Quantitative Indices for the Autecological Biogeography of a *Rhizobium* Endophyte of Rice at Macro and Micro Spatial Scales

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Abstract

Rhizobium leguminosarum bv. *trifolii* E11 is a natural root endophyte of rice isolated in the Nile delta where rice and berseem clover have been rotated since antiquity. Its autecological biogeography is being examined at two spatial scales: one at a macro scale relevant to its proposed use as a plant growth-promotive biofertilizer in rice-berseem clover agroecosystems, and a second at a micro scale relevant to its colonization of rice roots. Here we introduce two new indices to measure the prevalence in distribution of strain E11 within a defined spatial domain. An autecological biogeography index is described to map the distribution of a specific strain of rhizobia on a macro scale based on immunofluorescence microscopy of nodule occupants on legume trap hosts. A cluster index is introduced to analyze the *in situ* spatial pattern of root colonization by the bacteria at single-cell resolution using CMEIAS (Center for Microbial Ecology Image Analysis System) software for computer-assisted microscopy. When sampled at multiple

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georeferenced sites (i.e., at known Cartesian x,y coordinates relative to a landmark origin), these two indices provide values that are suitable for general use as the Z variate in spatial geostatistical analyses to model bacterial dispersion and colonization behavior, and to produce interpolated maps of the continuous distribution of the microbial symbiont within the defined spatial domain of the geographical region or root substratum, including areas that cannot be physically sampled. Both indices also have the potential for broad applications in microbial ecology.

1. Introduction

Recent studies on many continents have established that *Rhizobium*, the well-known nitrogen-fixing root-nodule endosymbiont of legume plants, also develops a natural, beneficial endophytic association with cereal roots growing in the same crop rotation (Yanni et al., 1997; Yanni et al., 2001 and references therein). This was first described in studies of *Rhizobium leguminosarum* bv. *trifolii* indigenous to the Egyptian Nile delta where berseem clover and rice have been cultivated in continuous rotation since antiquity (Yanni et al., 1997). Several field inoculation trials have indicated that certain strains of these rice-adapted clover rhizobia can promote vegetative and reproductive growth of the rice crop, resulting in significant increases in grain yield and agronomic fertilizer N-use efficiency, with less dependence on chemical N-fertilizers (Yanni et al., 2001). These *Rhizobium*-rice associations are highly strain/variety specific, with some combinations resulting in beneficial and others detrimental outcomes (Yanni et al., 1997; Yanni and Dazzo, 2000; Perrine et al., 2001; Yanni et al., 2001). Recently, several other naturally occurring, endophytic rhizobia-cereal associations have also been described for field-grown wheat, barley, wild rice, maize, sorghum, and millet rotated with legumes in Canada, Morocco, Senegal, Mexico, Kenya, India, and elsewhere in Egypt (Biederbeck et al., 2000; Chaintreuil et al., 2000; Englehard et al., 2000; Hartmann et al., 2000; Hilali et al., 2000; Lupway et al., 2000; Matiru et al., 2000; Gutierrez-Zamora and Martinez-Romero, 2001; Yanni et al., 2002).

As part of our continuing efforts towards gaining a better understanding of the underlying mechanisms of this intimate plant-bacterial association and in order to exploit its benefits for sustainable agriculture, we are investigating the spatial ecology of *Rhizobium leguminosarum* bv. *trifolii* strain E11. This rhizobial strain is a natural rice root endophyte indigenous to the Nile delta; it promotes the growth of selected varieties of rice, corn, and wheat, and performs

well as a candidate biofertilizer inoculant for rice under field conditions in that region (Yanni et al., 1997; Yanni et al., 2001). These studies of autecological biogeography (i.e., the ecology and prevalence in distribution of a single organism at various spatial scales) are being performed at two different spatial scales. One is at the kilometer (macro) scale relevant to field-inoculation studies where the distribution of this selected strain is being mapped in numerous rice-clover field sites scattered throughout the Nile Delta plus other sites in Egypt located southwest of Cairo where cereals are cultivated in a fertile area surrounded by desert. The second is at the micrometer (micro) scale which is directly relevant to bacteria-bacteria and bacteria-plant interactions where spatial patterns of rhizobial colonization of rice roots are defined *in situ* at single-cell resolution.

The most comprehensive method of ecological distribution analysis makes use of geostatistics, where a user-defined parameter of spatial abundance (e.g., localized density of colonized microbes) is measured at various sampling points within the spatial domain and then analyzed to produce a mathematical model for spatial autocorrelation that accurately defines various spatially dependent relationships derived from regionalized variable theory, plus makes optimal, statistically rigorous interpolation (kriging) maps of the parameter measured within that spatial domain, even for points not physically sampled (Robertson and Gross, 1994). Geostatistical analysis and krig mapping require that the relevant parameter being analyzed (the so-called "Z variate") is a quantitative (non-binary) value that is continuously distributed over the spatial domain and that the sampling sites are georeferenced, i.e., located at known Cartesian x,y coordinates relative to a landmark origin position within that domain. So in order for microbial biogeography studies to take full advantage of the awesome predictive power of geostatistical techniques to model the spatial distribution of the bacterial strain of interest, a single quantitative value of the Z variate (in this case, the localized density of the bacterial strain of interest relative to its nearest neighbors) must be assigned to each georeferenced site sampled.

Our autecological biogeography studies use various types of microscopy to locate the rhizobial strain of interest. For the macro field-scale studies, we use routine immunofluorescence microscopy to locate the specific bacterial strain as a root-nodule occupant on uninoculated berseem clover plants at various sample sites. Although the genotype diversity of rhizobia may differ when isolated directly from soil and from legume nodules (Louvier et al., 1996; Hartmann et al., 1998), we prefer the legume trap approach since the nodules contain large rhizobial populations that can be easily sampled directly in the field, preserved, transported intercontinentally and imported into the USA with proper APHIS permits, processed in the lab, and analyzed without a large background community of highly diverse microbes. The selective use of trap

host plants for ecological distribution studies of rhizobia has the added advantage in that only those strains most relevant to agriculture will be selected and strains present in low numbers may be detected if they are competent nodulators (Handley et al., 1998).

Since the mere presence or absence of the rhizobial strain of interest in the nodule is a binary score, it alone does not fulfill the requirement of being a quantitative, continuously distributed measure of prevalence as the Z variate for the geostatistical analyses. On the other hand, a score of percent nodule occupancy per plant or per sample site can overestimate the real prevalence of the strain of interest (especially with small sample sizes) since this value is unaffected by commonly occurring multiple nodule occupancies and/or partial antigenic relatedness of the rhizobial occupants present. For the root colonization studies at single cell-resolution, scanning electron microscopy and immunofluorescence microscopy of cereal roots are the methods of choice for gnotobiotic and field-scale studies, respectively, followed by computer-assisted digital image analysis of the georeferenced image quadrats sampled. Consequently, an appropriate measure of the spatial clustering of bacteria during root colonization is needed to serve as this Z variate. The objective of this study was therefore to develop quantitative measures of the prevalence of a rhizobial strain at sampling sites that are appropriate for use as the Z variate in geostatistical analysis of its autecological biogeography at micro and macro spatial scales. Portions of this work were presented at the 9th International Symposium on Nitrogen-Fixation with Non-Legumes, September 1–5, 2002 at Leuven, Belgium (Yanni et al., 2002).

2. Materials and Methods

Production of polyclonal antibodies to somatic antigens of R. leguminosarum bv. trifolii strain E11 and their use in immunofluorescence microscopy

Cells were grown for 5 days at 30°C on B3 agar plates supplemented with a 1:5 (v/v) dilution of filter-sterilized exudate of axenically grown rice roots (4 seedlings grown hydroponically for 4 days in half-strength Hoagland's medium [Sigma Chemical Co., St. Louis, MO, USA]) and a 1:5 dilution of Nile delta soil extract (100 g soil plus 1% $CaCO_3$ extracted with 100 ml deionized water for 1 hr at 100°C). Cells were suspended in phosphate buffered saline (PBS containing 10 mM KH_2PO_4-K_2HPO_4, 138 mM NaCl, pH 7.2), steamed for 1 hr at 100°C to inactivate non-somatic antigens, and washed by centrifugation in PBS. Rabbit polyclonal antisera were prepared against the immunodominant somatic antigens of these steamed and washed cells, and cells of the same population were used to prepare slides for titering the strain-specific

immunoreactivity of the antibody (Dazzo and Wright, 1996; McDermott and Dazzo, 2002).

Processing of root nodules and immunofluorescence microscopy of rhizobial occupants

Nodules on roots of uninoculated (naturally nodulated) berseem clover (*Trifolium alexandrinum*) were sampled in the Nile delta at five field sites near the Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt where this forage crop has been rotated with rice since antiquity (Yanni et al., 1997). Nodules were washed free of soil in running water, blotted dry, stored desiccated over cotton/$CaCO_3$ and transported to Michigan State University. They were rehydrated in sterile water, surface-sterilized using 0.1% (w/v) $HgCl_2$, rinsed several times in sterile water, squashed directly into wells of Teflon-coated microscope slides, gently heat-fixed, and stored dry until analyzed. For indirect immunofluorescence microscopy, samples on slides were pretreated with alkaline-hydrolyzed gelatin to suppress non-specific staining, then with a 1:1,000 dilution of the anti-E11 polyclonal antibody followed by a 1:20 dilution of affinity-purified FITC-conjugated goat anti-rabbit IgG immunoglobulin (Sigma Chemical Co.), and mounted in Vectashield photobleaching retardant. Extensive testing of this antiserum at the specified conditions indicated no immunofluorescence cross-reactivity with a diversity of 9 other rice-adapted strains of *R. leguminosarum* bv. *trifolii* isolated from the same region. Slides were examined with a Zeiss Research Photomicroscope 1 equipped with an HBO 100 lamp and FITC epifluorescence optics. The scoring of immunofluorescence results relative to the background covered a range of increasing immunoreactivity and fluorescence intensity from 0 to 4+ as previously described (Dazzo and Wright, 1996; McDermott and Dazzo, 2002).

Scanning electron microscopy and CMEIAS image analysis

Gnotobiotic cultures were grown for 4 days under growth chamber conditions, and 4 mm root segments were processed for SEM as previously described (Yanni et al., 2001). Georeferenced digital images were spatially calibrated, converted to binary in Adobe Photoshop, and analyzed using our custom CMEIAS image analysis system (Liu et al., 2001; Dazzo et al., 2001) to extract spatial distribution data from every foreground bacterial object of interest. CMEIAS ver. 1.27 will soon be available as a free internet download at <http://cme.msu.edu/Cmeias/>. Spatial autocorrelation semivariograms and kriging interpolation maps of the CMEIAS-acquired georeferenced data were created using GS+ Geostatistics software (Robertson, 2002).

3. Results and Discussion

Here we illustrate two new indices for autecological biogeographic studies of selected strains of rhizobia: one using immunofluorescence microscopy and the other using scanning electron microscopy combined with computer-assisted image analysis. These indices can be used as the Z variates in geostatistical analysis of the spatial ecology of the rhizobial strain of interest [in this case, a rhizobial endophyte strain evaluated for use as a candidate biofertilizer for rice] that is sampled at multiple georeferenced sites.

First, we introduce a quantitative Autecological Biogeography Index (ABI) to serve as a Z variate indicating the relative abundance of the rhizobial strain in root-nodule symbiosis with its legume trap host at various sampling sites. The formula to compute this macro scale index takes into account the number of nodules analyzed at each site sampled, the proportion of nodules containing occupants that immunoreact with the specific antiserum at each site sampled, and the relative intensity of immunofluorescence brightness of nodule occupants (0 to 4) indicative of the presence of multiple strains within the nodule and their degree of antigenic relatedness to the strain against which the antibody was prepared.

This ABI index is calculated according to the following equation:

$$ABI = [(n_1/N) 1 + (n_2/N) 2 + (n_3/N) 3 + (n_4/N) 4] \tag{1}$$

where N = sample size (number of nodules scored at each sample site), n_1 = number of nodules with occupants at the 1+ immunoreactivity level, n_2 = number of nodules with occupants at the 2+ immunoreactivity level, n_3 = number of nodules with occupants at the 3+ immunoreactivity level, and n_4 = number of nodules with occupants at the 4+ immunoreactivity level.

A lower, valid immunofluorescence result of "±" for the nodule squash occupants (cells with slightly brighter immunofluorescence than background but insufficient intensity for photographic recording using ASA 400 film) is given a score of "0" here and thus does not contribute to the computed Z variate of strain abundance at that georeferenced site.

To illustrate the use of this ABI index in autecological studies of rice-adapted rhizobia, we examined the prevalence of strain E11 in 218 nodules collected at 5 field sites near the Sakha Agricultural Research Station, Kafr El-Sheikh, Egypt. The immunofluorescence microscopy results are summarized in Table 1. Fig. 1 presents the computed ABI index values from these immunofluorescence data, and for comparison, the percent nodule occupancy calculated from these same data at the same sample sites. Because the ABI index is weighted by the presence and relative serological relatedness of multiple nodule occupants, it provides a more realistic measure of the prevalence of this strain of interest at the sites sampled rather than does %
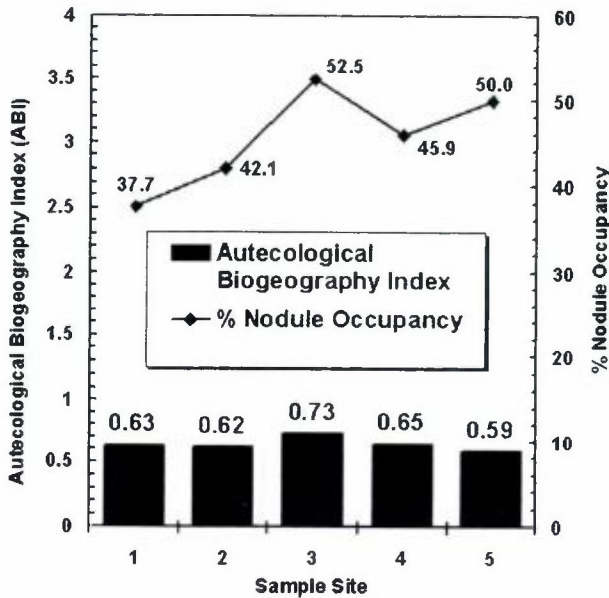
Figure 1.   Autecological biogeography of *R. leguminosarum* bv. *trifolii* E11 at 5 sample sites near Sakha, Kafr El-Sheikh, Egypt. The autecological biogeography index provides a more realistic representation of the relative abundance of the rhizobial strain at each sampling site than does the traditional parameter of % nodule occupancy, which tends to overestimate the relative abundance.

Table 1.   Relative abundance measurements to compute the autecological biogeography index of *R. leguminosarum* bv. *trifolii* strain E11 at 5 sample sites near Sakha, Kafr El-Sheikh, Egypt.

Abundance parameter	Sample site near Sakha				
	1	2	3	4	5
# of nodules examined per sample site (N)	53	57	59	37	12
# of nodules with 1+ immunoreactive occupants (n_1)	8	16	20	10	5
# of nodules with 2+ immunoreactive occupants (n_2)	11	5	10	7	1
# of nodules with 3+ immunoreactive occupants (n_3)	1	3	1	0	0
# of nodules with 4+ immunoreactive occupants (n_4)	0	0	0	0	0

nodule occupancy, which tends to overestimate its prevalence. This set of data is part of a larger ongoing study of the autecological biogeography of this strain

that includes a total sample size of 7,550 nodules obtained from uninoculated berseem clover plants at 47 sites (40 sites scattered throughout the Nile delta [35 from 3 different collection transects in the Nile delta and the 5 analyzed here that were collected near the Sakha Agricultural Research Station, Kafr El-Sheikh] and 7 other sites in a fertile area surrounded by desert southeast of Cairo). When obtained, all of these 47 ABI values will be used as the Z variate for each georeferenced sampling point in a geostatistical analysis of the prevalence in distribution of strain E11 in various rice-clover agroecosystems in Egypt.

Another index intended for use in studies on a micro scale of the spatial distribution of bacteria colonized on root surfaces is the Cluster Index (CI), calculated by the following equation:

$$CI = [1st\,NND]^{-1} \qquad\qquad\qquad (2)$$

where CI is the cluster index, and $[1st\,NND]^{-1}$ is the inverse of the distance between the centroid x,y position of every bacterium found within the spatial domain and its 1st nearest bacterial neighbor. Fig. 2A is an example of an image quadrat sampled by scanning electron microscopy on a rice root 4 days after incubation with strain E11 in gnotobiotic culture. Note the slightly clustered (non-uniform, non-random) distribution of the bacteria on the root surface, with their prevalence at junctions between epidermal cells providing potential portals of entry into the root interior. In this case, the centroid Cartesian x,y coordinates (relative to the $0,0$ landmark origin set at the image's lower left corner) and the CI values are extracted from each bacterial cell by CMEIAS image analysis.

Figure 2.   Geostatistical analysis of the spatial ecology of *Rhizobium leguminosarum* bv. *trifolii* strain Ell in an image quadrat of the root surface of Sakha 102 rice. A) Scanning electron micrograph showing a slightly clustered colonization pattern of bacterial cells particularly at epidermal root cell junctions. Note the potential portals of bacterial entry into the root. Bar scale is 5 µm. B) Auto-correlation semivariogram showing the best-fit isotropic model that indicates spatial dependence in distribution of the rhizobia colonized on the rice root surface in image A above, using the newly introduced Cluster Index as the Z variate. C) A 2-dimensional kriging interpolation map of the clustered distribution of rhizobial cells based on the semivariance autocorrelation model depicted in image B above. This method provides a statistically rigorous, continuous interpolation of the Cluster Index values within the spatial domain, even in areas not physically sampled (e.g., beneath overlying root hairs). The isopleths (continuous contour intervals as used in a weather map) are more distinct when the Z variate ladder is pseudocolored instead of grayscale.
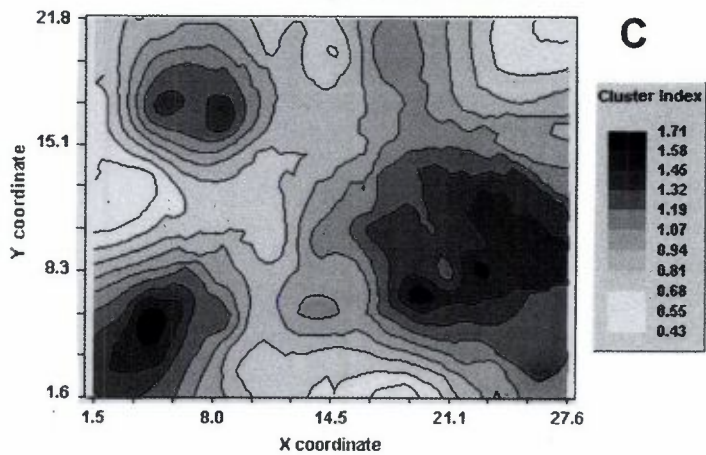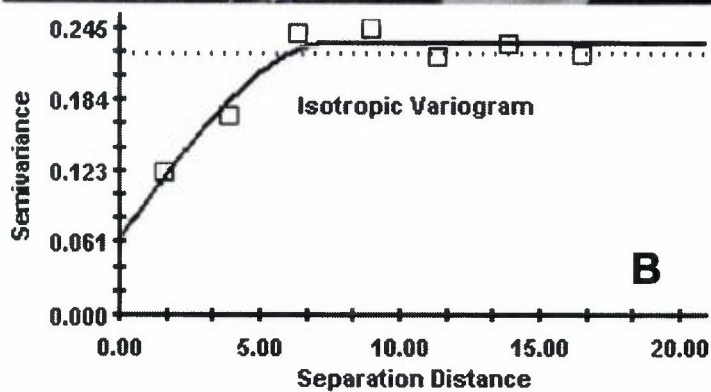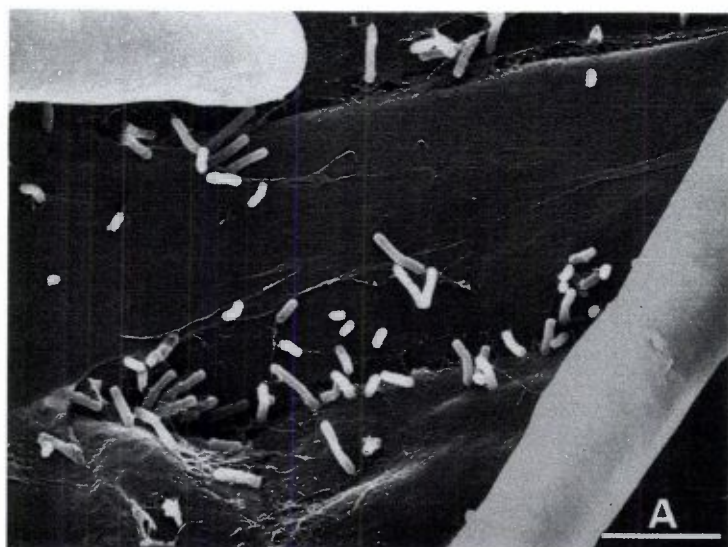
Figure 2.

This CI value serves as the Z variate in this geostatistical analysis of root colonization, and its value at each bacterial sampling point increases in proportion to the local clustered (increased proximity) distribution of the colonized bacteria. This powerful spatial modeling technique tests whether bacterial colonization is autocorrelated, i.e., has spatial dependence, and if so, it can then (i) define the spatial scale of separation distance at which bacterial interactions influence their spatial distribution, (ii) predict the most probable pattern of colonization behavior, and (iii) produce a statistically defensible interpolation (kriging map) of the continuous spatial distributions of each organism's influence on colonization by its nearest bacterial neighbors, even in areas of the root epidermis that cannot be physically sampled (in this case, areas obscured beneath overlying root hairs). The semivariogram produced by the geostatistical analysis of this image provides unambiguous evidence of spatial dependence in bacterial distribution colonized on the root surface, with an exponential isotropic model making the best fit to the autocorrelation data of this CI-Z variate (Fig. 2B), and the corresponding 2-dimensional kriging interpolation map of the influence of clustering on bacterial colonization of the root in this image quadrat (Fig. 2C).

In summary, the two quantitative indices of microbial prevalence introduced here are admirably suited as Z variates for the geostatistical analysis of the distribution of a rhizobial strain of interest at micro and macro spatial scales. Their use provides a fundamentally new quantitative approach in autecological biogeography to examine the spatial ecology of rhizobial strains selected for superior performance as candidate cereal biofertilizers. Information ultimately derived from this study should assist the biofertilization strategy program in predicting how effective will rhizobial inoculation be to enhance cereal growth and performance in the corresponding Egyptian agroecosystems. Also, both indices introduced here have the potential for even broader applications in microbial ecology, including studies of surface biofilm development.

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