Tansley review

Evolving ideas of legume evolution and diversity: a taxonomic perspective on the occurrence of nodulation

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Summary

Legumes evolved about 60 million years ago (Ma), and nodulation 58 Ma. Nondodulation remains common in Caesalpinioideae, with smaller numbers in Mimosoideae and Papilionoideae. The first type of infection by bacteria may have been at junctions where lateral roots emerged, followed by formation of infection threads to confine bacteria and convey them to some cells in the developing nodule, where they were generally released into symbiosomes. Infection threads were a prerequisite for root-hair infection, a process better controlled by the host, leading to a higher degree of specificity between symbionts. An alternative process, dating from the same time and persisting in about 25% of legumes, did not involve infection threads, bacteria entering a few host cells, surrounded by an undefined matrix. These cells divided repeatedly to give uniform infected tissue, with bacteria released into symbiosomes. Such legumes may have less stringent control of nodulation processes, and are found mainly in tropical and warm temperate areas. In each type of nodule, meristems may or may not be retained, leading to indeterminate or determinate forms. Nodule morphology and structure are host-determined, but the effectiveness of nitrogen fixation is largely controlled by the bacterial symbionts, which vary greatly in genotypic and phenotypic characters.


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I. When and where did legumes evolve?

It used to be thought that legumes evolved, probably in the humid tropics, in the late Cretaceous. The exact time was difficult to pinpoint because of the paucity and uncertain affinities of many fossils. Recent molecular studies have helped to improve this situation. Lavin et al. (2005) carried out an extensive analysis in which they rooted molecular data with well characterized fossils. In this way they produced an overall chronogram for the family and related this to phylogeny. The general conclusion was that legumes probably evolved approx. 60 million years ago (Ma), early in the Tertiary period. This was after the K/T extinction, and a time at which many other angiosperm families also first appeared (see discussion in Schrire et al., 2005a). In terms of the position of land masses, the older hypothesis of legume evolution suggested a location in western Gondwana (austrotropical), whereas a more recent evolution is consistent with a northern, Laurasian origin. In terms of spread of legumes, ideas changed from direct movement between South America and Africa (ruled out because these continents separated long before legumes evolved) to a 'boreal hypothesis' in which legumes spread northwards from the Americas, via the boreotropics and then southwards to reach Africa and Asia. These ideas are discussed fully by Doyle & Luckow (2003). Although the boreal hypothesis explains many observations, it still leaves important questions unanswered, in particular geographical disjunctions within and between genera. Its underlying rationale is that legume distribution is largely explained in terms of movement of land masses. Schrire et al. (2005a, 2005b) tested this hypothesis against their very detailed analysis of the biomes in which extant legumes are found. Their conclusion was that legume distribution is best explained as beginning with an origin in the region to the north of the Tethys Sea, a shallow stretch of salt water that separated the two main groups of land masses. Here conditions were semiarid, and the first legumes would have belonged to a succulent biome (also known as seasonally deciduous dry forests) with later spread into other biomes, including tropical rainforests. Current distribution is considered to be related more to biogeographical factors such as rainfall and temperature than to rafting of land masses. This, in itself, poses some interesting questions as to how legumes moved between continents; suggestions that would once have been unthinkable, such as movement between small islands as some sea levels dropped (Pennington et al., 2006 and references therein) and movement in extreme events such as tropical cyclones (Nathan, 2006), are now emerging as strong possibilities. These arguments will not be discussed here, except as they relate to nodulation and nitrogen fixation.

II. Evolution of legumes and nodulation

Apart from *Gunnera*, all flowering plants having symbiotic N-fixing symbioses fall in a single clade, Rosid I (Soltis et al., 2000). Figure 1 shows an outline of this clade. Plants that nodulate with the filamentous bacterium *Frankia*, termed actinorhizal plants, are scattered throughout the clade and are found in many different families. Within families, closely related genera may (Alnus) or may not (Betula) nodulate. Plants that nodulate with unicellular bacteria, collectively known as rhizobia, occur only in the Fabales, and within that group the Leguminosae, with the single exception of *Parasponia* (Ulmaceae). A detailed comparison of the nodules in these two plant groups can be found in Pawlowski & Sprent (in press). Although it is now known that many of the genes for nodulation have been acquired following duplication of those with related functions (Sprent, in press and references therein), it is not known whether all legumes have these extra genes and, if so, why they are not expressed in nonnodulating forms. In this review, the terminology of Lewis et al. (2005) is adopted, using Leguminosae, rather than Fabaceae, for the whole family, divided into three subfamilies: Caesalpinioideae,
Mimosoideae and Papilionoideae. All genera are as given by Lewis et al. (2005), and specific names are those accepted by the International Legume Database & Information Service (www.ildis.org). Bacteria that nodulate legumes are classified as α- or β- according to the branch of the Proteobacteria to which they belong. The former are further subdivided into ‘classical’ and ‘other’. Classical rhizobia are all those whose generic names include rhizobium, e.g. *Bradyrhizobium*, *Mesorhizobium* and *Rhizobium*. Sometimes, when exact genera are not known, the older phenotypic designations of fast- or slow-growing will be used.

For many years it was thought that the three subfamilies evolved in the order Caesalpinioideae, Mimosoideae and Papilionoideae, and the fact that nodulation is uncommon in the caesalpinioids, more common in mimosoids and very common in papilionoids reflected this sequence (Allen & Allen, 1981). Although there have been many new reports since 1981, the general distribution of nodulation among the subfamilies has not changed significantly; the currently known occurrence of nodulation in all tribes is given in Table S1 in Supplementary Material. However, the sequential evolution of the subfamilies is not consistent with recent evidence (Lavin et al., 2005). In this work, molecular data are aligned with well established fossil data. Evolutionary patterns are divided into ‘stems’ and ‘crowns’, the former being periods of relatively little diversification and the latter bursts of diversification. Assuming legumes to have appeared first approx. 60 Ma, there is a very short stem of 1–2 Ma followed by a crown in which elements of caesalpinioid and papilionoid legumes occur, with mimosoids branching from the caesalpinioid stem rather later (40 Ma). Figure 2 outlines some of the relations between the various stems and crowns as they relate to nodulation. Details of the ages of the nodes are given in Table S2. It must be stressed that these data will be refined as more information becomes available, and that the number of genera used is small relative to the whole family. Further, methodologies for dating such clades may change, leading to changes in their relative positions. Nevertheless, the paper of Lavin et al. (2005) is a seminal piece of work and, coupled with the volume edited by Lewis et al. (2005), which deals with all currently recognized legume genera, allows some tentative conclusions to be drawn about the origin and significance of the infection of legume roots by rhizobia and the subsequent formation of root nodules. As no fossil nodules are known, interpretations are based on current structures (Figs 3 and 4), which may have changed since the relevant genera first evolved.

III. Taxonomic assessment of nodulation

1. Caesalpinioideae

Lewis et al. (2005) divide Caesalpinioideae into four tribes (Table S2). Nodulation occurs in two, Cassiaeae and Caesalpiniaeae. Fewer genera are included in the study of Lavin et al. (2005), but comparison of the two sets of data shows that two of the groups that do not nodulate, roughly corresponding to tribes Cercideae and Detarieae, are well separated from the others (Fig. 2a, top branch) Although their nodes are relatively recent, 34 and 29 Ma, their stem clade ages show them to be early divergent lineages in the Caesalpinioideae. They are currently found principally in tropical rainforests, possibly by spreading there after evolving in dry deciduous (succulent biome) habitats (Schrire et al., 2005a, 2005b). In tribe Cassiaeae, the subtribe Cassiinae has three genera, *Cassia*, *Senna* and *Chamaecrista*—only the latter nodulates, and in the analysis of Doyle et al. (1997) it was found to be distinct from the other two. *Chamaecrista* is a large genus, with c. 333 species. Although mainly tropical, unusually for the subfamily, its species have extended more recently into temperate areas where herbaceous forms can be found, for instance, in grassy habitats of New England. Most species are shrubby and a few are trees. This range of habit and habitat is paralleled by a range in the structure of infected cells. In tree species, N-fixing bacteroids are retained within modified infection threads, known as fixation threads (Fig. 4d); in shrubby species, the walls of these threads are thinner; and in herbaceous species, bacteroids are released into symbiosomes (Naisbitt et al., 1992) as in all mimosoid and most papilionoid legumes (Fig. 4b).

According to Lavin et al. (2005), *Chamaecrista* is included in a crown node of 54.5 Ma, as are some members of the large tribe Caesalpiniaeae, which contains all other caesalpinioid genera confirmed to nodulate. These are *Campsiantra*, *Dimorphandra*, *Erythrophleum*, *Melanoxylon*, *Moldenhauera* and *Tachigali*, which now includes the nodulating genus *Sclerolobium*. Depending on the particular molecular characters scored, the relatedness of the nodulating genera appears to vary considerably. In the analysis of Haston et al. (2003), based on the *trnL* intron of the chloroplast genome, *Tachigali*, *Sclerolobium* and *Moldenhauera* are close, with *Dimorphandra* near and *Melanoxylon* further away, but Haston et al. (2005) note that future molecular data may result in there being closer grouping of the nodulated genera. No nodulating genus is included in the analysis of Lavin et al. (2005). All nodulated genera in the Caesalpiniaeae are trees, and all have fixation threads in their active nodules. All are South American, with the exception of *Erythrophleum*, which is found in Africa, Asia and Australia. Thus it appears that nodulation in these genera may be an ancient character. Because of the unique nodule structure, it is most parsimonious to assume that a single nodulation event occurred, as suggested in Fig. 2a. The limited evidence available suggests that caesalpinioid legumes can nodulate with a variety of classical rhizobia (de Lajudie et al., 1998 for *Chamaecrista*; Moreira et al., 1998 for *Dimorphandra*; Parker, 2000 for *Tachigali*).

If it is assumed that possession of arbuscular mycorrhizas (AM) is ancestral to angiosperms (Fitter & Moyersoen, 1996 and references therein), then we may infer that all early legumes were AM. Alexander (1989) looked at the distribution of AM
Fig. 2 Position of the major nodulating groups in the Leguminosae, as shown in the chronological diagrams of Lavin et al. (2005). (a) Caesalpinioideae and Mimosoideae; (b) Papilionoideae.
and ectomycorrhizas (ECT) in legumes, and found that, of the tribes of Caesalpinioideae recognized at that time, Cassieae, Caesalpinieae, Cercideae and Detarieae were AM and Amherstieae mainly ECT. The latter tribe is no longer recognized, with genera being included in Detarieae by Lewis et al. (2005).

In the molecular analysis of Lavin et al. (2005), a number of known ECT genera are grouped as a branch of caesalpinoid crown node 3 dated at 29.2 Ma (Fig. 2a). However, some of these genera are also able to form AM, usually at low levels. The extent of formation of AM is location-dependent (Moyersoen & Fitter, 1999), a point that will be considered later. Some recent evidence suggests that ectotrophic mycorrhizas in legumes may be a more ancient character than generally thought (Alexander, 2006).

2. Mimosoideae

The mimosoid crown node is dated by Lavin et al. (2005) at approx. 42 Ma. However, there is a rather long stem (approx. 15 Ma) before the crown group starts to proliferate into most of the tribes recognizable today. Tribal phylogeny is less well resolved than in the other two subfamilies, making it difficult to map the occurrence of nodulation (Sprent, 2001). The fact that some of the putative mimosoid fossils may belong to extinct lineages adds a further complication (Lavin et al., 2005).

Three tribes are recognized, with the former Parkieae and Mimozygantheae being included in tribe Mimoseae (Luckow, 2005; Luckow et al., 2005a), which still remains one of convenience. Most of the older groups in Lavin et al. (2005) appear not to nodulate, exceptions being Entada and some species of Pentaclethra. The remaining members of tribe Mimoseae and tribes Acacieae and Ingeae (dating from approx. 30.5 Ma) are uniformly nodulated, apart from a few that have lost their nodulating ability.

Compared with the Papilionoideae, nodule characters are very uniform, all nodules studied being indeterminate with varying degrees of branching; and (with a few exceptions, detailed later) infection is through root hairs. As this involves
infection threads, and as these are also the norm in caesalpinioideanodules, nodules in mimosoid legumes may have arisen from the same event as in the Caesalpinoideae, but this leaves a major problem in that the apparently most basally branching genera cannot nodulate (Fig. 2a). Species of tribe Mimosae may be nodulated by β-rhizobia (Barrett & Parker, 2005; Elliott et al., 2007).

The taxonomy of Acacia has been in a state of flux for many years. For convenience, most people have used three subgenera, Acacia, Aculeiferum and Phyllodineae, to separate what have been known for a long time to represent at least three different genera, with Aculeiferum being polyphyletic. Molecular evidence puts Phyllodineae firmly in tribe Ingeae, with the other two retained in tribe Acacieae. At the 2005 Botanical Congress in Vienna, it was agreed by a very narrow vote to retain the name Acacia for Phyllodineae, opening up the recognition at generic level of the names Vachellia (= subgenus Acacia), Senegalia (= much of subgenus Aculeiferum), Acaciella and two as-yet unnamed genera. This overturns usual procedures in taxonomy and has upset many who believe that subgenus Acacia, rather than Phyllodineae, should have retained this name. For further details of this controversy see Lewis (2005); Luckow et al. (2005b); www.worldwidewattle.com). In this review, the earlier classification into subgenera will be retained. From a symbiotic rather than a purely nodulation point of view, there are clear differences between the largely Australian subgenus Phyllodineae and the other subgenera, as many of its species are able to form both AM and ECT, even when grown in Africa or Brazil, whereas local ‘acacias’ do not. This may be one reason for their being so invasive in parts of the world such as South Africa (Sprent, 2001). Although acacias can nodulate with a wide range of α-rhizobia, there are differences among the constituent subgenera (Pueppke & Broughton, 1999), each having its own spectrum of symbionts.

Fig. 4 Major types of nodule. (a) Determinate nodules as found in Millettoid tribes and the Lotaeae (Fig. 3b). Size varies from 2 to 5 mm in diameter. (b) Symbiosomes: these membrane-bound vesicles may contain one or more bacteroids (the nitrogen-fixing form of rhizobia), usually rod-shaped but sometime spherical or pleiomorphic. (c) Indeterminate nodule: the most common form of nodule. May be unbranched, or extensively branched, as here, annual or perennial; vary in size from 3 mm to several centimetres long. (d) Broken fixation thread through which bacteroids can be clearly seen. Found in caesalpinioideanodules. (e) Infected tissue containing a mixture of infected and uninfected cells (Fig. 3a,b). (f) Infected tissue containing only infected cells (Fig. 3c–e).
and a wide range of nodule effectiveness (e.g. Burdon et al., 1999 for *Phyllodineae*. Ben Romdhane et al., 2006 for *Acacia tortilis* ssp. *radiana* (*Aculeiferum*). Some species of subgenus *Aculeiferum*, section *Monacanthea*, most of which are scrambling shrubs found at the edge of thickets in South America, Texas and parts of Africa, have lost their ability to nodulate (Sprent, 2001). All the nonnodulating species used in the phylogenetic analysis of Miller et al. (2003) form a very distinct clade, suggesting a single event leading to loss of nodulation: this illustrates the type of disjunction of related species found between continents (Schrire et al., 2005a, 2005b). The only nonnodulating species included by Lavin et al. (2005), *Acacia greggii*, is dated at approx. 15–20 Ma.

Many acacias worldwide, particularly members of subgenera *Aculeiferum* and *Acacia*, are now found in low-nutrient, dry and sometimes saline soils. Nitrogen is only one of many factors limiting plant growth, and any N fixation that does occur is restricted to periods where soil moisture is present. Where the soil is deep and sandy, such as in large parts of Africa (e.g. Sudan, Senegal), woody plants first develop extensive tap-root systems, before producing much shoot new matter, and certainly before producing root nodules. For example, *Acacia senegal* seedlings can have tap roots nearly 2 m long when 2 months old. In dry soils, nodules do not form even when the soil contains large numbers of compatible rhizobia, with potentially quite high rates of N fixation (Fagg & Allison, 2004 and references therein). This species, and others such as *Acacia polyacantha* and *Acacia mellifera*, belong to subgenus *Aculeiferum*, but are genetically distinct from the group that has lost the ability to nodulate (Harrier et al., 2002). Plants from these two groups may grow in close proximity, for example *Acacia brevispica* (nonnodulating) and *A. mellifera* (nodulating) in low-N soil of the semiarid Loruk region of Kenya (Odey et al., 2002). In these conditions, is the ability to nodulate of relatively little value? Does it matter that the plant bears nodules with a range of efficiencies? Potential rates of photosynthesis are high, so that any carbon drain on the plant by essentially parasitic nodules may not be significant. In order to nodulate with a range of rhizobia, plant roots must exude a wide range of compounds that can induce the relevant bacteria to produce their own nodule-inducing signals for transmission to the host. There is some evidence that this is the case in acacias (Shaw et al., 1997). As far as the rhizobia are concerned, it may be sufficient for them to find a niche where they get protection from adverse soil conditions, as well as a source of nutrients.

### 3. Papilionoideae

In Lewis et al. (2005), 28 tribes are retained for this subfamily. As with the other subfamilies, there are likely to be changes in the tribal affiliations in the next few years. In the analysis of Lavin et al. (2005), Papilionoideae is divided among distinct groups, and these will form the basis of the present discussion (Fig. 2b). The phylogeny and chronology are not fully congruent, so precedence will be given here to chronology, as it raises some interesting questions and also provides some answers about nodulation.

The papilionoid crown node is dated as 58.6 Ma. Before the next major crowns, the genistoid and dalbergioid, there is a rather ill-defined group of genera, most of which cannot nodulate, containing members of various tribes, including several from the Swartziaeae. However, there is a distinct *Swartzia* node, dated at approx. 49 Ma, which contains all the genera (*Ateleia*, *Bobgunnia*, *Cythostegia* and *Swartzia*) in tribe Swartziaeae that are confirmed to nodulate. From a nodulation point of view, these genera sit uncomfortably here, as almost all other genera in the Papilionoideae (except a few that have lost the ability) can nodulate. Intuitively, it seems unlikely that there was a separate nodulation event in a small group of swartzioid legumes. It is possible that, with sampling of more species and more detailed analysis, preferably including nodulation as a character, the positions of these branches may change.

Between the genistoid and dalbergioid nodes, dated at 56.4 and 55.3 Ma, respectively (Lavin et al., 2005) are two genera, *Hymenolobium* and *Andira*, which have recently been excluded from the dalbergioid clade for many reasons, mainly molecular, but also because they do not have the aescynomoid type of nodule that typifies the clade (Lavin et al., 2001). Rather, they have nodules where bacteroids are retained in fixation threads (Fig. 4d), as in the Caesalpinioideae. Three other papilionoid genera share this character: *Poecilanthe* and *Cyclolobium*, both formerly in tribe Millettieae, now in Brongniartaeae (Ross & Crisp, 2005), but in the genistoid crown of Lavin et al. (2005); and *Dahlstedtia*, which is retained in tribe Millettieae (Schrire, 2005). With the exception of *Andira inermis*, which is also found in West Central Africa, all five papilionoid genera with fixation threads are trees or shrubs native to South America, as are all but one of the known nodulating caesalpinioideae genera. They are found in a range of habitats, from very dry to seasonally flooded. It would be interesting to sample more species of these genera to see if nodules of the shrubby species have thinner-walled fixation threads than the tree species, as found in *Chamaecrista*.

The genistoid group consists of several tribes and parts of tribes. In terms of nodule characters it is rather diverse, but some patterns are beginning to emerge. The genus *Ormosia* (tribe Sophoreae) is separated from the rest, and it has large, often profusely branched, indeterminate nodules. In addition to *Poecilanthe* and *Cyclolobium* (see above), tribe Brongniartaeae contains eight genera with unknown nodule structure. Studies of nodules on these genera could help resolve taxonomic problems. In tribe Thermopsideae, nodules are indeterminate and some have structures in which bacteroids are grouped in ‘thin-walled threads’ or other forms where they not fully released into symbiosomes (Sutherland et al., 1994). These are reminiscent of intermediates between fixation threads and symbiosomes, seen in various species of *Chamaecrista*. Tribe Crotalarieae has nodules that are typically indeterminate, with
apical meristems that may branch or, in some species of Lupinus, grow around the root as in lupin (Corby, 1988; Fig. 3c). At least some genera appear to lack infection threads and have infected cells that can divide and give rise to patches of uniform tissue, lacking uninfected cells (Crotalaria juncea, de Rothschild, 1963; Lupinus bainisii, E. K. James, pers. comm.; Fig. 4f). These features, including indeterminate growth, are common to the next tribe, Genisteae, which includes Lupinus, a genus that has been widely studied for its nodulation characters and which is also unique in being the only legume genus known that is not mycorrhizal. In both Lupinus and Chamaecytisus (now included in Cytisus; Polhill & van Wyk, 2005), infection does not involve infection threads and, instead, infection threads are rarely seen (Lupinus) or transient (Chamaecytisus) (James et al., 1997; Vega-Hernandez et al., 2001). Infection in Lupinus albus occurs between epidermal cells adjacent to the bases of root hairs (González-Sama et al., 2004). Data on species of Cytisus, Genista, Pteretia, Sarothamnus (a synonym of Cytisus) and Ulex (Lechtova-Trinka, 1931; Sajnaga et al., 2001; Kalita et al., 2006; J.I.S., unpublished data) suggest that these features may be typical of the tribe. They are nodulated by species of Bradyrhizobium with broad host range (Sajnaga et al., 2001; Kalita et al., 2006), consistent with the suggestion made below for dalbergioid legumes, that a nonhair infection process may be less well controlled.

No member of tribe Podalyrieae is included by Lavin et al. (2005), but this tribe is currently considered to be close to tribes Crotalarieae and Genisteae (van Wyk, 2005). However, nodules on Cyclopia genistoides, although indeterminate, have infection threads and a mixture of infected and uninfected cells in the central region (E. K. James, pers. comm.; Fig. 4e).

Recent studies have shown that species of three genistoid genera from Africa, Cyclopia, Crotalaria and Lottonis, can nodulate with nonclassical rhizobia. The first nodulates with Burkholderia from the β-rhizobia (Kock, 2004) and the other two with Methylbacterium from the α-group (Sy et al., 2001; Jaftha et al., 2002). A report that Aspalathus carnosa (Crotalarieae) nodulates with the β-rhizobium Burkholderia tuberum (Moulin et al., 2001) has not been confirmed.

In the dalbergioid group there are two early branches. There is little information about the smaller branch, dated at approx. 36.9 Ma, but several genera, for example Porothonnus, are known to have indeterminate nodules, often extensively branched (Sprent, 2001). The larger group is typified by aeschynomennoid nodules, with a ‘crack’ infection pathway in which bacteria enter via breaks where lateral (adventitious in some stem nodulating species) roots emerge. Infection threads are never formed, and infected cells are not interspersed with uninfected ones. This form of infection bypasses some of the complex processes involved in infection via root hairs (Goormachtig et al., 2004), and could be regarded as primitive. The ancient origin of the genistoid and dalbergioid groups makes more sense in terms of nodulation processes than their former positions, which were thought to be derived (Polhill, 1981).

As far as is known, nodulation is universal, with the possible exception of Grazielodendron and the certain exception of two closely related genera of woody climber, Chaetocalyx and Nisolia, dated at 8.5 Ma (Lavin et al., 2005), which appear to have lost their nodulating ability. Aeschynomennoid nodules are generally small (up to 5 mm in diameter), determinate in growth and short-lived, meaning that they must be replaced regularly. One advantage of this type of nodule is that it can be formed on old roots as long as they retain the ability to form lateral roots. This could be important for large trees, such as some species of Dalbergia and Pterocarpus, because it would allow a wider distribution of nodules across the root system. On the other hand, it could be argued that not having a root-hair infection could lead to a higher level of occupancy by less effective bacteria. There is some evidence to support this suggestion. In the economically important species Arachis hypogaea (groundnut or peanut) there is a considerable degree of promiscuity, leading to problems for introducing improved inoculant strains (Sprent, 1994b), and Rasolomampianina et al. (2005) have recently isolated seven different bacterial genera from nodules of Dalbergia spp. in Madagascar.

The smaller mirbelioid crown node originated slightly more recently (48 Ma bp). It consists of the endemic Australian tribe Bossiaeeae and the Mirbelieae, which is also found in Papua New Guinea. However, there is a comparatively long stem, the main radiation of the crown starting approx. 30 Ma, after Australia became an island (32 Ma bp), but peaking in the latter 10 Ma when the continent became increasingly arid (Crisp et al., 2004). Many of the soils that they grow in are very low in nutrients, which may explain why, outside some species of Acacia (see above), members of these tribes are the only known legumes to be dual mycorrhizal (AM + ECT), some also having cluster roots (Sprent, 1994a). All appear to have indeterminate nodules, and it is now emerging that many are nodulated by a wide range of α- and β-rhizobia, a factor thought to be advantageous in these environments (Watkin et al., 2005). Lawrie (1981) found abundant deformed root hairs on Aotus ericoides (Mirbelieae), but did not find infection threads in them. However, as threads were found in developing nodules, it was thought that a hair infection must occur. However, as discussed later, this does not necessarily follow. Not in the analysis of Lavin et al. (2005), but generally agreed to be associated with the mirbelioid clade, is the monogenic South African tribe Hypocalypteae (Lewis et al., 2005). Although this genus is known to have indeterminate nodules (Sprent, 2001), there is no information on nodule structure or their endophytic bacteria.

Tribe Indigofereae is represented by a small crown node that appears to have separated from other papilionoid groups at least 50 Ma bp. It is mainly African, although the large type genus Indigofera has a pantropical distribution and indeterminate, often branched nodules. It has been suggested that the Indigofereae and all subsequent tribes share a polyploid event (Pfeil et al., 2005).
The millettioid crown node, dated 45 Ma BP, has two parts, one of which contains much of tribe Millettia and the other Phaseoleae, Desmodieae and Psoraleae. This division is entirely consistent with nodule characters. With the removal of Poecilanthe and Cyclolobium to the genistoid clade (see above), the remaining genera in the older part of the millettioid node all have indeterminate nodules, often much branched and, in the one case that has been studied, Lonchocarpus muehlbergianus, infection is not via root hairs (which were absent), probably occurring between epidermal cells. Infection threads are formed later, and the central tissue has both infected and uninfected cells (Cordeiro et al., 1996). The infected tissue of *Milletia laurentii* is similar (T. H. A. Kiam, pers. comm.).

The generally younger part of the group, tribes Desmodieae, Phaseoleae and Psoraleae, show a range in habit, but tend towards shrubby and herbaceous forms, with some genera such as *Phaseolus* being relatively recent (<10 Ma). All that have been examined typically have determinate nodules, with prominent lenticels and central tissue containing a mixture of infected and uninfected cells (Figs 3b,4a) and, where tested, the typical products exported from *N* fixation in nodules are ureides rather than amides (Sprent, 2001). There are occasional reports of genera with two types of nodule, for example *Cajanus* and *Kennedia* (Corby, 1988). The woody genus *Erythrina* is anomalous in this group on some nodule and other characters, such as nodule morphology and low levels of exported ureides (Sprent & Parsons, 2000). It is likely that the determinate nodule form has been derived from indeterminate. Its advantages and disadvantages have been discussed by Sprent (1980). Briefly, they develop rapidly and initially use the products of fixation (ureides) to fuel bacterial division and host-cell enlargement. Ureides are economical with carbon, compared with amides, but are less soluble and hence may be better for warmer regions. The lifespan of determinate nodules is usually said to be short (a few weeks), but this may apply particularly to the annual crop species that have been studied most widely. In perennial plants, nodules may last for months and also may become lobed, indicating that their meristems remain active (Sprent, 2001). When they are ephemeral, as in soybean (*Glycine max*), and are killed by stress, they must be replaced by new nodules. This is not true of indeterminate nodules, the apical meristems of which can survive considerable stress and may live for years, resuming growth within days of restoration of suitable growing conditions. Perhaps it is not surprising that the vast majority of all legume nodules, worldwide, are indeterminate. Rhizobia presently known to nodulate the ureide-exporting legumes are all classical rhizobia, mainly slow-growing types.

The remainder of the Papilionoideae have their origin in the Hologalegina crown node, dated at 51 Ma BP. Like the millettioid group, it is split into two main parts. The older robinoid crown node (48 Ma BP) has three sections of interest for nodule characters. First, the genus *Sesbania*, now included in a monogenic tribe, *Sesbaniae* (Lavin & Schrire, 2005), one species of which, *Sesbania rostrata*, has been studied intensively as it can nodulate on both stems (associated with adventitious roots) and roots, using different infection pathways (crack entry and root-hair infection) depending on growth conditions (Goormachtig et al., 2004). Nodules may appear very similar to the aescychnomenoid type, or grow out and be indeterminate. One of its nodulating bacteria is *Azorhizobium caulinodans*, a classical rhizobium that can grow *ex planta* on nitrogen gas as its sole N source. Sister to *Sesbaniae* is tribe Loteae, which now includes former tribe Coronilleae. From a nodulation point of view, this change is a retrograde step, but the molecular evidence in its favour is very strong. Members of old tribe Loteae have determinate nodules, morphologically and structurally very similar to those of the Phaseoleae and related tribes described in the previous section, but exporting amides rather than ureides as a product of fixation. It appears that determinate nodules have arisen twice during legume evolution. Members of former tribe Coronilleae have indeterminate nodules. The Loteae as now understood is relatively young, herbaceous and temperate in location. The third part of this group is tribe Robineae, a complex group of woody legumes mainly in tropical and warm temperate regions, with indeterminate nodules.

The second part of this large clade is distinguished by the loss of the inverted repeat in the chloroplast genome (Doyle, 1995), and is known as the inverted repeat loss clade (IRLC). It is rooted at approx. 39 Ma. A small group of its genera, including the familiar ornamental genus *Wisteria*, is still included in tribe Millettia, pending further research into its correct position. The rest of the IRLC consists of tribes Astrapaleae, Galegeae, Hedysareae, Trifolieae, Cicerae and Fabeeae (formerly Viteae). These tribes are largely warm temperate through temperate to arctic, and have indeterminate nodules, often branched, with root-hair infection. They generally nodulate with fast-growing α-rhizobia and usually show a high degree of specificity between symbiotic partners.

**IV. What triggered the development of nodulation?**

Assuming an origin of the Leguminosae at 60 Ma, the next few million years were a time of great climatic change, particularly with respect to carbon dioxide and temperature. At approx. 55 Ma, several linked changes occurred. There was an abrupt rise in temperature of 5–10°C over a wide range of latitudes (Bowen et al., 2004; Sluijs et al., 2006), accompanied by a ‘catastrophic’ release of both methane and carbon dioxide from sea-floor sediments. Evidence from mid-latitudes suggests that the climate also became more humid at this time and that there was enhanced cycling of carbon through terrestrial ecosystems (Bowen et al., 2004). In certain parts of the neotropics, at least, there was also a significant increase in plant biodiversity as shown by fossil records (Jaramillo et al., 2006); this diversity later decreased. Could the high CO₂ levels mean that N would become limiting for plant growth,
Thus favouring evolution of N fixation? Certainly this period marked the origin of two major groups of nodulating legumes, the genistoids and dalbergioids, as well as groups of caesalpinioiids that include nodulating genera. If nodulated legumes evolved under conditions of high CO2, then it might be expected that they would be favoured by current rises in atmospheric concentrations of this gas. Data from the many experiments looking at effects of enhanced CO2 have been inconclusive, but it is worth noting that one of the more recent publications suggests that response may vary with legume species (West et al., 2005). Unfortunately, their most responsive genus, Lespedeza, is also the most recently evolved of those used. Rogers et al. (2006) suggested that at high CO2 levels it would be more efficient for soybean (closely related to Lespedeza) to fix N into ureides than to assimilate nitrate, thus giving them an advantage over nonnitrogen-fixing species.

Subsequently, CO2 levels dropped, until they started to rise again in recent times. One of the ways in which plants adapted to this drop was by developing C4 photosynthesis, a process that has evolved on many occasions, but never, it appears, in legumes (Sage, 2004). Why this is so, when many legumes have sclerophyllous characters rather like those of some C4 plants, is completely obscure.

More recently, the earth began to cool and ice began to form first at the South and then the North Pole. Legumes colonized all the emerging cooler areas, extending into arctic regions (Sprent, 2001). This move towards higher latitudes was accompanied by an increasing interdependence of the legumes and rhizobia (Perret et al., 2000; Sprent, 2003), increasing specificity.

Specificity is one of the oldest branches of study in legume nodulation, driven by the needs of the inoculant industry which supported Australasian and US agriculture. Although varying greatly, specificity appears to be more consistently stringent in temperate than in tropical legumes, as exemplified by the numerous studies on members of tribes Trifolieae and Faboeae (Vicieae). Although the term strictly refers to the initial interactions between bacteria and host, leading to infection (infecitivity), a high degree of specificity is usually followed by a formation of a highly effective N-fixing nodule.

This is best illustrated by the classic of plant genetics, the garden pea, Pisum sativum, and its symbiont Rhizobium leguminosarum bv. viciae. Using a variety of molecular, biochemical and immunological methods, Ludwig et al. (2003) showed that in order to for the bacteria to reduce N and the plant to assimilate the products, the two partners exchange messages in the form of amino compounds. Mutations that block the production or transport of messages result in nonfunctional nodules. This is one extreme in host–symbiont specificity. At the other extreme, there are two possibilities: first, a single bacterial genotype that nodulates many hosts; and second, a single host genotype that is nodulated by many bacteria (Sprent, 2001, 2003). Between these extremes lies an infinite variety of outcomes. The recognition of nonclassical rhizobia has added a further level of complexity.

Which partner is in charge – bacterium or plant? In terms of nodule morphology and structure, the legume is the dominant partner, which is why nodules can be such a useful taxonomic tool (Sprent, 2000). However, when it comes to extent of N fixation, the bacteria become the more significant component. There is much current interest in the control of mutualistic associations. In the case of legume nodules, the most detailed work has been carried out on particular symbioses, with a focus on how effectivity (rather than infectivity) is maintained. Denison and coworkers carried out experimental and simulation studies to arrive at their ideas on how soybean nodules keep out ‘cheaters’ by various means, including withholding essential nutrients (Denison & Kiers, 2004 and references therein). In more general terms, Foster & Wenseleers, (2006) put forward a model based on cost–benefit analysis, and in another paper Foster & Kokko (2006) suggest that cheating can stabilize cooperation by maintaining variation in the system. Such a cost–benefit analysis has not been carried out on any of the less effective legume nodule symbioses, but costs of keeping options open are incurred in terms of the genetic information needed to code for the signal molecules and the actual production of these molecules by both plant and bacteria (Long, 1989). These costs need to be set against the potential benefit of the formation of some effective nodules under a variety of environmental conditions. Is it best for a particular host plant, or a specific strain of bacteria, to take a chance on finding a compatible partner, when diversity of both is high, as in many tropical ecosystems?

The case of acacias in dry areas has already been considered, when it was argued that N fixation may not be a high priority for plants in low-nutrient soils. A different system is found in the Bayang-Mbo rainforest in Cameroon, where most of the legumes (trees plus some lianas) present cannot nodulate, but where those that can nodulate profusely and produce pink nodules, usually a sign of effectiveness (Sprent, 2005). It could be argued here that a few highly specific and highly active nodulated legumes are a better strategy for the forest, as a functional unit, than more nodulated species with a range of nodule effectiveness. This could require exchange of nutrients (in both directions) between trees via AM. Although in this forest the nonnodulating legume trees include those known to be either AM or ECT (Alexander, 1989), the latter may be able to produce enough AM infections (Moyersoen & Fitter, 1999) to participate in such an exchange of nutrients with nodulated trees (Högbarg & Alexander, 1995; Sprent, 2005; Selosse et al., 2006). Perhaps the key to such a system working effectively is a plentiful supply of water and rapid nutrient cycling through litter layers.

V. Nonclassical rhizobia

Work to date on these has largely been concerned with the taxonomy of the bacteria, rather than their ecology or that of their host plants. There have been a plethora of reports of
bacteria isolated from nodules, but comparatively few of these isolates have been shown to nodulate the plants from which they were isolated. Indeed some, such as Labrys neptuniae, isolated from Neptunia oleracea, appear unable to do so (Chou et al., in press). A genus that seems to prefer β-rhizobia is Mimosa, which has c. 500 species, widely distributed in the neotropics, with centres of endemism in Mexico and parts of Brazil, but with significant numbers of species in parts of Asia and Madagascar (Luckow, 2005). It is the only genus with species that have been conclusively shown to be nodulated effectively by fully characterized β-rhizobia. Mimosa pudica and Mimosa diplotricha nodulate with Ralstonia (now renamed Cupriavidus) taiwanensis (Chen et al., 2003) and Mimosa pigra with Burkholderia spp. (Chen et al., 2006), one of which, Burkholderia mimosarum, a newly described species, has been found in nodules from Taiwan, Brazil and Venezuela (Chen et al., 2006). Elliott et al. (2007) showed that Burkholderia phymatum, which can also fix nitrogen ex planta, nodulates 30 Mimosa spp. representing all continents where the genus occurs. As far as is known, it is native to the soils where the plants are endemic. Interestingly, strains of rhizobia reported to nodulate Mimosa spp. in Papua New Guinea in 1980 have now been shown to be Burkholderia phymatum (Elliott et al., 2007). Preliminary evidence suggests that nodulating strains of both Burkholderia and Ralstonia may have obtained nod genes from local rhizobia (Chen et al., 2005). Although vertical DNA transmission is the norm in bacteria, some genera, including Bradyrhizobium and Ralstonia, appear particularly prone to be involved in lateral (horizontal) gene transfer (Kunin et al., 2005).

Several preliminary publications, in addition to the work on Mimosa (cited above), indicate that nodulation of legumes by β-rhizobia may be widespread, at least in the tropics. Hung et al. (2005) showed that a putative Burkholderia strain nodulated Obwia caudata (as Catenaria caudatum) in Taiwan; Rasolomampianina et al. (2005) found a strain of Burkholderia cepacia (a plant pathogen) that formed ineffective nodules on Dalbergia loulouvi, and we have strong evidence of nodulation of several species of Cyclopia (but no species of Aspalathus) with Burkholderia tuberum (G. N. Elliott, pers. comm.). These three genera are from different tribes of the Papilionoideae. We are currently checking nodules from the Caesalpinioideae for presence of β-rhizobia. Whether β-rhizobia or ‘other’ α-rhizobia are particularly significant in certain ecosystems remains to be determined. One factor that may be relevant is their ability to survive in particular soils in the absence of host plants.

VI. Conclusions
Although there are still many unknowns, based on the information above, development of a possible scenario for legume nodulation in a temporal and spatial context will be attempted.

The first event in development of the symbiosis would have been communication between the partners, followed by bacteria gaining entry without triggering defence responses. The simplest way of achieving these two aims could have been by bypassing some of the complex signalling required for root-hair infection (Long, 1989) and gaining entry instead between epidermal cells or via wounds, such as where lateral roots emerge. The most widely studied infections of this type are the formation of aeschynomenoid nodules and those associated with root initials on stems of S. rostrata. In the first of these, infection threads are not formed, yet the apparently most primitive type of nodule has bacteria retained within modified infection threads (Fig. 4d). Although it is sometimes assumed that if infection threads are found in nodules, they must be involved in rhizobial entry (e.g. Lawrie, 1981), this is not necessarily the case. The best documented case of a crack infection leading to development of infection threads is in the aquatic mimosoid species Neptunia natans (Subba-Rao et al., 1995). Here, following initial infection, development conforms to the normal pattern of indeterminate nodules. If, however, in the primitive case, the plant did not fully accept the bacteria as beneficial, then it may have retained them in fixation threads, as seen in caesalpinioi and a few papilionoid nodules. The logical sequence from this is as found in Chamaecrista, where there is a gradation in structure of infected cells from fixation threads to thinner-walled threads and finally release of bacteria into symbiosomes (Naisbitt et al., 1992). In this scenario, root-hair infection would be a later development, requiring the evolution of a more sophisticated signalling system. However, some legumes that normally use a hair infection retain the ability to use what could be regarded as a default position. Examples are Trifolium repens, where modification of the signalling system leads to formation of nodules at the site of lateral root emergence (Mathiesius et al., 2000), and species of Lotus that, under some conditions, can be infected via or between enlarged epidermal cells (James & Sprent, 1999). Further, not all extant legumes produce root hairs, and those that do may not produce them under all growing conditions (Sprent, 2001). In the absence of root hairs, there may be crack infection (as in some species of Neptunia), or infection between epidermal cells (for example in Mimosa sabrellia, de Faria et al., 1988 and L. muehlbergianus, Cordeiro et al., 1996). In all these cases, bacteria are distributed to N-fixing cells by infection threads. den Herden et al., 2006) give a very detailed account of the formation of infection threads following crack infection in flooded roots of S. rostrata and suggest that this adaptation evolved several times in different species in response to flooding. From the evidence reviewed here, it seems more likely that legumes may have retained the ability to nodulate using the more primitive process when conditions dictate this.

None of this explains how dalbergioid and some genistoid nodules manage without either infection threads or uninfected cells in the infected region, the latter having an important metabolic function, at least in ureide-exporting legumes (Sprent, 1980). The main difference between nodules on
these two important groups of legumes is whether their growth is determinate (dalbergioids, Fig. 3c) or indeterminate (genistoids, Fig. 3d), giving the latter the flexibility of all indeterminate nodules of being able to resume growth after periods of stress. This may also have allowed them to invade more temperate, strongly seasonal areas than the dalbergioids.

Although the majority of publications on legume nodulation (e.g. den Herden et al., 2006) suggest that ‘most legumes’ have a root-hair infection, evidence collected for this review suggests that at least 25% of all legume genera may have a nonhair infection and nodule development lacking infection threads. As far as is known (and there is no information for the Caesalpinioideae), all other groups of legumes have a root-hair infection. The necessary condition for this is that infection threads are formed. This has the potential advantage of allowing the plant more control, using the various systems whereby the bacteria and plants recognize each other; but, as indicated earlier for Acacia, this advantage is not always taken. However, we may speculate that the recently evolved vicioid legumes have capitalized on this, nodulating with a small number of bacteria and being very reliant on N fixation. They tend to grow in rather more fertile soils, with respect to phosphorus, potassium and trace elements, than many native legumes of lower latitudes, and except for problems of low pH and high aluminium with some species such as white clover (T. repens), they are likely to gain significant competitive advantage from being able to fix N. Why some of the Loteae developed determinate nodules is a mystery, as indeterminate nodules are far more versatile.

Figure 5 summarizes these ideas, but leaves open a number of questions, perhaps the most important of which is how many different nodulation events occurred in legume evolution. The main problem here is the significant number of basal papilionoid and mimosoid legumes that are unable to nodulate (Fig. 2). From their current phylogenetic positions, it seems unlikely that they would have lost the ability to nodulate. There have been occasional reports in the literature that rhizobia can enter roots of some caesalpinioid legumes, most frequently Gleditsia (Allen & Allen, 1981), but also Peltophorum (Bryan et al., 1995), forming infection thread-like structures but without leading to the formation of nodules. Recent studies with a cytokinin mutant of Lotus japonicus showed abundant production of infection threads but no formation of nodules (Murray et al., 2006). This separation of the processes of infection from those of nodulation could be significant in evolutionary terms. In some advanced papilionoid legumes, at least, the host has all the necessary information to form determinate (Tirichine et al., 2006) and indeterminate (Gleason et al., 2006) nodules in the absence of rhizobia. At what stage this information was acquired is not known. Studies on a wider range of legumes, particularly in the Caesalpinioideae, are needed to link the evolution of the processes of infection and nodulation. It would be very interesting to look at some of the nonnodulating genera for genes known to be involved in the nodulation process and see what is missing – genes or the processing thereof. Inclusion of such molecular information in future phylogenetic analyses could lead to a reassessment of the position of these genera and help to resolve the question.
of the number of separate nodulation events. It has been noted earlier that nodulation can be a useful taxonomic tool (Sprent, 2000), using both presence and absence of nodules, and their morphology and structure. So far the latter has proved useful in helping to determine the dalbergioid clade (Lavin et al., 2001), but data reviewed here for the genistoid legumes suggest that future studies on these genera might usefully include nodule characters.

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References


Supplementary Material

The following supplementary material is available for this article online:

**Table S1.** Nodulating genera in subfamilies and tribes of legumes, as given by Lewis *et al.* (2005), except that Mimozygantheae is incorporated into Mimosoideae.

**Table S2.** Estimated ages of crown nodes of legumes that have importance for nodule characters.

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