denote 16S rDNA species groups (Figure 6).

5.2.3. Multilocus sequence analysis using concatenated sequences

The multilocus sequencing approach to define bacterial species has recently been proposed as an alternative to defining bacterial species by DNA-DNA hybridisation (Gevers *et al.*, 2005).

The partition homogeneity test or incongruence length difference test (ILD) (Cunningham, 1997) was applied to check whether it was appropriate to combine the data (Bull *et al.*, 1993). Parsimony step-length homogeneity was supported for the three partition *atp*D+*rec*A+*gln*A test (P=0.16), but

rejected for other partitions involving 16S rDNA or ITS or both (P<0.001). These results permit us to combine the *atp*D, *rec*A and *gln*A data, but suggest that the 16S rDNA and ITS data should be treated separately.

Thus, in order to determine the most consensual placement of the two candidate species (groups **A1** and **A2**) within the genus *Mesorhizobium*, the sequences of the three protein-coding genes were concatenated and used to construct a global tree combining the *atp*D, *rec*A and *gln*A gene sequences. The six isolates from group **A1** and isolate 90-Évora from group **A2** were used, besides the rhizobia type strains. This concatenation approach to phylogenetics has been used before in eukaryotes (Baldauf *et al.*, 2000) and prokaryotes (Casiraghi *et al.*, 2005; Godoy *et al.*, 2003; Gundi *et al.*, 2004; Kotetishvili *et al.*, 2005; Thompson *et al.*, 2005) and even in rhizobia (Silva *et al.*, 2005; Vinuesa *et al.*, 2005a; Young *et al.*, 2006).

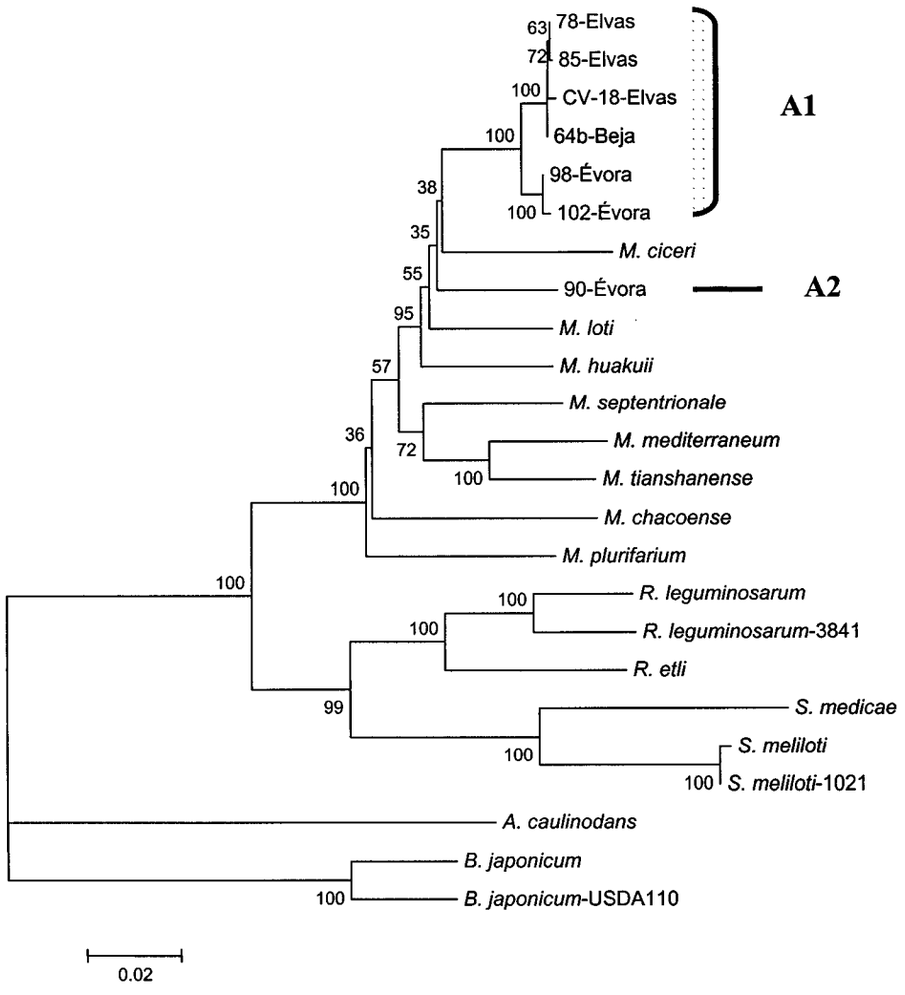

Figure 27. Neighbour-Joining phylogeny based on *atp*D, *rec*A and *gln*A sequences from seven isolates of the 16S rDNA groups **A1** and **A2**, the two putative new species (alignment length 1814 bp).

Percentage bootstrap support is shown on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Letters A1 and A2 denote 16S rDNA species groups.

The phylogeny based on the concatenated sequences of the three protein coding genes (Figure 27), supports the existence of a new *Mesorhizobium* species (group **A1**) that includes a number of closely related strains. Neither this new species nor the single isolate of the second putative new species (group **A2**) are specifically related to any other described *Mesorhizobium* species.

The candidate species **A1** has an uncertain position within the genus *Mesorhizobium*, since most bootstraps values are low and do not allow us to conclusively place this species. Group **A1** isolates are individually closer to *M. loti* with sequence similarity values between 91.5% and 92.7% followed by *M. ciceri* with sequence identities ranging 90.8% to 92%. Isolate 90-Évora (group **A2**) shares 90.3 % sequence identity with *M. loti*, and 90.2% with both *M. ciceri* and *M. septentrionale*.

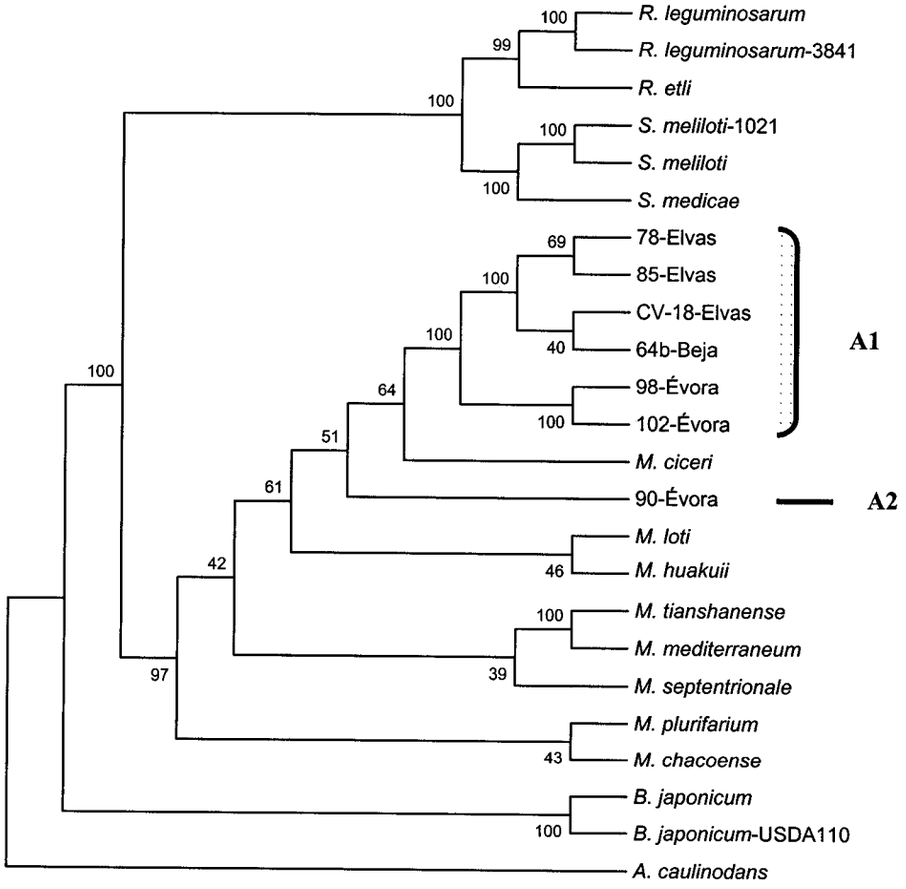

Figure 28. Maximum Likelihood phylogeny (consensus tree) based on *atp*D, *rec*A and *gln*A sequences from seven isolates of the 16S rDNA groups **A1** and **A2**, the two putative new species (alignment length 1814 bp). Percentage bootstrap support is shown on internal branches (100 replicates). Letters A1 and A2 denote 16S rDNA species groups.

Phylogenetic analysis using the maximum likelihood method (Figure 28) has shown a similar topology than with the neighbour-joining method (Figure 27).

The bootstrapping procedure implemented in PHYLIP does not perform the analysis on the tree inferred from original dataset. Instead, Felsenstein (2006) has argued that one reasonable tree would be the one recovered from the random samples. However, the original inferred tree and the tree produced by bootstrapping are usually pretty similar.

However, at least five branches within the genus *Mesorhizobium* have real low bootstrap support (below 50%) and thus the phylogenetic positions defended by these trees may not hold up for the majority of the genome.

5.2.4. 16S rDNA extended phylogeny

With the purpose of determining whether the two new species, supported by the phylogeny based on the five different loci, had more isolates, the analysis of 16S rDNA partial sequence data was performed for nine additional isolates belonging to cluster A of the 16S rDNA RFLP dendrogram (Figure 5).

GenBank accession numbers for the 16S rDNA sequences determined in this study are DQ787130 to DQ787138.

Five additional isolates, namely 83-Elvas, CV-1-Elvas, CV-11-Elvas, 92-Évora and 96-Évora, were found to belong to the new species from group **A1**. Thus, this new species can be described with a total of eleven isolates.

Two additional isolates clustered in new species group **A2** that includes four isolates, all from Évora: 89a.-Évora, 90-Évora, 94-Évora and 101-Évora. Further examination of these isolates and search for more isolates is required to warrant their classification as a new species, since it has been proposed that descriptions of new species should be based on a minimum of five isolates (Christensen *et al.*, 2001).

Unexpectedly, isolate 87-Évora does not cluster with either of the two putative new species groups. It seems to be close to *M. huakuii* with 99.6% sequence identity. Alternatively, it could be that it belongs to a third hypothetical new species. Further studies are needed to confirm the phylogenetic position of this particular isolate.

Also unpredictably, isolate 79-Elvas is close to the groups of *M. ciceri* (**D2**) and *M. loti* (**D1**). It shares 99.8% sequence similarity with *M. ciceri* and 99.6% with *M. loti*. Further studies are needed to determine whether isolate 79-Elvas belongs to the *M. ciceri* or the *M. loti* group.

The resolution within groups of this extended 16S rDNA based phylogeny (Figure 29) is poorer than the previously presented 16S rDNA phylogeny for the 21 isolates (Figure 6), since a shorter

region of the 16S rDNA was used (845 bp). However, this region served our purposes, given that it is enough to separate the six groups, generating the same species groups as the complete 16S rDNA sequences (Figure 6).

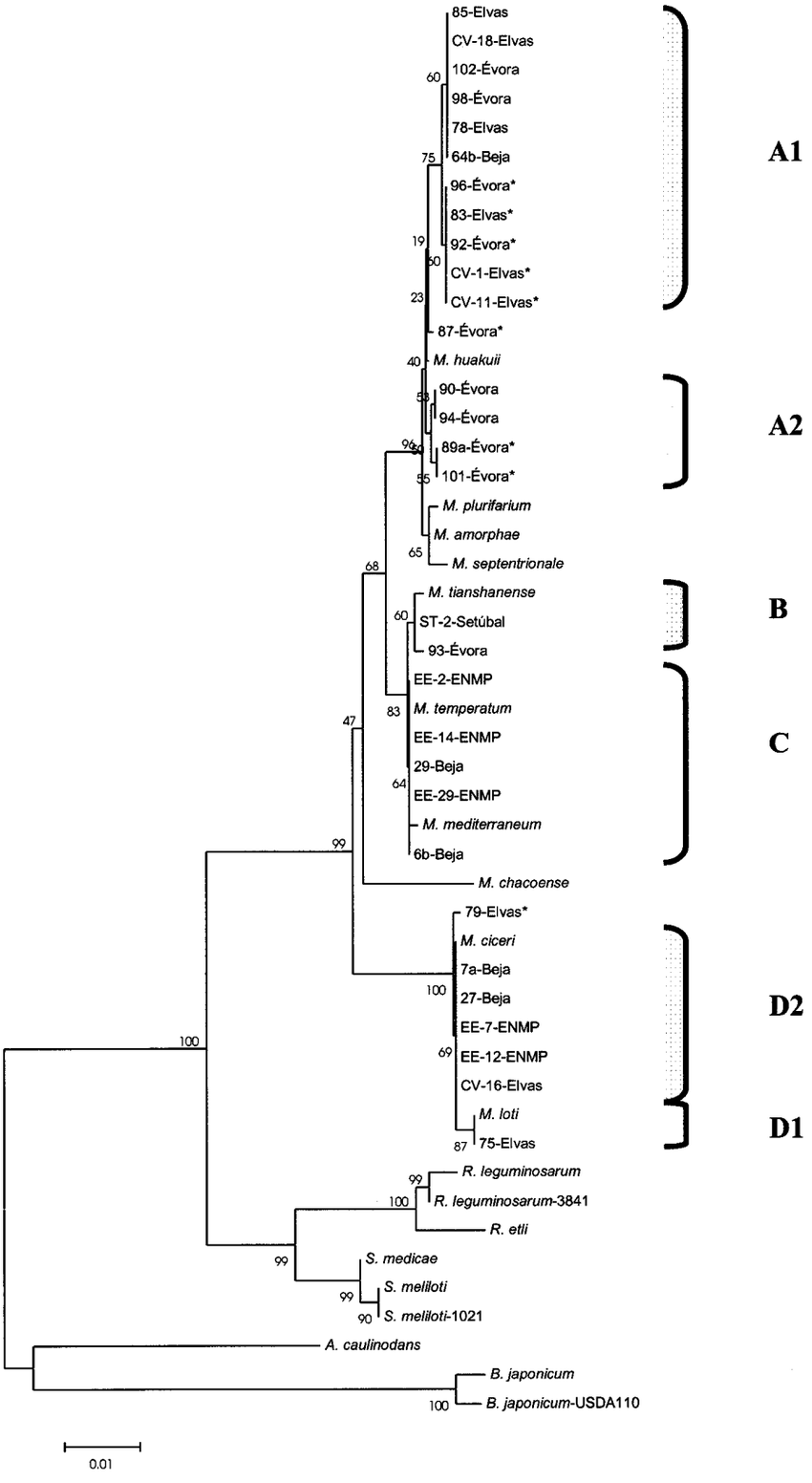

Figure 29. (on opposite page) Neighbour-Joining phylogeny based on 16S rDNA partial sequences (alignment length 845 bp) from the 21 isolates plus nine additional isolates from 16S rDNA RFLP cluster A (Figure 5), marked with an *. Percentage bootstrap support is indicated on internal branches (1000 replicates); scale bar indicates 0.01 substitutions per site. Letters A1, A2, B, C, D1 and D2 denote 16S rDNA species groups.

5.2.5. DNA base composition

DNA base composition was determined for isolate 64b.-Beja, as representative of the putative new species from group **A1**, and for 94-Évora, as representative of the putative new species from group **A2**. The GC content of isolate 64b.-Beja is 62.7%, and of 94-Évora is 60.3%.

5.3. Discussion

Single-gene phylogenies may not reflect the evolution of the genome as a whole and it is therefore necessary to adopt a multilocus approach for species delineation. A combination of population genetics, meaning the genetic diversity within and between different populations, and phylogenetics is recommended (Lan & Reeves, 2001).

The identities of the twenty-one chickpea rhizobia isolates and their relationship to known rhizobia species were established by comparative analysis of the 16S rDNA, the ITS region, and *atp*D and *recA* gene sequences. Native chickpea rhizobia isolates clustered with the genus *Mesorhizobium*, either as members of known species or as putative new species.

The topology of the four phylogenetic trees provides strong support for the monophyly of the genus *Mesorhizobium*. The four trees are globally congruent pointing out the species assignment of the Portuguese chickpea isolates, already indicated in chapter 3. They all support the existence of two putative new species with six and two isolates, respectively. Furthermore, they support the identification of two isolates as *M. tianshanense*, five isolates as *M. mediterraneum*, one isolate as *M. loti* and five isolates as *M. ciceri*.

Phylogenetic analyses of all loci (16S rDNA, ITS region, *atp*D, *rec*A and *gln*A) revealed identical topologies with neighbour-joining (Figure 6, Figure 23, Figure 24, Figure 25 and Figure 26), minimum evolution (data not shown) and maximum likelihood methods (data not shown).

Some species groups appear associated in all four trees, namely groups **A1** (new) and **A2** (new), groups **B** (*M. tianshanense*) and **C** (*M. mediterraneum*), and groups **D1** (*M. loti*) and **D2** (*M. ciceri*), with the exception of the *rec*A tree in which groups **A1** and **A2** are separated. This is in agreement

with the relatedness among species within the genus *Mesorhizobium* as established by previously reported phylogenetic analyses of the 16S rDNA, *atp*D, *gln*A and *rec*A, using only mesorhizobia type strains (Gao *et al.*, 2004; Gaunt *et al.*, 2001; Kwon *et al.*, 2005; Turner & Young, 2000). Nevertheless, this is the first study that uses all ten *Mesorhizobium* type species for phylogenetic purposes.

The analysis of ITS, *atp*D and *rec*A sequences fully support the *Mesorhizobium* clade as well as its placement in the rhizobial phylogeny based on 16S rDNA sequence data, but not all species relationships within the genus. Relationships among the species are not the same when comparing the four phylogenetic trees, which suggests that there may be recombination of chromosomal genes between *Mesorhizobium* species. For example, the *M. loti* type strain is closely related to *M. ciceri* in terms of 16S rDNA and *gln*A, but seems to be closer to group **A2** in the ITS, *atp*D and *rec*A trees.

The *rec*A phylogeny (Figure 25) is the most similar to the 16S rDNA phylogeny (Figure 6), but it shows very low bootstrap values in the deeper branches within the genus *Mesorhizobium*. Only group **D1** (*M. loti*) is disrupted, and all other species groups received between 53% and 100% bootstrap support.

In the ITS phylogeny (Figure 23), groups **A2** and **D1** are disordered and the other four species groups have high bootstrap values (78-99%).

The *atp*D sequence analysis (Figure 24) is the one that provides more differentiation between isolates. However, only groups **A1** (new species), **C** (*M. mediterraneum*) and **D2** (*M. ciceri*) remain unaffected and with high bootstrap values (65-78%). The groups **A1** (new species), *M. tianshanense* (**B**) and *M. loti* (**C**) appear separated and the positions of their isolates is not always clear, since bootstrap values are lower.

Regarding the *gln*A phylogeny (Figure 26), candidate species **A1** is supported by a bootstrap value of 100%. However, its closest type strain is that of *M. ciceri*, as in the *rec*A phylogeny (Figure 25), which was not the case for the 16S rDNA (Figure 6), ITS (Figure 23) and *atp*D (Figure 24) phylogenies, where candidate species **A1** is closer to *M. amorphae*, *M. huakuii*, *M. plurifarium* or/and *M. septentrionale*.

Our ITS, *atp*D and *rec*A data reinforce the view that, at the genus level, the SSU based phylogeny holds for the majority of the genome, but this is not completely true at the species level. *Mesorhizobium* forms a closely knit clade, but relationships among the species are not the same for all genes. The *M. loti* and *M. ciceri* type strains are very similar in their 16S rDNA sequence, but this is not true for any of the other genes. This is consistent with the finding that *Mesorhizobium* strains that are virtually identical in 16S rDNA sequence may be unrelated in terms of DNA-DNA hybridisation (Sullivan *et al.*, 1996) and suggests that there may be recombination of chromosomal genes between *Mesorhizobium* species.

Evidence for recombination within *Mesorhizobium* species has been provided before, but the genus is genetically isolated from other rhizobia (Gaunt *et al.*, 2001).

The existence of isolates belonging to one or two putative new species had been suggested in chapters 3 and 4, based on 16S rDNA sequences, DAPD fingerprinting, SDS-PAGE total protein profiles and also on Intrinsic Antibiotic Resistance patterns (Alexandre *et al.*, 2006).

Each isolate from the group **A1** putative new species can be differentiated in terms of either DAPD, total protein and plasmid profiles (chapter 4), as well as according to the auxanographic tests results (chapter 4).

Thus, to confirm the identity of the new species a fifth gene (*gln*A) was sequenced, since the sequencing of housekeeping protein coding genes has been increasingly defended, in detriment of more subjective analyses, such as DNA-DNA hybridisation.

The group of isolates that apparently forms one or two new species probably includes other members as suggested by the 16S rDNA RFLP partial sequence, namely from Elvas and Évora: 79-Elvas, 83-Elvas, CV-1-Elvas, CV-11-Elvas, 87-Évora, 89a.-Évora, 92-Évora, 96-Évora and 101-Évora.

Thus, a new species is described based on the eleven isolates group **A1**, and the name *M. lusitanum* is proposed. However, not much can be concluded about the phylogeny of this group within the genus, as the bootstrap values for the individual genes and even for the concatenated tree are low (Dirk Gevers, personal communication).

The putative new species corresponding to group **A2** has only four isolates, and therefore its formal description is not proposed in the present work.

A standardised format for the description of a new species has been proposed (Kämpfer *et al.*, 2003), however this is still largely based on DNA-DNA hybridisation, which is hardly comparable between different laboratories. On the other hand, sequences are portable data that can easily be exchanged and compared worldwide.

The technique of DNA-DNA hybridisation has several disadvantages that have been discouraging towards its use. It is not easily implemented and it is hardly comparable between different laboratories, particularly if different protocols are used. Furthermore, the laborious nature of pairwise cross hybridisations and the impossibility of establishing a central database make the use of fast and easily obtained portable sequence data much more attractive.

Isolates from the group **A1** new species have a high symbiotic effectiveness. Furthermore, they are also acid tolerant (Brígido *et al.*, *In Press.;* Rodrigues *et al.*, 2006) and show an elevated survival rate when subjected to heat shocks (our unpublished results). On the other hand, isolate 64b.-Beja is very competitive when used together with other strains (L. Alho, personal communication). All these are important features of strains that could be used as potentially effective field inoculants.

This is the first molecular phylogenetics study of mesorhizobia that used a multilocus sequence analysis approach for species delineation. Multilocus sequence analyses are hereby shown to be very useful in rhizobial molecular systematics when they are combined with sufficient taxon sampling and advanced phylogenetic inference methods.

The genus *Mesorhizobium* was created to recognize a group of species with distinctive phenotypic properties and forming a clearly defined clade in the 16S rDNA phylogeny that was outside the fast-growing rhizobia, *Rhizobium-Sinorhizobium*, cluster (Jarvis *et al.*, 1997). Our analyses of 16S rDNA, ITS, *atp*D, *rec*A and *gln*A sequences fully support both the integrity of the *Mesorhizobium* clade and its placement in the phylogeny, as had been reported before (Gaunt *et al.*, 2001; Turner & Young, 2000). It has been shown in the present work, the importance of carefully choosing adequate core loci for the inference of the underlying species phylogeny, as suggested by Daubin and co-workers (2002). These should be loci that have mostly remained stable during evolution and have not been prone to recombination and horizontal gene transfer.

Before the present work only two species had been described to form effective symbioses with chickpea, namely *M. ciceri* (Nour *et al.*, 1994a) and *M. mediterraneum* (Nour *et al.*, 1995). Thus, this study made a significant contribute to the knowledge of rhizobia species able to nodulate chickpea, extending the current view of chickpea microsymbionts to isolates with a chromosomal background that corresponds to *M. loti* (group **D1**), *M. tianshanense* (group **B**), two candidate species (groups **A1** and **A2**) and maybe *M. huakuii* (Figure 29).

Here we present a well resolved mesorhizobia species phylogeny based on combined 16S rDNA, ITS, *atp*D and *rec*A sequences, using several strains from each species.

Furthermore, we describe a new species of mesorhizobia based on 16S rDNA, ITS, *atp*D, *rec*A and *gln*A sequences, as well as on different phenotypic and molecular approaches.

5.3.1. Description of *Mesorhizobium lusitanum* sp. nov.

Mesorhizobium lusitanum (lu.si.ta'num L. neut. adj. *lusitanum* Portuguese, implying that the strains were isolated from Portugal).

Gram-negative, aerobic, non-spore-forming rods. Isolates from root nodules of *Cicer arietinum* L. Colonies are visible after 3-4 days in YMA, at 28°C, and have a creamy appearance. Do not acidify nor alkalinize the medium. All strains exhibited very similar total protein profiles obtained by SDS-PAGE. Strains have distinct plasmid profiles. *M. lusitanum* strains display distinct DAPD fingerprints, although some patterns are identical between different strains. In general, they form highly effective symbiosis with chickpea. Most strains are resistant to polymycin B. Furthermore, each strain has a distinct IAR pattern.

The type strain is 64b.-Beja with a GC content of 62.7%, and it has the characteristics described for the species. It will be deposited in two culture collections, namely in the "Colección Española de Cultivos Tipo" (CECT), Spain, and in the Culture Collection of the University of Helsinki (HAMBI), Finland.

6. EVIDENCE FOR LATERAL GENE TRANSFER BETWEEN *Mesorhizobium* SPECIES BASED ON PHYLOGENY OF SYMBIOSIS GENES

This chapter is based on the following manuscripts:

Laranjo, M., Alexandre, A., Rivas, R., Velázquez, E., Young, J. P. W. and Oliveira, S. Phylogenetic analysis of symbiosis genes supports gene transfer within the genus *Mesorhizobium*. *Manuscript in preparation.*

Velázquez, E., Rivas, R., Laranjo, M., Mateos, P. F., Oliveira, S. and Martínez-Molina, E. Strains of *Mesorhizobium amorphae* and *M. tianshanense* carrying symbiotic genes of common chickpea endosymbiotic species constitute a novel biovar (ciceri) of these species able to nodulate *Cicer arietinum*. *Submitted to Letters in Applied Microbiology.*

6.1. Introduction

The bacterial symbiosis genes, which are essential for the establishment of an effective symbiosis, can be divided into two main categories: nodulation genes (*nod*, *noe* and *nol*), which are responsible for nodulation, and nitrogen fixation genes (*nif* and *fix*), that are involved in atmospheric nitrogen fixation.

In response to the release of flavonoids by host plants, rhizobia synthesise and secrete lipochitooligosaccharides, the Nod factors. The first step in Nod factor assembly is performed by an *N*-acetylglucosaminyltransferase, a chitin synthase encoded by *nodC* (Geremia *et al.*, 1994), which is responsible for the chain length of the Nod factor (Perret *et al.*, 2000). Then the deacetylase NodB removes the *N*-acetyl moiety from the non-reducing terminus of the *N*-acetylglucosamine oligosaccharides (John *et al.*, 1993). Finally, an acyltransferase encoded by *nodA* links an acyl chain to the acetyl-freeC-2 carbon of the nonreducing end of the oligosaccharide (Debellé *et al.*, 1996). There are several sites on the chitin backbone than may carry substituents, which are the major determinants of host specificity. For instance, *S. meliloti nod*H$^-$ mutants make unsulphated Nod factors and lose their ability to nodulate alfalfa, but acquire the ability to nodulate vetch. On the other hand, *nod*Q$^-$ mutants nodulate both alfalfa and vetch (Roche *et al.*, 1991).

It has been suggested that *nod*A and *nod*C are involved in host-specific nodulation (Roche *et al.*, 1996). NodA varies in its specificity for different fatty acid substrates, thus contributing to the host range. NodC is also a determinant of the length of the Nod factor backbone and thus of host specificity (Kamst *et al.*, 1997).

The *nod*A, *nod*B, *nod*C, *nod*D, *nod*I and *nod*J genes are present in all rhizobia. Other nodulation genes, such as *nod*V and *nod*W and particularly *noe* and *nol* genes, are only found in one or some rhizobial strains (Schlaman *et al.*, 1998).

The common nodulation genes, *nod*A, *nod*B and *nod*C, are present in a single copy in all known rhizobia (Schlaman *et al.*, 1998), whereas there are usually one to five copies of the *nod*D gene in rhizobial genomes (Perret *et al.*, 2000).

The *nif*H gene codes for one of the subunits of the nitrogenase complex, the dinitrogenase reductase or Fe protein. One copy has been reported for mesorhizobia (Haukka *et al.*, 1998). Reiteration of the *nif*H gene has been reported for *A. caulinodans* and *R. etli* (González *et al.*, 2006), and also for *Rhizobium* sp. NGR234 (Freiberg *et al.*, 1997). All copies code for functional Fe proteins, but mutagenesis experiments have shown that none is indispensable for nitrogen fixation (Quinto *et al.*, 1985). High conservation of their nucleotide sequences indicates that they have been acquired by gene duplication (Kaminski *et al.*, 1998). Extra copies may provide better adaptation and rapid differential response to environmental conditions, higher nitrogen fixation ability (Valderrama *et al.*, 1996), or may present a reductase activity unrelated to nitrogen fixation (Kaminski *et al.*, 1998).

Several strains of *B. japonicum*, *B.* spp. and *R. leguminosarum* can display nitrogenase activity in the absence of the host and there have been some reports in the past that provide evidence of non-symbiotic nitrogen fixation (Kaminski *et al.*, 1998).

nod genes are unique to rhizobia. On the other hand, *nif* genes are found in many bacteria besides rhizobia.

In *Rhizobium* sp. NGR234 evolution of nitrogen fixation genes is different from the evolution of nodulation genes, as evidenced by their different GC content (Freiberg *et al.*, 1997).

Chickpea has been grown since ancient times, its cultivation is restricted to the Mediterranean area, Middle East, west Asia, Ethiopia and South America (Nour *et al.*, 1994b). Despite it is the third most widely grown crop legume in the world (Saxena & Singh, 1987), there are only a few studies on its symbionts. Chickpea has been traditionally considered a restrictive host for nodulation (Broughton & Perret, 1999) and only two species, *M. ciceri* (Nour *et al.*, 1994a) and *M. mediterraneum* (Nour *et al.*, 1995), have been described to effectively nodulate chickpea.

However, our results (chapter 3) have shown that isolates close to *M. loti*, *M. tianshanense* and from two putative new species within the genus *Mesorhizobium* are able to effectively nodulate chickpea.

Other recent studies have also shown that diverse *Mesorhizobium* species may effectively nodulate chickpea (Rivas *et al.*, *In Press.;* Rivas *et al.*, *Submitted.*).

The main aim of the present study was to compare the different phylogenetic histories for both housekeeping and symbiosis loci among, previously identified (chapters 3 to 5), diverse mesorhizobia that nodulate chickpea. The final purpose was to investigate evolutionary relationships among rhizobia that, although chromosomally divergent, nodulate the same host, and to elucidate whether chickpea is a restrictive host for nodulation.

The *nif*H gene was chosen as a nitrogen fixation marker, because a large number of rhizobial sequences are available for comparison. The *nod*C gene was selected, since it is essential for nodulation in all rhizobial strains, and is also host determinant.

6.2. Results

6.2.1. Phylogenies of symbiosis genes

6.2.1.1. *nif*H phylogeny

Phylogenetic studies have been conducted on different nitrogen fixation genes, but *nif*H has been the most widely used.

*nif*H gene was amplified by PCR with two heterologous primers, *nif*H-1 and *nif*H-2 (Eardly *et al.*, 1992), which amplify a 600 bp fragment. The PCR products were restricted with several endonucleases, according to the manufacturer's instructions, but *Alu*I was the only discriminating one.

Table 19. *nif*H RFLP type obtained for the 41 isolates. n.dt.-not determined.

ISOLATES	*nif*H TYPE	ISOLATES	*nif*H TYPE
4a.-Beja	A	**EE-2-ENMP**	n.dt.
6b.-Beja	A	**EE-3-ENMP**	A
7a.-Beja	B	**EE-7-ENMP**	A
9-Beja	B	**EE-9-ENMP**	A
21-Beja	B	**EE-11-ENMP**	A
27-Beja	A	**EE-12-ENMP**	A
29-Beja	A	**EE-13-ENMP**	A
31-Beja	B	**EE-14-ENMP**	n.dt.
54b.-Beja	A	**EE-20-ENMP**	n.dt.
64b.-Beja	A	**EE-29-ENMP**	A
75-Elvas	A	**87-Évora**	A
77-Elvas	A	**89a.-Évora**	A
78-Elvas	A	**90-Évora**	A
79-Elvas	A	**92-Évora**	A
83-Elvas	A	**93-Évora**	A
85-Elvas	A	**94-Évora**	A
CV-1-Elvas	n.dt.	**96-Évora**	A
CV-11-Elvas	n.dt.	**98-Évora**	A
CV-16-Elvas	n.dt.	**101-Évora**	A
CV-18-Elvas	A	**102-Évora**	A
		ST-2-Setúbal	n.dt.

Two different *nif*H RFLP patterns were obtained with the analysed isolates.

The same twenty-one isolates from all species groups were chosen for sequencing.

GenBank accession numbers for the *nif*H sequences determined in this study are DQ431732 to DQ431752.

Repeated attempts to amplify and sequence the *nif*H gene of *B. japonicum*, *M. amorphae* and *M. huakuii* type strains were unsuccessful.

Figure 30. Neighbour-Joining phylogeny based on *nif*H sequences from the 21 isolates (alignment length 562 bp). Percentage bootstrap support is shown on internal branches (1000 replicates); scale bar indicates 0.02 substitutions per site. Letters a, b and c indicate subgroups.

Regarding the phylogeny based on the *nif*H sequences (Figure 30), the chickpea symbionts form an independent branch, which received 100% bootstrap support. This branch includes the type strains of *M. ciceri* and *M. mediterraneum*, the two known chickpea microsymbionts. All other species from the genus *Mesorhizobium*, including *M. amorphae*, *M. loti* and *M. tianshanense*, are found outside this cluster. There are three subgroups (a, b and c), all with high bootstrap support.

6.2.1.2. *nod*C phylogeny

GenBank accession numbers for the *nod*C sequences determined in this study are DQ431753 to DQ431773.

Repeated attempts to amplify and sequence *B. japonicum*, *M. huakuii*, *M. plurifarium*, *R. leguminosarum* and *S. meliloti* type strains *nod*C were unsuccessful.

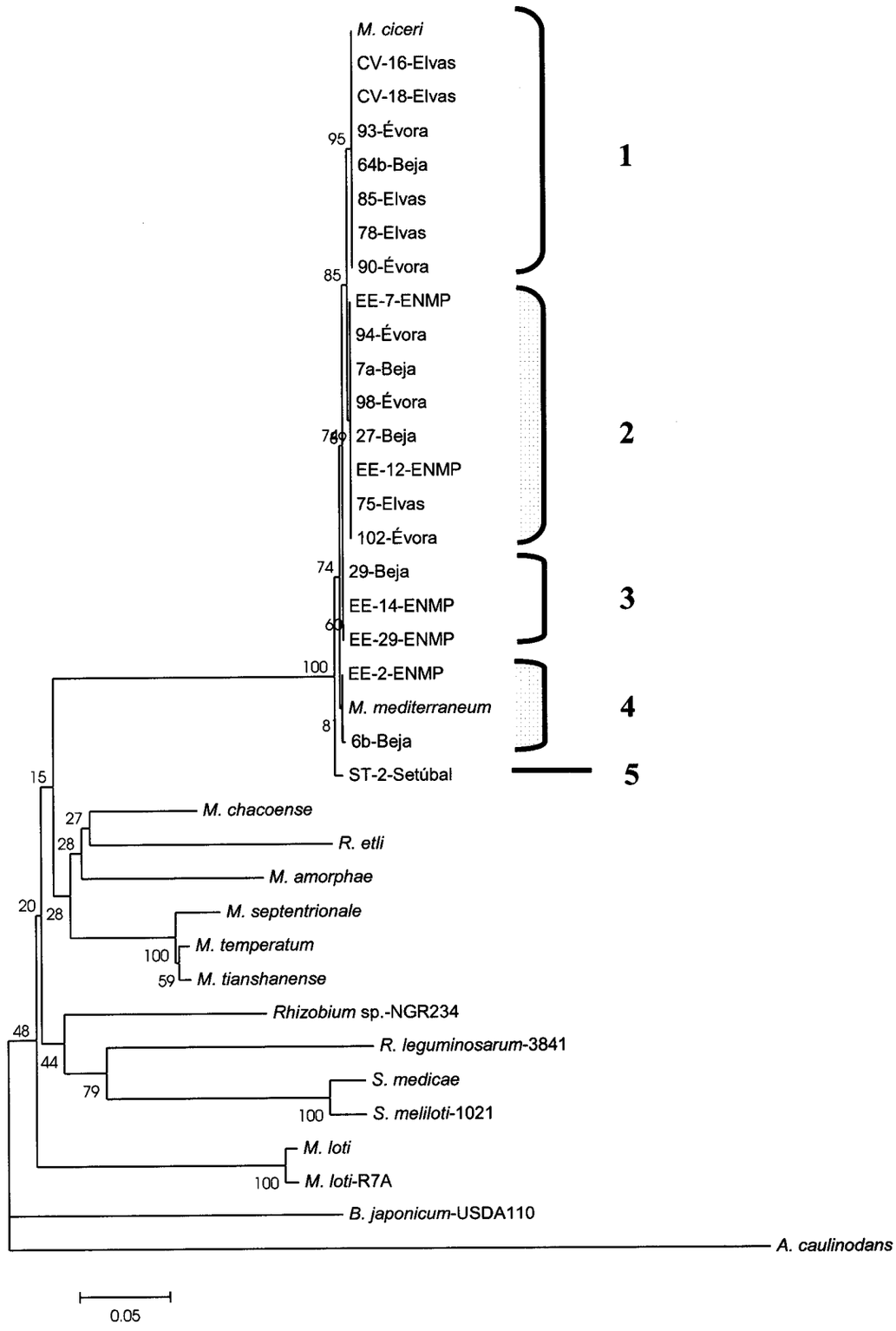

Figure 31. Neighbour-Joining phylogeny based on *nod*C sequences from the 21 isolates (alignment length 678 bp). Percentage bootstrap support is shown on internal branches (1000 replicates); scale bar indicates 0.05 substitutions per site. Number 1 to 5 indicate subgroups.

In the *nod*C based phylogeny (Figure 31) all chickpea symbionts are in the same cluster with 99% bootstrap support, which again includes the type strains of *M. ciceri* and *M. mediterraneum*, and can, in this case, be divided into five subgroups. All other *Mesorhizobium* species are outside this branch.

Phylogenetic analysis revealed identical topologies with neighbour-joining (Figure 30, Figure 31) and maximum likelihood methods (data not shown).

All chickpea rhizobia isolates carry almost identical *nif*H (Figure 30) and *nod*C (Figure 31) genes, despite their identification as different species based on 16S rDNA (Figure 6). So, different chromosomal backgrounds harbour similar symbiosis genes reflecting, in this case, host specificity towards chickpea.

Some common features are evident from analysis of the trees based on the two symbiosis genes, namely the separate position of isolate ST-2-Setúbal and the clustering of isolates 29-Beja, EE-14-ENMP and EE-29-ENMP. Furthermore, there is also a significant correlation between *nif*H and *nod*C groups (χ^2=42.000, df=8, P<0.001), which may suggest that these two symbiosis genes might have co-evolved. For instance, *nif*H group c corresponds to *nod*C group 3.

*nif*H sequences seem to be generally more conserved than *nod*C sequences. Indeed, chickpea microsymbionts *nif*H sequences are 99.1% to 100% identical, while the sequence identity between their *nod*C sequences is 98.3% to 100%.

There is no correlation between SE and the symbiosis genes, *nif*H and *nod*C, subgroups.

Table 20 shows the accession numbers of rhizobia reference and type strains.

Table 20. Rhizobia reference and type strains GenBank accession numbers or RhizoBase records of the sequences used in the phylogenetic analyses. Sequences in bold were determined in this study. n. dt.-not determined.

SPECIES	STRAIN	*nif*H	*nod*C
Azorhizobium caulinodans	ORS 571^T	M16709	L18897
Bradyrhizobium japonicum	USDA 6^T	n.dt.	n.dt.
B. japonicum	USDA 110	blr1769	blr2027
Mesorhizobium amorphae	ACCC 19665^T	n.dt.	AF217261
M. chacoense	LMG 19008^T	**DQ450927**	**DQ450937**
M. ciceri	UPM-Ca7^T	**DQ450928**	**DQ450938**
M. huakuii	CCBAU 2609^T	n.dt.	n.dt.
M. loti	NZP 2213^T	**DQ450929**	**DQ450939**
M. loti	R7A	ML0303	ML0132
M. mediterraneum	UPM-Ca36^T	**DQ450930**	**DQ450940**
M. plurifarium	ORS 1032^T	**DQ450931**	n.dt.
M. septentrionale	HAMBI 2582^T	**DQ450932**	**DQ450941**
M. temperatum	HAMBI 2583^T	**DQ450933**	**DQ450942**
M. tianshanense	A-1BS^T	**DQ450934**	**DQ450943**
Rhizobium sp.	NGR234	y4vK	y4hG
R. etli	CFN 42^T	RESP0005F	RESP0032F
R. leguminosarum	USDA 2370^T	**DQ450935**	n.dt.
R. leguminosarum bv. *viciae*	3841	pRL100162	pRL100187
Sinorhizobium medicae	A-321^T	**DQ450936**	**DQ450944**
S. meliloti	USDA 1002^T	M55229	n.dt.
S. meliloti	1021	SMa0825	SMa0866

6.2.2. Localization of symbiosis genes by Southern analysis

In order to determine the copy number of symbiosis genes, *nif*H and *nod*C, total DNA RFLP gels were transferred to nylon membranes and hybridised with corresponding probes.

Figure 32 shows a gel of a RFLP of total DNA and the corresponding Southern hybridisation with a *nif*H probe.

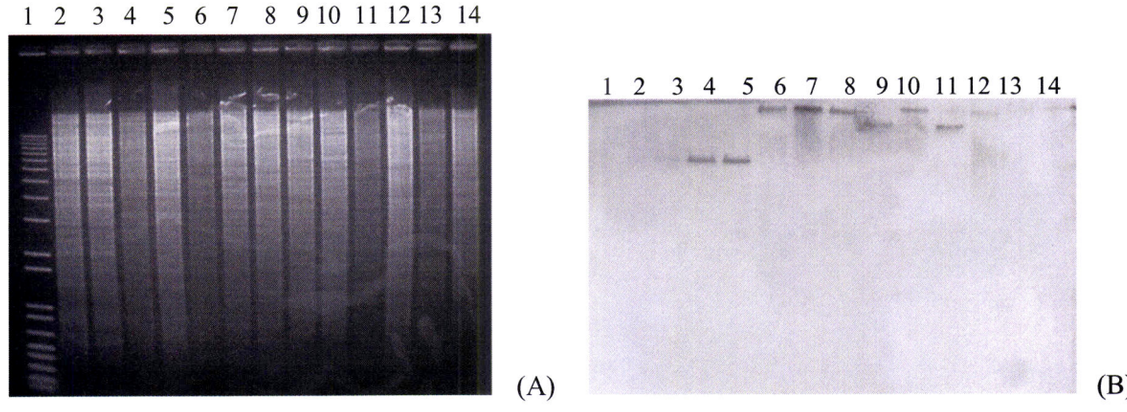

Figure 32. (on opposite page) RFLP of total DNA with *Bam*HI (A) and Southern hybridisation of total DNA with a *nif*H (*M. mediterraneum* UPM-Ca36^T) probe (B). Lane 1-1Kb DNA Plus Ladder (Invitrogen), lane 2-*S. meliloti*-1021, lane 3-*R. etli*-CFN42^T, lane 4-*M. ciceri*-UPM-Ca7^T, lane 5-*M. mediterraneum*-UPM-Ca36^T, lane 6-7a.-Beja, lane 7-29-Beja, lane 8-75-Elvas, lane 9-85-Elvas, lane 10-EE-7-ENMP, lane 11-EE-29-ENMP, lane 12-93-Évora, lane 13-98-Évora, lane 14-ST-2-Setúbal.

Hybridisation of the *nif*H probe with total DNA digested with endonucleases (Figure 32) indicated the presence of a single hybridisation signal in all analysed isolates. The absence of an hybridisation signal in *S. meliloti* and *R. etli* might be due to differences in gene sequence or to insufficient amount of DNA transferred to the membrane.

Plasmid gels were transferred to nylon membranes and hybridised with *nif*H and *nod*C probes in order to determine the location of symbiosis genes. Figure 33 and Figure 34 show plasmid profiles of isolates and hybridisation analysis with the two probes.

In most isolates, no hybridisation signal was detected in any plasmid, indicating chromosomal location of symbiosis genes, location in unresolved large megaplasmids or insufficient transfer of DNA to the membrane. Despite a heterologous probe was used, all analysed *nif*H sequences, including those of *M. ciceri* and *M. mediterraneum*, share more than 99% homology. With isolates 6b.-Beja, 29-Beja and 94-Évora, hybridisation with *nif*H probe revealed one signal and, with isolate 101-Évora. two signals were detected (Figure 33). With the *nod*C probe two signals were detected in isolates 29-Beja and EE-29-ENMP and one signal in isolate 6b.-Beja, (Figure 34).

Contrary to Southern hybridisation using plasmid gels, hybridisation of the *nod*C probe with total digested DNA (data not shown) revealed the existence of a single hybridisation signal. For isolates 6b.-Beja, 29-Beja and EE-29-ENMP, we can not exclude the hypothesis that the obtained single hybridisation signal might result from hybridisation to two fragments of the same molecular weight, both harbouring the *nod*C gene and generated by restriction of distinct plasmid DNA.

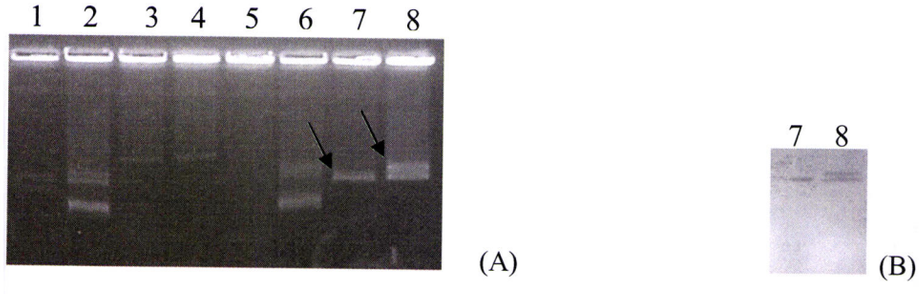

Figure 33. Plasmid profiles gel (A) and Southern hybridisation (B) of some isolates with a *nif*H (29-Beja) probe. Lane 1-6b.-Beja, lane 2-29-Beja, lane 3-78-Elvas, lane 4-85-Elvas, lane 5-EE-7-ENMP, lane 6-EE-14-ENMP, lane 7-94-Évora, lane 8-101-Évora.

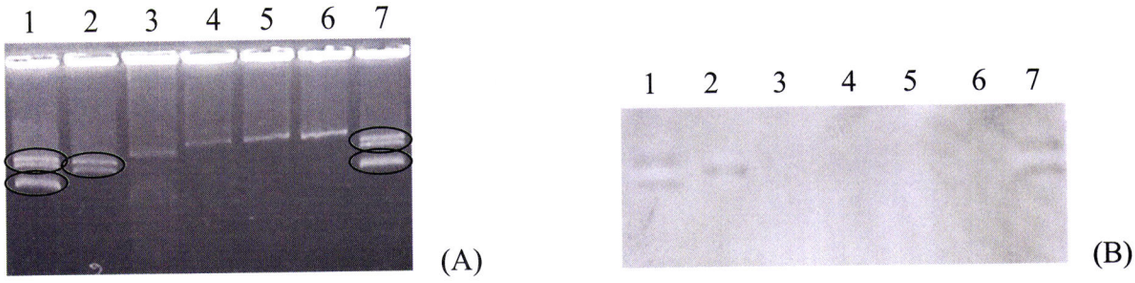

Figure 34. Plasmid profiles gel (A) and Southern hybridisation (B) of some isolates with a *nod*C (29-Beja) probe. Lane 1-29-Beja, lane 2-6b.-Beja, lane 3-7a.-Beja, lane 4-64b.-Beja, lane 5-78-Elvas, lane 6-CV-18-Elvas, lane 7-EE-29-ENMP.

6.3. Discussion

The symbiosis genes, *nif*H and *nod*C, from 21 chickpea mesorhizobia isolates were sequenced. Regardless of their species affiliation, all chickpea nodulating isolates, used in the present study, contained *nif*H and *nod*C sequences that cluster together in highly supported clades in the corresponding gene trees.

The *nif*H and *nod*C genes from the isolates used in this study show more than 99% and 98% identity, respectively, among them and with the type strains of *M. ciceri* and *M. mediterraneum*, which suggests a monophyletic origin of these symbiosis genes for the whole cluster of chickpea symbionts. The *nif*H and *nod*C are located more than 200 kb apart from one another on the chromosomal symbiosis island of sequenced strains *Mesorhizobium* sp. MAFF303099 and *M. loti* R7A (Kaneko *et al.*, 2000a; Sullivan *et al.*, 2002). It is probable that these genes have a common phylogenetic origin and were acquired together by lateral gene transfer (LGT) between different species enabling effective nodulation of chickpea.

LGT of symbiosis genes seems to be the most plausible hypothesis to explain the obtained incongruence between phylogenies based on symbiosis and core genes, such as 16S rDNA, as suggested before (Lan & Reeves, 2000; Sullivan & Ronson, 1998; Wernegreen & Riley, 1999). LGT might explain the similarity among *nif*H and *nod*C sequences from chickpea mesorhizobia belonging to different species. LGT among diverse rhizobia has been proposed before to explain the dispersion of symbiosis genes (González *et al.*, 2006; Haukka *et al.*, 1998; Laguerre *et al.*, 2001; Lindström *et al.*, 2002; Moulin *et al.*, 2001; Parker *et al.*, 2002; Sawada *et al.*, 2003; Sullivan *et al.*, 1995; Suominen *et al.*, 2001; Vinuesa *et al.*, 2005a; 2005b; 2005c). Symbiosis plasmids detected in the present study are smaller than, for example, p*Sym*A and p*Sym*B of *S. meliloti* (Downie & Young, 2001), which might

favour their easier exchange, explaining their dispersal among so many different *Mesorhizobium* species.

The occurrence of lateral gene transfer events across species in the *Mesorhizobium* genus, has been reported before for *Lotus corniculatus* and *Astragalus sinicus* symbionts (Sullivan *et al.*, 1996; Turner *et al.*, 2002).

The close position of all chickpea rhizobia in *nif*H and *nod*C phylogenies might also reflect the distribution of this legume mostly within the Mediterranean region. Besides the *nif*H and *nod*C from Portuguese isolates of the present study, all other *nif*H and *nod*C sequences available in the GenBank database, from chickpea rhizobia isolated in countries of the Mediterranean area, namely Spain (Rivas *et al.*, *In Press.;* Rivas *et al.*, *Submitted.*), Morocco (Sanjuan, J., unpublished results) and Iran (Asgharzadeh, A. and Hosseini-Mazinani, S. M., unpublished results), fall into the same clusters in the phylogeny of these two genes (data not shown).

Both *nif*H and *nod*C phylogenies evidence a close relationship among rhizobial strains nodulating the same host, however some cases of incongruence have been detected before (Laguerre *et al.*, 2001).

There must be some reason that could have constrained *nif*H relatively to *nod*C, as it seems they show a similar phylogeny for chickpea rhizobia, despite no plausible reason exists for *nif*H to be host related. The hypothesis is that plasmid symbiosis genes are horizontally transferred together, due to plasmid transfer, so that, although this transfer might be host conditioned, the genes responsible for nitrogen fixation are also transferred. It is possible that the *nod*C gene is chickpea rhizobia specific, since this gene has already been reported as host determinant (Roche *et al.*, 1996).

The *nif*H gene may have existed long before the symbiotic association between rhizobia and host legumes was established, and its phylogeny has been reported to be broadly more compatible with that based on 16S rDNA (Terefework *et al.*, 2000; Ueda *et al.*, 1995b; Young, 1992a), when *nif*H sequences from all groups of nitrogen-fixing organisms are considered. Similarly, the phylogenies for NifD, NifK and NifA have also been reported to agree with NifH (Young, 1993; Young, 2003b). However, Haukka and collaborators (1998) have looked at the genera of rhizobia and suggested that, at this level, the *nif*H phylogeny resembles the *nod*A phylogeny.

The phylogeny obtained with the *nod*C gene is in general agreement with the phylogenies reported by other authors for nodulation genes, such as *nod*A, *nod*B, *nod*C, *nod*I, *nod*J, *nod*Z, *noe*L, *nol*L (Moulin *et al.*, 2004; Suominen *et al.*, 2001; Wernegreen & Riley, 1999; Zhang *et al.*, 2000), which suggests that nodulation genes have had a common evolutionary origin.

The rhizobial host range is determined by several specific nodulation genes that confer them specificity for a given legume (Roche *et al.*, 1996). Rhizobia show various degrees of promiscuity (Perret *et al.*, 2000). The promiscuity of a given legume is related to the number of Nod factors it can interact with, rather than the chromosomal diversity of rhizobia, which are able to nodulate this legume (Downie, 1998). However, rhizobia that nodulate legumes of the tribes Cicereae, Trifolieae

and Vicieae are more specialised and, thus have more restricted host ranges (Broughton & Perret, 1999).

Chickpea has been considered a narrow-host range legume (Broughton & Perret, 1999; Gaur & Sen, 1979), mainly because it cannot be nodulated by broad host range rhizobia, such as *Rhizobium* sp. NGR234 (Broughton & Perret, 1999). Nevertheless, our studies, as well as others (Rivas *et al.*, *In Press.;* Rivas *et al.*, *Submitted.*), show that chickpea is able to establish symbioses with several species of *Mesorhizobium*, although these carry identical *nodC* and *nifH* genes. Therefore, our results suggest that chickpea can be considered a narrow host legume, since it is nodulated by rhizobia with similar symbiosis genes, which could lead to the production of similar Nod Factors. Similar results have been reported by others with *Bradyrhizobium* species (Jarabo-Lorenzo *et al.*, 2003).

Our results suggest that strains from different species, namely *M. loti* and *M. tianshanense*, as well as from two putative new species, acquired symbiosis genes similar to the ones of the known chickpea rhizobia species, *M. ciceri* and *M. mediterraneum*, and are thus able to effectively nodulate chickpea. They also show that distinct symbiosis genes may have been acquired by different strains from the same species depending on the legume hosts present in various geographical locations.

The number of rhizobia in the soil increases in the presence of a compatible host legume or after it has been cultivated (Young *et al.*, 2003). Thus, the occurrence of the *M. loti* related isolate 75-Elvas in the soil of Elvas might be related to the existence of *Lotus corniculatus* in Portuguese soils (Talavera *et al.*, 1999). However, this is more unlikely for isolates of *M. tianshanense*, since *Glycyrrhiza pallidiflora*, the host legume from which the first strains were isolated, does not exist in the Portuguese soils used in this study (Talavera *et al.*, 1999).

The GC content of accessory genome is generally lower than that of the core genome (Downie & Young, 2001; Galibert *et al.*, 2001; González *et al.*, 2006; Kaneko *et al.*, 2002a; Kaneko *et al.*, 2000a). The accessory genome has at least two distinct components (high and low GC) (J. Peter W. Young, personal communication). It has a history of horizontal gene transfer, but resides in a rhizobium long enough to acquire its characteristics. Symbiosis loci such as *nif*H and *nod*C belong to the accessory genome and have usually an evolutionary history independent of other loci. *nif*H usually has a higher GC content than *nod*C gene, at least in the available rhizobial complete genomes. The chickpea symbionts mean GC content is 60.2% for the *nif*H gene and 56.1% for the *nod*C gene (data not shown). As suggested before by Freiberg and collaborators (1997), it is possible that nodulation genes have evolved separately from nitrogen fixation genes. Hennecke (1985) and co-workers have pointed out that the GC content of the *nif*H genes is typical of the genomes that carry them.

Chitinases are plant enzymes responsible for Nod factors cleavage in the rhizosphere. Different chitinases inactivate specific Nod factors (Perret *et al.*, 2000). In the present work, the rhizobia phylogeny based on *nod*C nucleotide sequences correlates with the host phylogeny based on legume class III chitinases nucleotide sequences (data not shown). More legume chitinases sequences are needed, namely from the original hosts of mesorhizobia, in order to confirm this result, since only a

few sequences are available until now. These results agree with other studies that have been reported similar correlations between *nod*C and host phylogenies, based on leghemoglobin, ITS sequences and other data (Dobert *et al.*, 1994; Suominen *et al.*, 2001; Ueda *et al.*, 1995a; Wernegreen & Riley, 1999).

This broad correspondence between the *nod*C gene and legume phylogeny indicates that the association between chickpea and its microsymbionts has been stable in evolution, as suggested before (Wernegreen & Riley, 1999). However, rhizobia are able to grow as saprophytes in the soil, without being in symbiosis with any legume. Therefore, each rhizobium may come across different legume species and thus the opportunity for changes in host range is huge (Young & Johnston, 1989).

Symbiosis loci are unsuitable characters for the definition of species, but may be used in the description of biovars. These biovars should not be mistaken with new species based on their symbiosis genome or phenotype (Graham *et al.*, 1991; Lan & Reeves, 2001), since symbiosis plasmids or islands are easily exchangeable between bacteria.

Based on the present phylogenetic analyses of symbiosis *nif*H and *nod*C sequences, and on plant-infection experiments, we probably found one new biovar for chickpea isolates, namely *M. loti* bv. *ciceri*. However, a formal description is not proposed, since it only has one representative, namely isolate 75-Elvas, and more isolates are needed to substantiate this biovar.

Furthermore, our results have contributed to the proposal of the new biovar *M. tianshanense* bv. *ciceri*, suggested by Rivas *et al.* (*Submitted.*), that includes five chickpea rhizobia strains isolated in both Portugal and Spain.

The results obtained with the Southern hybridisation experiments regarding the *nif*H gene generally agree with the results previously obtained by PCR amplification of the *nif*H gene from plasmid DNA. In most isolates no hybridisation signal was observed, suggesting chromosomal location of symbiosis genes. One hybridisation signal was detected with the *nif*H probe in three isolates (6b.-Beja, 29-Beja and 94-Évora). In isolates 6b.-Beja and 29-Beja, the *nif*H gene had been previously detected in two plasmids by PCR amplification from plasmid DNA. It is possible that one of the amplification results was a false positive. However, the hybridisation signal obtained for these two isolates is diffuse and the hypothesis that it may correspond to hybridisation of the *nif*H probe to the two plasmids, closely located in the gel, can not be excluded. Nevertheless, in this case, the hybridisation signal should be stronger.

The existence of more than one symbiosis plasmid in chickpea rhizobia has been suggested before. However, the presence of the same symbiosis genes in more than one plasmid is uncommon (Barbour *et al.*, 1985; Harrison *et al.*, 1988; Rosenberg *et al.*, 1981). Reiteration of symbiosis genes generally occurs within the same replicon, plasmid (Freiberg *et al.*, 1997; Prakash & Atherly, 1984) or chromosome (Barbour *et al.*, 1985; Freiberg *et al.*, 1997; Prakash & Atherly, 1984; Quinto *et al.*, 1982; Quinto *et al.*, 1985). Extra copies of symbiosis genes have been proposed to be either pseudogenes, that do not confer any obvious selective advantage, in nodulation or nitrogen fixation, to the isolates that carry them, or functional genes induced by various environmental conditions (Scolnik

& Haselkorn, 1984). Rastogi and collaborators have reported reiteration of functional *nif*E and *nod*C genes (1992).

Interestingly, our results also suggest the existence of two copies of the *nod*C gene, in isolates 29-Beja and EE-29-ENMP.

The plasmid profiles of both 29-Beja and EE-29-ENMP are similar, and given the same hybridisation pattern observed in both isolates with the *nif*H (data not shown) and the *nod*C probe (Figure 34), we may speculate that these two isolates may indeed carry the same plasmids. This is even more interesting, since they are from different origins, but from the same species, which may suggest that plasmids do not move randomly among distinct species in the population. The association of certain plasmids to particular species is not totally unexpected, since it has been reported as a constant feature for *R. leguminosarum* strains (Young, 1992a), which could possibly be a sign of coevolution of plasmid and chromosomal bacterial genomes and host plant (Young & Wexler, 1988).

Since data for only a few isolates are available, we did not attempt to correlate SE with number of copies of both *nif*H and *nod*C. However, it is interesting to notice that isolate 29-Beja, the one with more than one *nif*H and *nod*C copies, shows a very high SE (71%), which may suggest that these could be functional copies.

In the present work the phylogenetic analysis of the symbiosis and accessory genome in *Mesorhizobium* has been performed. This study has contributed to the elucidation of the molecular bases of host specificity. However, some questions have also arisen from the results obtained and future work will include the investigation of the host range of these chickpea isolates.

7. GENERAL DISCUSSION

7.1. Phylogenies based on housekeeping loci and symbiosis genes

Sequencing of the 16S rRNA gene from 21 rhizobia revealed a diversity of sequences, despite being all isolated from *Cicer arietinum* L.

According to the 16S rDNA phylogeny (Figure 6), the 21 chickpea mesorhizobia isolates belong to six different species groups: six isolates to candidate species I (group **A1**), two isolates to candidate species II (group **A2**), two isolates to *M. tianshanense* (group **B**), five isolates to *M. mediterraneum* (group **C**), one isolate to *M. loti* (group **D1**), and five isolates to *M. ciceri* (group **D2**).

The presented 16S rDNA based phylogeny (Figure 6) is generally well supported, since all six species groups show high bootstrap values (68-98%).

Until recently, only two species were known to effectively nodulate chickpea, namely *M. ciceri* (Nour *et al.*, 1994a) and *M. mediterraneum* (Nour *et al.*, 1995). Chickpea rhizobia isolates different from *M. ciceri* and *M. mediterraneum* had been isolated before, but their species affiliation had not been investigated (Nour *et al.*, 1994b). Up to this work, *M. loti* and *M. tianshanense* strains were not known to nodulate chickpea.

From the analysis of the phylogenetic tree based on 16S rDNA sequences presented in Figure 6, it is clear that no branch of the evolutionary tree exclusively carries root nodule bacteria (Martínez-Romero & Caballero-Mellado, 1996; Young, 1996; Young & Haukka, 1996), which supports their polyphyletic origin. Furthermore, the great diversity in chromosomal backgrounds of rhizobia nodulating the same host indicates there has been a high level of LGT of symbiosis genes between rhizobia (Dobert *et al.*, 1994; Haukka *et al.*, 1998; Laguerre *et al.*, 2001; Moulin *et al.*, 2004; Normand & Bousquet, 1989; Souza & Eguiarte, 1997; Suominen *et al.*, 2001; Ueda *et al.*, 1995a; Young & Wexler, 1988; Young & Haukka, 1996; Zhang *et al.*, 2000).

A full length 16S rDNA sequence is now a pre-requisite for the description of a new bacterial species (Kämpfer *et al.*, 2003; Stackebrandt *et al.*, 2002). However, for a few years it has become clear that this molecule poses some problems and the use of its sequence in phylogenetic inferences therefore presents some limitations. In most bacteria, the presence of several rRNA operons, sometimes heterogeneous in sequence, has been reported (Cilia *et al.*, 1996; Eardly *et al.*, 2005; Hashimoto *et al.*, 2003; van Berkum *et al.*, 2003). However, 16S rRNA gene copies with different sequences does not seem to be a problem in rhizobia, since the sequences of 16S rRNA genes of rhizobial complete genomes are identical (Capela *et al.*, 2001; Galibert *et al.*, 2001; González *et al.*, 2006; Kaneko *et al.*, 2000a; Young *et al.*, 2006). Recombination among 16S rRNA sequences as well as transfer of 16S rDNA between strains or even species has been reported (Eardly *et al.*, 1996; Hashimoto *et al.*, 2003; Young & Haukka, 1996). A third problem of the use of 16S rDNA is that the resolution power of the gene is not enough to resolve relationships between closely related species

(Fox *et al.*, 1992; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002). Thus, sequencing of additional genes is necessary to assess phylogeny at the species level.

The phylogenetic trees of rhizobia isolates based on ITS, *atp*D, *rec*A and *gln*A are globally congruent pointing out the species assignment of the Portuguese chickpea isolates, already indicated by 16S rDNA sequence analysis, and provide strong support for the monophyly of the genus *Mesorhizobium*. They all support the existence of two putative new species (groups **A1** and **A2**), within the genus *Mesorhizobium*. However, the relationships between the different species within the genus are not the same for all loci. In particular, the positions of *M. chacoense* and *M. plurifarium* are cloudy, since they mostly seem to be apart from all other *Mesorhizobium* species, and, at the same time, their position is poorly supported, with bootstrap values mostly below 50%, specially in the protein-coding genes phylogenies. The position of candidate species **A1** within the genus *Mesorhizobium* also differs from tree to tree: it is closer to *M. ciceri* in the concatenated tree (Figure 27, Figure 28) and to *M. huakuii* in the 16S rDNA phylogeny (Figure 6).

Diversity and phylogeny of symbiosis genes *nif*H and *nod*C, were studied by sequence analysis. The resulting phylogenies were compared to each other, to the 16S rDNA phylogeny and to the host plant phylogeny.

Although the sequences of *nif*H and *nod*C genes were the only analysed, these can be considered as representative of the most common symbiosis genes, namely nitrogen fixation and nodulation genes.

The symbiosis genes of all chickpea mesorhizobia appear to be similar. Sequence analysis of the symbiosis genes *nif*H and *nod*C from all 21 isolates has revealed that independently of their chromosomal background, all chickpea nodulating isolates harbour similar symbiosis genes, which may suggest the occurrence of LGT among different *Mesorhizobium* species. This is quite expectable when their usual location in plasmids or islands is considered. In chapter 6 it was shown that some chickpea mesorhizobia can carry their symbiosis genes in p*Sym*, which had not been reported before for chickpea symbionts. Nevertheless, the possibly that some of our isolates carry their symbiosis genes in the chromosome, was not excluded.

The sequence analysis of the symbiosis *nif*H and *nod*C genes has shown that all isolates share very similar sequences, which may suggest the existence of lateral gene transfer between the different species in the soil.

The phylogenies for *nif*H and *nod*C genes from chickpea rhizobia are very similar supporting the occurrence of transfer as plasmids or symbiosis islands. All chickpea rhizobia were placed in the same large *nif*H and *nod*C sequence branches. Although it has been suggested that *nif*H phylogeny closely follows the 16S rDNA phylogeny at higher taxonomic levels (Hennecke *et al.*, 1985; Young, 1992b), whereas the phylogeny of *nod* genes is more closely related to the phylogeny of host plants (Dobert *et al.*, 1994; Ueda *et al.*, 1995a), our isolates *nif*H sequences cluster in a single branch, independently of their chromosomal background, supporting the existence of LGT.

The results obtained in the present study indicate that several factors may influence the phylogeny and evolution of symbiosis genes, namely host plant, geographical origin, chromosomal background and lateral gene transfer.

Our studies are similar to the ones by Zhang and co-workers (Zhang *et al.*, 2000), who have demonstrated that the nodulation genes of *M. huakuii* strains that nodulate *Astragalus sinicus* are conserved despite chromosomal diversity. This provided evidence for LGT of symbiosis genes among members of the genus *Mesorhizobium.*

Plasmids and chromosomal gene islands are key mobile elements involved in LGT, which leads to rapid evolution of bacterial genomes (Bushman, 2002), as well as fast adaptation and colonisation of different ecological niches (Souza & Eguiarte, 1997). The symbiosis genes of *Rhizobium* and *Sinorhizobium* are located on symbiosis plasmids and transfer of these plasmids between strains has been demonstrated in laboratory conditions (Hynes *et al.*, 1986; Kaijalainen & Lindström, 1989; Martínez *et al.*, 1987; Rogel *et al.*, 2001), in cultivated soils (Laguerre *et al.*, 1992; Louvrier *et al.*, 1996; Young & Wexler, 1988) and in natural soils (Wernegreen & Riley, 1999). On the contrary, the symbiosis genes are located in the chromosome, in a "symbiosis island", in *Bradyrhizobium* and *Mesorhizobium*, with the exception of *M. amorphae* (Wang *et al.*, 1999a) and *M. huakuii* (Zhang *et al.*, 2000). Other authors, have shown the presence of symbiosis genes in a chromosomal "symbiosis island" with the ability to transfer in the *M. loti* strain R7A (Sullivan & Ronson, 1998). Additionally, two putative "symbiosis islands" have been revealed by comparative genome sequence analysis in the complete genomes of *M. loti* strain MAFF303099 (Kaneko *et al.*, 2000a; Sullivan *et al.*, 2002) and *B. japonicum* strain USDA110 (Kaneko *et al.*, 2002a; Moulin *et al.*, 2004). However, the mobility of these two putative "symbiosis islands" has not yet been demonstrated.

Probably the isolates related to *M. loti* and *M. tianshanense* harbour the symbiosis genes of chickpea symbionts, and due to that are able to nodulate chickpea, since they do not appear to possess symbiosis genes identical to their type strains. *M. tianshanense* was originally isolated in China from *Glycyrrhiza pallidiflora* (Chen *et al.*, 1995), and this is the first time that it isolated from European soils and from a different host. It is also the first time that *M. loti*, which was originally isolated in New Zealand from *Lotus corniculatus* (Jarvis *et al.*, 1982), is recovered from chickpea nodules.

Since the host range of *M. loti* and *M. tianshanense* is not known, the type strains of these species were used to inoculate chickpea, and it was observed that they were unable to nodulate *Cicer arietinum* (data not shown).

In those isolates where the symbiosis genes, *nif*H and *nod*C, could not be detected in plasmids, but only in the whole genomic DNA, the hypothesis is that there has been an integration of symbiosis plasmid into the chromosome of the host bacteria. This has been reported before (Mavingui *et al.*, 2002) and reveals the plastic genome organisation of rhizobia. Another hypothesis could be that in these cases symbiosis genes are located in the chromosome in "symbiosis islands", which are also able to transfer between bacteria and may even transform a saprophyte in a symbiont (Sullivan *et al.*, 1995;

Tan *et al.*, 2001a). Thanks to these transfers, the integration of symbiosis functions in new bacteria enable a better adaptation to local environmental conditions and hosts specificities (Sessitsch *et al.*, 2002), and it is probably what happened with the strains of *M. loti* and *M. tianshanense* to effectively nodulate chickpea.

Insertion sequences that commonly flank symbiosis genes regions (Freiberg *et al.*, 1997; Mazurier *et al.*, 1996; Meneghetti *et al.*, 1996; Radeva *et al.*, 2001) may play an important role in the mechanisms of LGT of symbiosis genes. The symbiosis island of the *M. loti* strain R7A appears to function in a way similar to the integrative plasmids of other bacteria or pathogenesis islands (Sullivan & Ronson, 1998).

Recently, strains of *M. amorphae* and *M. tianshanense* able to nodulate chickpea have also been isolated in Spanish soils (Rivas *et al.*, *In Press.;* Rivas *et al.*, *Submitted.*).

There may be two new biovars, namely *M. loti* bv. *ciceri* and *M. tianshanense* bv. *ciceri*. However, the number of isolates characterised for each group, namely one and two isolates, respectively, does not allow the formal description to be taxonomically valid.

One of the most interesting findings of this work is that *nif*H and *nod*C genes from the common chickpea endosymbionts, *M. ciceri* and *M. mediterraneum*, are harboured by isolates of the six species groups. This means that, although nodulated by several different species, chickpea is indeed a restrictive host for nodulation, as suggested before (Broughton & Perret, 1999), because all these species share the same symbiosis genes. Chickpea probably only recognises a minority of Nod factors (Downie, 1998), which are encoded by the symbiosis genes of *M. ciceri* and *M. mediterraneum*.

Naturally, for a rhizobium to produce Nod factors that will be recognised by chickpea, so that the bacterium can enter its root and form nodules, several other nodulation genes are needed, besides *nod*C, such as the common nodulation genes *nod*A and *nod*B.

The diversity of *nod* genes carried by a rhizobium determines the structural variation of Nod factors, which, in turn, determines the host range of legumes that a particular strain is able to nodulate (Downie, 1994; Downie, 1998). A single strain can produce a diversity of Nod factors and the number of Nod factors produced may be proportional to the number of hosts it can nodulate (Downie, 1994). Therefore, the isolate related to *M. loti* (75-Elvas) and the two isolates from *M. tianshanense* (93-Évora and ST-2-Setúbal) might originally have their own set of symbiosis genes. Later, they acquired the symbiosis genes from one of the common chickpea endosymbionts by LGT, and their nodulation range was either changed or extended to chickpea. In order to test this hypothesis nodulation tests would have to be performed with these strains on the original hosts of *M. loti* and *M. tianshanense*, *Lotus corniculatus* and *Glycyrrhiza pallidiflora*, respectively. We can expect that they are not able to nodulate the original hosts, otherwise they should carry the required symbiosis genes. However, the possibility that they have additional copies of *nif*H and *nod*C, that would allow them to effectively nodulate these hosts, can not be excluded.

The chromosomal background of a rhizobium may also influence nodulation (Roest *et al.*, 1997). The hypothesis of different regulation of the same symbiosis genes in different chromosomal backgrounds, namely different transcription levels, would explain why rhizobia harbouring similar or even the same symbiosis genes may exhibited such different SE values on the same host, chickpea.

This is the first molecular phylogenetics study of mesorhizobia that used a multilocus sequence analysis approach for species delineation. It is also the first report on evidence of LGT in chickpea mesorhizobia.

The present work has changed the current view of chickpea symbionts and contributed to the further knowledge of rhizobia phylogeny in general. Variation in core-symbiosis genes associations in rhizobia might constitute a problem for the classification of rhizobia. Until now rhizobial species descriptions have always included important accessory characteristics, such as symbiotic abilities. However, these are encoded by genes that are prone to LGT, and this study has contributed further to the knowledge that rhizobia with different chromosomal backgrounds may carry similar symbiosis genes. This may explain why a restrictive host for nodulation, such as chickpea, is nodulated by several different rhizobia species.

7.2. Diversity of chickpea mesorhizobia

Strains of different mesorhizobia species able to effectively nodulate chickpea have been isolated from the four studied soils from the Portuguese Alentejo region.

Several methods were used to characterise 41 Portuguese chickpea rhizobia strains and to evaluate diversity within and between different natural populations: 16S rDNA RFLP analysis (chapter 3), auxanographic tests, symbiotic effectiveness (SE), DAPD fingerprinting, SDS-PAGE total protein patterns and plasmid profiles (chapter 4).

The methods of DAPD, SDS-PAGE total protein and plasmid profiles have been shown to be useful for the evaluation of rhizobial genetic diversity. However, DAPD analysis is a better strain differentiation method, since it has shown good resolution at the species level. Furthermore, DAPD profiles are a fast, easy and relatively cheap method to routinely implement in the laboratory. Total protein profiles, contrary to the results obtained by other authors (de Lajudie *et al.*, 1994; Nick *et al.*, 1999a; Velázquez *et al.*, 2001), have a low resolution power at the species level and does not seem to be an appropriate method for strain differentiation in mesorhizobia.

If a single method had to be chosen for evaluation of genetic diversity of rhizobia populations and strain differentiation, that method would be DAPD fingerprinting.

Although the increased SE of isolates from 16S rDNA sequence species group **A1** can not be related to the presence of a particular plasmid, all isolates from this group have one megaplasmid,

which has approximately the same molecular weight in four isolates (64b.-Beja, 78-Elvas, 85-Elvas and CV-18-Elvas), with the exception of isolate 102-Évora, in which no plasmids were detected.

Cryptic plasmids in rhizobia are also known to influence nodulation and competitiveness for nodule occupation (Bromfield *et al.*, 1985; Pankhurst *et al.*, 1986; Toro & Olivares, 1986).

Nitrogen fixation has been related with cryptic plasmids (Baldani *et al.*, 1992; Brom *et al.*, 1992; Hynes & McGregor, 1990; Kuykendall *et al.*, 1994; Pankhurst *et al.*, 1986; Thurman *et al.*, 1985). Some authors have reported that the presence of cryptic plasmid inhibits nitrogen fixation (Barbour & Elkan, 1989; O'Connell *et al.*, 1998; Velázquez *et al.*, 1995), while others have described a positive effect of cryptic plasmid on nitrogen fixation (Martínez *et al.*, 1987).

Furthermore, plasmid number has been reported to be negatively correlated with SE (Harrison *et al.*, 1988; Thurman *et al.*, 1985). Although most of the highly effective isolates studied in this work have one or no plasmids, isolate 29-Beja, which has six plasmids, is the exception, since it has a SE of 71% (chapter 4).

7.3. New species of *Mesorhizobium*

Entering a new era, where the term "prokaryote" no longer has a place (Sapp, 2005), it is time to re-evaluate how to define bacterial species (Gevers *et al.*, 2005; Kämpfer *et al.*, 2003; Stackebrandt *et al.*, 2002). A biological species concept has been suggested as probable good approach for defining bacterial species as a group of strains that share a common gene pool (Cohan, 2002; Dykhuizen & Green, 1991).

The gene coding for 16S rDNA is at the moment still the most commonly used molecule for phylogenetic characterisation in bacteria. However, there are some limitations to its use, namely the existence of several copies (Cilia *et al.*, 1996; Clayton *et al.*, 1995; Coenye & Vandamme, 2003) and the recognition that it may be subjected to LGT (van Berkum *et al.*, 2003). Therefore, alternative phylogenetic markers have been searched for. Genes to include in comparative sequence analyses should, if possible, be widely distributed, not be frequently transmitted by LGT, be present in single copy, be long enough to contain sufficient information and short enough to allow easy sequencing, be neither too conserved nor too variable (Gevers & Coenye, In Press). Nevertheless, few genes will be found that can be used universally and match these criteria. Thus, group specific markers will probably be the most successful approach.

The currently described species of rhizobia are distinctive by polyphasic analysis, including cultural characteristics, and the standard description criteria, DNA-DNA hybridisation and 16S rDNA sequence (Graham *et al.*, 1991). However, it has been suggested that these methods may not always provide accurate estimates of evolutionary relationships and new approaches should be tested (van Berkum & Eardly, 1998).

At least one new species of mesorhizobia able to nodulate chickpea is proposed based on 16S rDNA, ITS, *atp*D, *rec*A and *gln*A sequences, and named *Mesorhizobium lusitanum* (group **A1**).

Looking at the individual trees based on each of the five loci, it could be concluded that among the studied isolates there are at least two candidate species (**A1** and **A2**) within the genus *Mesorhizobium*. About the phylogeny of these new groups within the genus not much can be concluded, as the bootstrap values for the individual loci and even for the combined (*atp*D, *rec*A and *gln*A) tree are low.

*gln*A and *atp*D strongly support three new species, ITS and *rec*A support at least two new species, and, compared to this, the resolution of the 16S rDNA is limited, as was known before (Coenye *et al.*, 2005; Fox *et al.*, 1992; Hanage *et al.*, 2005; Kwon *et al.*, 2005; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002).

A formal description of a second putative new species (group **A2**) has not been done in the present work, because only four isolates belonging to this new group were found and, according to the most recent guidelines, species descriptions should include at least five isolates (Christensen *et al.*, 2001; Stackebrandt *et al.*, 2002).

Both new species are supported by both DAPD profiles and total protein profiles.

Isolates from *M. lusitanum* have a high symbiotic effectiveness. Furthermore, they are acid tolerant (Brígido *et al.*, *In Press.;* Rodrigues *et al.*, 2006) and show an elevated survival rate when subjected to heat shocks (our unpublished results). On the other hand, isolate 64b.-Beja is very competitive when used together with other strains (L. Alho, personal communication).

7.4. Perspectives for future studies

This study clearly demonstrated that genetically and phenotypically diverse strains are able to nodulate chickpea. Phylogenetic analysis based on symbiosis genes suggests LGT of symbiosis genes between chickpea endosymbionts.

The success of establishment of an inoculant strain in the soil depends, among other things, on the indigenous rhizobia population in that soil. Therefore, it seemed important to investigate the native populations of agricultural soils, and in particular, their symbiotic properties, where chickpea is to be cultivated and there is the need for inoculation with compatible rhizobia.

The proposed new species *M. lusitanum* isolates share several characteristics which are very interesting from the point of view of inoculants production. Stress response to different environmental conditions should be carefully investigated, and special attention should be paid to the nodulation competitiveness of some isolates in the field.

Further studies will be conducted on the four isolates of candidate species II (group **A2**) and new isolates belonging to this species will be searched for.

The molecular bases of symbiosis will be investigated using the symbiosis between chickpea and different mesorhizobia as a model. In particular, the hypothesis that the same nodulation genes would allow effective nodulation of several hosts will be investigated. The possibility that different transcription levels of the same nitrogen fixation genes might be responsible for different SE values in different strains will also be considered.

It will also be worthwhile to further examine the stress tolerance to different environmental conditions of symbiotically effective isolates, and to go a step forward towards the understanding of the molecular bases of stress response in rhizobia.

Improved knowledge on the symbiosis and stress characteristics of rhizobia at the molecular level, will allow the future use of successful field inoculants.

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