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Evolution of nitrogen fixation in spatially structured populations of *Rhizobium*

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Symbiosis between legumes and nitrogen-fixing bacteria is thought to bring mutual benefit to each participant. However, it is not known how rhizobia benefit from nodulation of legume hosts because they fix nitrogen only after differentiating into bacteroids, terminally differentiated cells that cannot reproduce. Because free-living rhizobia can reproduce, and may benefit from the increase of plant root exudates stimulated by nodulation, evolution of symbiotic nitrogen fixation may depend upon kin selection. However, unrelated nonmutualists may also benefit from increased plant exudates and nitrogen-fixing populations are therefore vulnerable to invasion by nonfixing, saprophytic *Rhizobium*. The access of nonfixing *Rhizobium* to the plant exudates associated with nodules depends upon the spatial structure of the *Rhizobium* populations within the soil. We investigate the influence of spatial structure on the evolution of N-fixation within a *Rhizobium* population using a mathematical model. Our model demonstrates that spatial structure is necessary for the evolution of N-fixation and that N-fixation is more likely to evolve with increasing degrees of spatial structure. In fact, we identify three dynamic outcomes that depend upon the relative strength of the costs of N-fixation relative to the degree of spatial structure and benefits resulting from nodulations. If the costs are relatively high, N-fixation will not evolve; if the costs are relatively low, N-fixing genes will fix in the population, but at intermediate conditions, a stable mixture of N-fixing bacteria and nonfixing bacteria will be maintained. The conditions for coexistence of N-fixing bacteria and nonfixing bacteria expand under a saturating relationship between nodule numbers and N-fixing genotype frequency.

Keywords: coevolution, kin-selection, mutualism, nitrogen-fixation, spatial structure, symbiosis.

Introduction

Symbiotic relationships between N-fixing bacteria and their eukaryotic hosts are thought to bring mutual benefit to each participant. However, little is known about how rhizobia benefit from nodulating legumes. In fact, detailed natural history of the legume–*Rhizobium* interaction points to a potentially severe cost to rhizobia as a consequence of symbiotic N-fixation. During nodulation, infecting rhizobia enter host meristem cells and differentiate into bacteroids. They can fix atmospheric dinitrogen only in this differentiated state (Caetano-Anollés & Gresshof, 1991); however, it is reached through a terminal developmental event during which bacteroids lose the ability to reproduce (Zhou *et al*., 1985). In hosts with determinate nodules, some bacteria may remain undifferentiated within the nodule and can reproduce when released into the soil during nodule senescence. However, in other hosts, especially those with indeterminate nodules, no viable undifferentiated bacteria survive nodule senescence (Sutton & Patterson, 1979; Zhou *et al*., 1985; Sprent *et al*., 1987). In either case, it appears likely that *Rhizobium* cells that fix nitrogen are genetically dead.

Rhizobia might nonetheless benefit from nodulation via benefits conveyed to their free-living kin (Jimenez & Casadesus, 1989; Olivieri & Frank, 1994). Host plant roots excrete many compounds into the rhizosphere that increase rhizobial growth, and nodulation may increase the rate of excretion into the rhizosphere (Boivin *et al*., 1991; Hartwig *et al*., 1991). Because rhizobia reproduce largely by asexual fission, bacteroids inside the nodule are likely to have genetically identical kin outside the nodule. Therefore, although a differentiated bacterium may have sacrificed its own reproduction, this altruistic action could be evolutionarily advantageous if the enhanced exudates of its host increase the fitness of its

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kin sufficiently to offset its own loss of reproductive capacity.

One problem with kin selection, however, is its vulnerability to cheaters. The rhizosphere is an intensely competitive environment (Lynch, 1990), and enhancing plant growth improves the rhizosphere environment for all microbes dependent upon plant resources. Unrelated nonmutualists can benefit from nodulation, and a generalized improvement of the rhizosphere does not guarantee an increase in the relative fitness of bacteroid kin. Kin may be more likely to receive the benefit of altruistic acts within a spatially structured environment (Wade, 1985). Soil, being a viscous matrix in which bacterial dispersal is limited (Wadisirisuk *et al*., 1989; Lowther & Patrick, 1993; Parco *et al*., 1994), is known to be a highly structured environment for soil organisms (Chanway *et al*., 1991; Bever *et al*., 1996). In such a structured system, there will be a greater probability than expected by chance that a reproductive N-fixing bacterium benefits directly from nodulation by its relatives. In this paper, we develop a model to describe the evolutionary dynamics of N-fixation within a spatially structured environment.

Model

Symbiotic nitrogen fixation is a complex genetic process. Successful nodulation requires active *nod* genes and additional genes, including *nif* and *fix* genes, are required for nitrogen fixation. In many rhizobia, however, many or most of these genes are carried on a single plasmid (Long, 1992) and coinherited (Broughton *et al*., 1987; Wexler *et al*., 1996). Consequently, we model the system with two asexually reproducing genotypes: a mutualistic genotype that fixes nitrogen, hence called N-fixing, and a saprophytic one that does not.

Nitrogen fixation could result in several costs to *Rhizobium*, including bacteroid sterilization and the energetic cost of carrying the symbiotic plasmid. Free-living N-fixing bacteria are notoriously slow growing on artificial media relative to saprophytic relatives (Anand & Dogra, 1997; Sarathchandra *et al*., 1997). In this model, we assume that free-living N-fixing bacteria incur a fixed cost in their basal fitness relative to saprophytic bacteria. The basal fitness of saprophytic bacteria is set to 1, while the basal fitness of the N-fixing genotype is $(1–c)$, where c is the realized cost in growth rate resulting from fixation ability, across all environments.

Net growth rates of free-living rhizobia depend upon their environment. Population densities are highest in the rhizosphere, the soil region immediately surrounding roots and which contains carbohydrates and other nutrients that leak from roots (Sprent, 1979; Chanway *et al*., 1991). In a N-limited soil, nodulation may increase the rate of excretion into the rhizosphere by improving host growth, resulting in the environment of reproductive rhizobia improving with increasing nodule number. Plant hosts can support a limited number of nodules, with numerous factors determining nodule number, including host genotype, soil physical characteristics, and nitrate supply (Sprent, 1979). However, for a given host genotype and soil type, the actual number of nodules the host produces is undoubtedly a function of the frequency of the N-fixing genotype. No nodules are possible in the absence of the N-fixing genotype and nodule number presumably rises with the frequency of the N-fixing genotype to a maximum when the N-fixing genotype reaches 100% frequency. The linearity of this function, however, is unknown. We therefore use a general function to describe the dependence of nodule number on N-fixing genotype frequency. Specifically, nodule number is assumed to increase as a function of $[p/(p + a\,q)]$, where p is the frequency of the N-fixing genotype, q is the frequency of the saprophytic genotype, and the parameter a alters the shape of the nodule number function (functional response). When the parameter a is less than 1, the functional response will be saturating, when a equals 1, the functional response will be linear, and when a is greater than 1, the functional response will be accelerating (Fig. 1). We then represent the total amount of carbon available to reproductive bacteria as $[1 + b\,p/(p + a\,q)]$, where '1' indicates the standing availability of carbon in the rhizosphere of unnodulated roots, and the second term represents the increased carbon resulting from nodulation. The parameter 'b' then represents the 'benefit' of nodulation to the free-living bacteria.

Access of free-living, reproductive bacteria to the benefits of nodulation depends upon the spatial structure

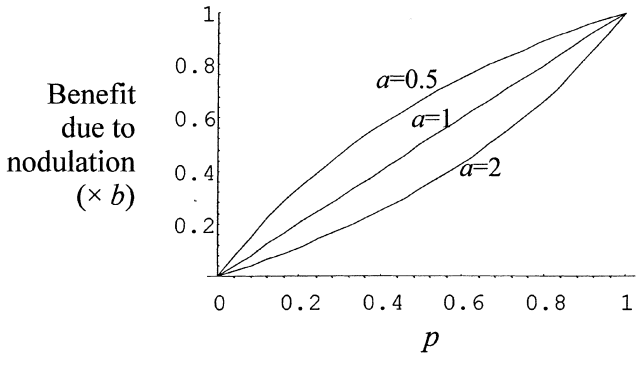

Fig. 1 The benefit to rhizosphere bacteria resulting from nodulation, plotted as a proportion of the maximum, is presented as a function of N-fixing genotype frequency for three values of the parameter, a. This functional relationship is saturating when $a = 0.5$, linear when $a = 1$ and accelerating when $a = 2$.

of their population. Nodules are initiated early in the season; the likelihood of a nodule forming at any particular location is dependent upon the genotype of *Rhizobium* present at that location. Carbon exuded after these nodules mature is then accessible only to reproductive rhizobia in close proximity to these nodules. In a thoroughly mixed environment, all reproductive bacteria would be equally likely to be near a nodule and thus have access to the benefits of nodulation. However, in a system with limited mixing, an N-fixing reproductive cell would be more likely to be near a nodule (produced by its kin) than a nonfixing reproductive. We describe this level of mixing using a convenient shorthand which describes the coefficient of relationship, Φ, between a reproductive bacterium randomly sampled from the rhizosphere and bacteroids in a nearby nodule (as in Simms & Bever, 1998). This coefficient then is the proportion of bacteria within the zone of influence of a nodule that are identical by descent to the bacteroids in the nodule. If the rate of bacterial movement in the soil is high relative to the rate at which nodules are formed and senesce, then the spatial relationship between bacteroids and reproductive bacteria may be random and $\Phi = 0$. $\Phi = 1$ when there is no bacterial movement; in this case, reproductives of the N-fixing genotype will always be spatially associated with a nodule occupied by their kin.

The access of reproductive bacteria to the benefits of nodulation is therefore determined both by the genotype frequencies and the rate of mixing of bacteria in the soil. For example, the probability that an N-fixing reproductive bacterium receives the benefit of nodulation is the sum of the probability of being identical by descent (Φ) and the probability of not being identical by descent ($1 - \Phi$) weighted by the proportion of N-fixing genotype in the environment (p). We therefore calculate the expected fitness of each bacterial genotype over a single season (a single round of nodulation) as the sum of its fitness with the benefits of nodulation weighted by the probability of receiving these benefits plus the weighted fitness of the bacterium in the absence of nodules. Hence,

$$w_{\text{N-fixing}} = (1-c)\left[1 + \left(\frac{b}{p+aq}\right)(\Phi + p(1-\Phi))\right] \qquad (1)$$

and

$$w_{\text{saprophytic}} = 1 + \left(\frac{bp}{p+aq}\right)(1-\Phi). \qquad (2)$$

The fitness of both genotypes increases with the frequency of the N-fixing genotype, but the fitness of the saprophytic genotype increases more steeply. The two fitness functions may cross to produce an internal equilibrium, (Fig. 2b). The change in frequency of the nitrogen fixation genotype with each generation is

$$\Delta p = p' - p = \frac{p w_{\text{N-fixing}}}{\bar{w}} - p$$
$$= \frac{pq}{w}\left[\left(\frac{b}{p+aq}\right) \times (\Phi - c(p+q\Phi)) - c\right], \qquad (3)$$

where $\bar{w}$ is the average fitness of the population and

$$\bar{w} = 1 + p\left[\left(\frac{b}{p+aq}\right)(1 - c(p+q\Phi)) - c\right]. \qquad (4)$$

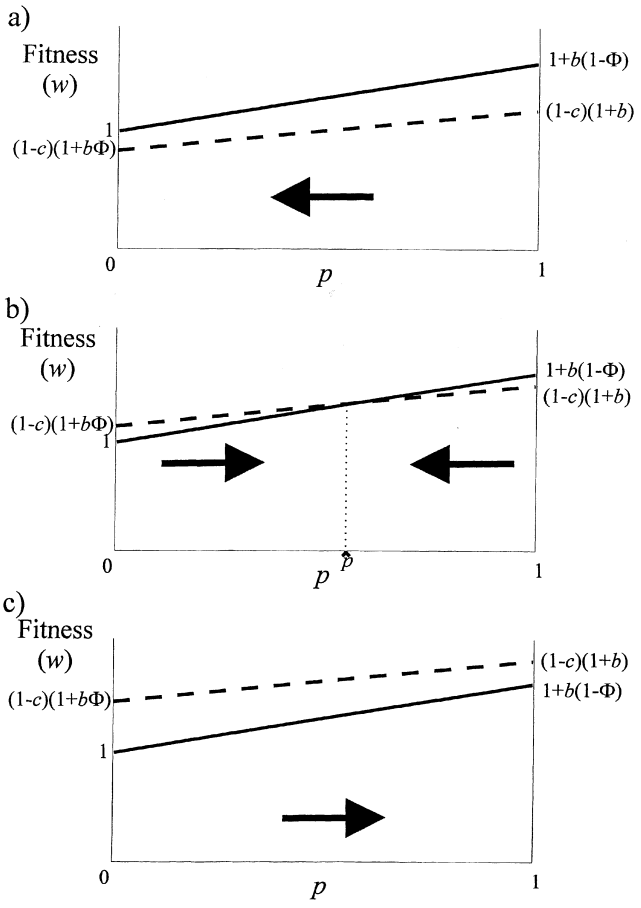

Fig. 2 Fitness of N-fixing and saprophytic genotypes as a function of N-fixing genotype frequency under intermediate levels of mixing, assuming a linear relationship between benefit to rhizosphere free-living bacteria and N-fixing genotype frequency ($a = 1$). The solid line depicts the fitness the saprophytic genotype, the dotted line depicts the fitness of the N-fixing genotype, and the arrows depict the direction of evolutionary change. Depending on the parameters, the population may fix at all saprophytic genotype (a), fix at all N-fixing genotype (c) or stably maintain both genotypes (b).

Equilibrium is reached when $p=0$, $p=1$, and also when:

$$\hat{p} = \frac{b\Phi - ac - cb\Phi}{c(1-a) + cb(1-\Phi)}. \quad (5)$$

Analysis of local stability indicates that the equilibrium at $p=0$ will be stable and the saprophytic genotype will be fixed when

$$\Phi < \frac{ac}{b(1-c)} \quad (6)$$

(Fig. 2a). The equilibrium at $p=1$ will be stable and N-fixation will be fixed when

$$\Phi > \frac{c(1+b)}{b} \quad (7)$$

(Fig. 2c). The internal equilibrium will exist and will be stable when neither inequality given in eqns (6) and (7) is true. Under these conditions the system exhibits negative frequency dependence, where pure populations of either the N-fixing or the saprophytic genotype can be invaded by the other (Fig. 2b).

Some degree of spatial structure is essential to establish the N-fixing genotype (Fig. 3). At intermediate levels of mixing, coexistence of the mutualistic and saprophytic genotypes is possible, with the equilibrium level of N-fixation increasing with increasing spatial isolation as well as increasing benefits of nodulation relative to the costs of N-fixation (Fig. 3b,c). The coexistence of N-fixing and saprophyte genotypes is not possible in the absence of mixing and the population would either be fixed at all N-fixing or all saprophyte depending on the efficiency of the N-fixing genotype as measured by the relative costs vs. benefits of nodulation (Fig. 3a).

Interestingly, the linearity of the relationship between nodule number and frequency of N-fixing genotype does not influence the conditions for fixation of the N-fixation genotype, but strongly influences the conditions for coexistence. Specifically, if the functional response of nodulation to N-fixing genotype frequency is saturating ($a < 1$), then conditions for the establishment of the N-fixing genotype expand (Fig. 3c). However, if this functional response is accelerating ($a > 1$), then conditions for the establishment of the N-fixing genotype narrow.

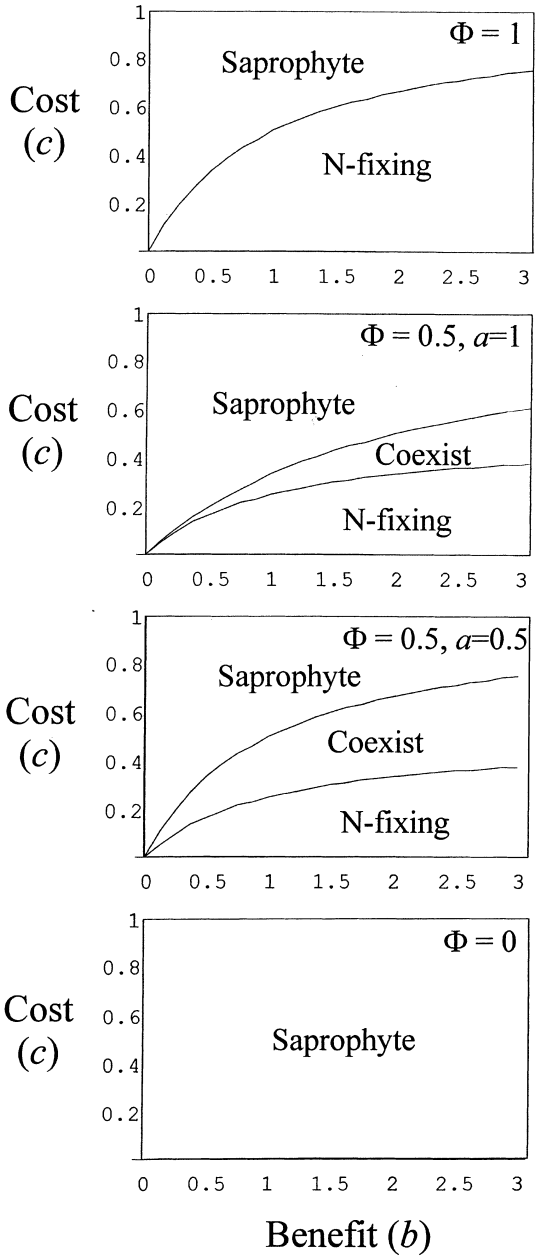

Fig. 3 Conditions for the evolution of N-fixation at three levels of spatial structure. With no mixing (a), the N-fixing genotype has the broadest conditions to evolve regardless of the linearity of the functional response of nodulation. At intermediate levels of mixing, the two genotypes can coexist under a restricted set of conditions (b). These conditions for coexistence expand under a saturating functional relationship between nodulation and N-fixing genotype frequency (c). N-fixation cannot evolve with complete mixing (d).

Discussion

Our model predicts that the evolution of nitrogen fixation in *Rhizobium* is dependent upon spatial structure (Fig. 3). Increasing spatial structure increases the likelihood that kin benefit from nodulation, thereby increasing the likelihood that N-fixation will evolve. Spatial structure had been identified previously as a

potential mechanism allowing kin selection and the evolution of mutualism (Wilson, 1983; Wade, 1985; Frank, 1994). The predictions of the present model are in general agreement with the simple condition for evolution by kin selection derived by Hamilton (1964) and applied to the evolution of N-fixation by Olivieri & Frank (1994). According to Hamilton's rule, N-fixation would evolve when $b > c/r$, where b and c are the benefits and costs of N-fixation as in the present model and r is the degree of relationship between bacteroids and reproductive bacteria, comparable to Φ in the present model. The minimal condition for the evolution of N-fixation by kin selection within our model, as obtained by rearranging eqn (6) to give

$$b > \frac{ac}{\Phi(1-c)} \tag{8}$$

is more restrictive than predicted by Hamilton's rule (when $a = 1$), as a result of the fixed cost of N-fixation incurred by the free-living, reproductive bacteria. This fixed cost encompasses both loss of reproductive bacteria arising from terminal events in nodulation and reduced growth rate due to carrying the large Nod + plasmid. Meeting the condition in eqn (8) will only guarantee that the N-fixing genotype can invade the saprophytic genotype; the present model identifies conditions under which the two genotypes stably coexist. Moreover, by rearranging eqn (7), we predict that N-fixation will fix within the population under the more restrictive condition of $b > \frac{c}{\Phi - c}$.

These strict spatial conditions for the initial establishment of nitrogen fixation depend upon the functional response of nodule number to N-fixing genotype frequency. If this relationship is saturating (depicted in Fig. 1), the conditions for establishment are relaxed, while the conditions for establishment are narrowed under an accelerating functional response (Fig. 3). Data are not available with which to identify the nature of this functional response, but we suspect that this relationship should be strongly saturating. To explain, while many biological factors will influence the functional response of nodule number, one factor that will cause it to be saturating is host control of maximal nodule number. Because plant physiology is known to exert strong influences on nodule number (Sprent, 1979), this factor alone should generate a saturating functional response. In addition, any factor that facilitates identification by the plant of N-fixing genotypes will allow the formation of more than expected when N-fixing genotypes are at low frequency, thereby generating a saturating response. Systems of active recognition are well documented and the molecular details of these systems are now being described (Long, 1992). Finally, if we modify the assumption in this model that benefit to rhizosphere bacteria is directly proportional to nodule number and also allow nodule size and its resulting benefit to vary, this action will likely increase the proportionate influence of N-fixing genotypes as well. Specifically, in this case, a host that produced few nodules as a consequence of low frequency of the N-fixing genotype, would subsequently be likely to invest more in these few nodules, resulting in large nodule size and large benefits to the nearby reproductive bacteria. It is interesting in this context to note that plant species vary in their production of nodules of indeterminant vs. determinant size (Sprent *et al.*, 1987). From the present model, we can suggest that plants that produce nodules of indeterminate size may better promote the initial establishment of N-fixing genotypes in *Rhizobium* populations. However, the model does not suggest that production of nodules of indeterminate size would influence the probability of fixation of N-fixing genotypes within the population.

The strict spatial conditions for the establishment of nitrogen fixation, could also be relaxed if the carbon excreted from nodules was accessible only to the N-fixing genotype. In fact, such a private carbon source can be produced by some *Rhizobium* genotypes in the form of rhizopine, and, interestingly, the genes for rhizopine production and catabolism are linked to the genes for N-fixation (Murphy *et al.*, 1988; Murphy & Saint, 1992; Wexler *et al.*, 1996). We previously demonstrated that evolution of rhizopine-production is more likely with increasing levels of mixing within the soil (Simms & Bever, 1998). Thus genotypes producing and catabolizing this private carbon source might be able to evolve nitrogen fixation under levels of mixing that would otherwise prevent the evolution of this mutualism. The importance of this mechanism remains to be demonstrated.

Given the frequent location of the genes required for N-fixation on a single plasmid, and the evidence for this plasmid's horizontal transfer between bacteria (e.g. Souza *et al.*, 1992), the appropriate unit of selection in the evolution of N-fixation may be the plasmid rather than bacteria. While the present model does not distinguish between these two units, as we assumed that the plasmid is inherited stably with bacterial genotype, this issue merits further consideration in future discussions and models of the evolution of N-fixation.

Explicit tests of the predictions of the model are not possible because of the absence of empirical estimates of the model parameters from the same system. The absence of these data identifies the need to estimate these critical parameters. Measurement of the probability that bacteria outside nodules is identical to the bacteroids inside the nodules, Φ, is particularly critical,

as this parameter is not only pivotal for the evolution of N-fixation, but also for the evolution of rhizopine (Simms & Bever, 1998).

Our model's demonstration of the dependence of the evolution of N-fixation on spatial structure has important implications for evolution within highly managed agricultural systems. Intensive management of such systems reduces the spatial structure of the soil, resulting in a homogenization of the *Rhizobium* populations (Souza *et al*., 1992; Wernegreen *et al*., 1997; Silva *et al*., 1999). Our model suggests that this reduction in soil spatial structure could contribute to the frequently observed decline of efficiency of the N-fixing bacteria within agricultural systems (e.g. Caldwell & Vest, 1970; Ham, 1980; Triplett, 1990).

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