1 **Genetic diversity of indigenous *Bradyrhizobium* nodulating promiscuous**

2 **soybean [*Glycine max* (L) Merr.] varieties in Kenya: Impact of phosphorus**

3 **and lime fertilization in two contrasting sites.**

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26

27 **Abstract**

28 While soybean is an exotic crop introduced in Kenya early last century, promiscuous
29 (TGx) varieties which nodulate with indigenous rhizobia have only recently been
30 introduced. Since farmers in Kenya generally cannot afford or access fertilizer or
31 inoculants, the identification of effective indigenous *Bradyrhizobium* strains which
32 nodulate promiscuous soybean could be useful in the development of inoculant strains.
33 Genetic diversity and phylogeny of indigenous *Bradyrhizobium* strains nodulating seven
34 introduced promiscuous soybean varieties grown in two different sites in Kenya was
35 assayed using the Polymerase Chain Reaction-Restriction Fragment Length
36 Polymorphism (PCR-RFLP) of the 16S-23S rDNA intergenic spacer region and 16S
37 rRNA gene sequencing. PCR-RFLP analysis directly applied on 289 nodules using *Msp* I
38 distinguished 18 intergenic spacer groups (IGS) I-XVIII. Predominant IGS groups were I,
39 III, II, IV and VI which constituted 43.9%, 24.6%, 8.3% 7.6% and 6.9% respectively of
40 all the analyzed nodules from the two sites while IGS group VII, IX, X, XI, XII, XIV,
41 XVI, XVII, XVIII each constituted 1 % or less. The IGS groups were specific to sites and
42 treatments but not varieties. Phylogenetic analysis of the 16S rRNA gene sequences
43 showed that all indigenous strains belong to the genus *Bradyrhizobium. Bradyrhizobium*
44 *elkanii, Bradyrhizobium* spp *and Bradyrhizobium japonicum* related strains were the
45 most predominant and accounted for 37.9%, 34.5%, and 20.7% respectively while *B.*
46 *yuanmigense* related accounted for 6.9 % of all strains identified in the two combined
47 sites.

48 The diversity identified in *Bradyrhizobium* populations in the two sites represent a
49 valuable genetic resource that has potential utility for the selection of more competitive

50 and effective strains to improve biological nitrogen fixation and thus increase soybean

51 yields at low cost.

52

53 **Key words:** Agro-ecological zones, genetic diversity, promiscuous soybean, polymerase

54 chain reaction- restriction fragment length polymorphism.

55

56 **Introduction**

57 Soybean [*Glycine max* L. (Merrill)] has recently gained prominence in Kenya due to its
58 potential as food, livestock feed, for soil fertility improvement and income generation for
59 small-holder farmers. As human food, it is rated as one of the most important sources of
60 high quality protein, edible oils and vitamins (GTZ 1996). In Kenya, soybean cultivation
61 is reported to have started in earnest on a small-scale in the early sixties in Nyanza and
62 Western provinces and by large-scale farmers for fodder in Trans-Nzoia, Uasin Gishu,
63 Laikipia and Nakuru (Njuguna 1985). Currently in Kenya, soybean production has only
64 reached a fraction of the demand of food processors and animal feed millers. While
65 demand is expected to rise to about 150,000 tons per year over the next ten years (TSBF
66 2003), local production is estimated at less than 10,000 tons. Soybean varieties currently
67 grown by farmers produce low yields and have low soil improving potential unless
68 inoculated with *Bradyrhizobium* bacteria to enhance their nodulation and nitrogen fixing
69 ability. Most parts of Western, Central and Eastern Kenya regions receive adequate
70 rainfall, have mostly well drained soils, moderate to high fertility with pH ranges of 5.5-
71 7.5 and are hence considered to have the highest potential for soybean production and
72 future expansion. Most of the soils in those areas have a high phosphorus (P) fixation
73 capacity necessitating the addition of P for adequate nodule establishment and function
74 (Sinclair and Valdez 2002). Inoculation success depends on both (abiotic) factors such as
75 extremes of pH, high temperature and desiccation as well as biotic stresses (Brockwell et
76 al. 1991).

77 Compatible populations of specific *Bradyrhizobium* species necessary for nodulation of
78 soybean are seldom present in soils where the crop has not been previously grown.

79 *Bradyrhizobium japonicum* inoculation is therefore required in order to achieve adequate
80 and effective nodulation of the crop when first introduced to many tropical soils that may
81 even contain high cowpea rhizobial populations (Caldwel and Vest 1968). The need to
82 inoculate was considered unfeasible because many African countries were not adequately
83 equipped to deal with problems associated with *Bradyrhizobium* inoculant use in the
84 tropics (Ayanaba 1977). This remains an important constraint in the production of
85 soybean genotypes with specific *Bradyrhizobium* requirements. To circumvent this
86 problem, the soybean breeding program at the International Institute for Tropical
87 Agriculture (IITA), Nigeria, developed soybean genotypes, designated TGx (tropical
88 glycine cross), which nodulate effectively with indigenous *Bradyrhizobium* spp
89 populations. These promiscuous varieties have been tested in many African countries
90 without N fertilizer or *B. japonicum* inoculation. Results from these trials indicate that
91 indigenous *Bradyrhizobium* populations do not always meet the N demand of the tested
92 TGx genotypes in many locations in Nigeria (Sanginga et al. 1996; Okereke and
93 Eaglesham 1992) and eastern and southern Africa (Mpepereki et al. 2000). The presence
94 in sufficient populations of effective indigenous soil bradyrhizobia can facilitate TGx.
95 varieties to derive nitrogen (N) through biological nitrogen fixation (BNF) and to
96 determine whether or not they will respond to added rhizobia or N fertilizer (Turk et al.
97 1993). Success of inoculation depends on several environmental (abiotic) factors such as
98 extremes of pH, high temperature and desiccation as well as biotic stresses (Brockwell et
99 al. 1991; Kahindi et al. 1997; Zahran 1999; Musiyiwa et al. 2005). Studies have indicated
100 that in some soils, as few as 10 competitive indigenous bradyrhizobia per gram of soil
101 can act as an efficient barrier to introduced strains (Thies et al. 1991a; Abaidoo et al.

102 2007). Indigenous rhizobia associated with leguminous crops are diverse. They exhibit
103 this diversity in their genetic constitution as well as competiveness and effectivity with
104 and between hosts (Padzemik et al. 1977; Pueppke and Broughton 1999).
105 A variety of methods exist for the assessment of genetic diversity in closely or distantly
106 related bacterial species. Traditionally, variation has been determined using
107 characteristics such as growth rate and colony morphology (size, shape, color, texture and
108 general appearance) and antibiotic resistance methods (Graham et al. 1991; Somasegaran
109 and Hoben 1994**)**. However, these methods are not sufficiently discriminative to account
110 for all the variation exhibited in the target species. They cannot delineate sources of
111 observed phenotypic variation into its components that may be due to environmental
112 factors or underlying genetic factors. In recent years, DNA techniques have been used to
113 detect sequence polymorphisms within and between strains of bacteria (Laguerre et al.
114 1994; Williams et al 1990; Vost et al. 1995; Nakamura et al. 1987; Lanham and Brenan
115 1999; Stern et al. 1984; Hulton et al. 1991; Virdi and Sachdeva 2005).
116 The application of PCR-RFLP analysis of the 16S-23 rDNA intergenic region and
117 sequence analysis of the 16S RNA gene are vital tools in clustering genetically related
118 rhizobia. These have been frequently used in microbial taxonomy to determine inter and
119 intra specific relationships (Vinuesa et al. 1998; Abaidoo et al. 2000; Doignon-Bourcier
120 et al. 2000; Sarr et al. 2005). In these methods, the generated PCR fingerprints are unique
121 to each isolate and are used to group them at strain level. Previous studies on the diversity
122 of bradyrhizobia from soybean have used the PCR-RFLP analysis, a high-resolution
123 genotypic fingerprinting technique based on the restriction of amplified fragments from
124 total genomic DNA (Laguerre et al. 1994). The PCR-RFLP technique was shown to

125 provide an insight into the extent of genetic diversity of indigenous *Bradyrhizobium*
126 isolates nodulating cowpea (Krasova-Wade et al. 2003). The genetic diversity of rhizobia
127 nodulating promiscuous soybean varieties has not been determined in Kenya.
128 Determination of the genetic diversity of indigenous *Bradyrhizobium* populations in
129 Kenyan soils will be a valuable first step in the development of cost effective strategies to
130 optimize biological nitrogen fixation and thus increase soybean yields.

131 The objectives of this study were (i) to assess the natural nodulation of seven
132 introduced promiscuous soybean varieties with indigenous *Bradyrhizobium* strains under
133 phosphorus and lime application in two diverse sites in Kenya (ii) to assess the genetic
134 diversity of these indigenous *Bradyrhizobium* strains based on PCR amplification and
135 restriction of the 16S-23S rDNA intergenic region and sequence analysis of the 16S
136 rRNA gene and (iii) to determine the genetic relatedness of the indigenous bradyrhizobia
137 to the reference strains in the GenBank.

138

139 **Materials and Methods**

140 **Experimental sites**

141 This study was implemented in Mitunguu (Latitude: N 00° 06' 00.5", Longitude: E 037°
142 47' 39.2") at an elevation of 959 m above sea level and Bungoma (Latitude: N 00° 76'
143 68.0", Longitude: E 034° 67' 05.7") at an elevation of 1648 m, selected in different agro-
144 ecological zones with high potential for soybean production in Eastern and Western
145 Kenya respectively. The predominant soils at Mitunguu (LM_3) and Bungoma (LM_1) are
146 ferralsols (FAO-UNESCO 1990). The Bungoma and Mitunguu sites received 545 mm

147 and 377 mm of rainfall respectively during the crop growing season and had no known
148 recorded history of soybean cultivation or inoculation with *Bradyrhizobium* strains.
149
150 **Soil sampling and analysis**
151 The top soil (0-15cm) from each site was sampled before fields were prepared for
152 planting by sampling randomly eight cores per replicate using a 3.5 cm soil auger,
153 bulked, sub-sampled and analyzed for organic carbon (OC) content, extractable P, soil
154 pH) analysis using ICRAF laboratory procedures (1999). Soil solution pH was measured
155 using a glass electrode in a suspension of 5.0 g soil in 10 ml water after equilibrating for
156 60 min.
157
158 **Soybean genotypes and fertilizer treatments**
159 In each site, TGx varieties were planted in a strip plot design with varieties allocated at
160 the main and fertilizer treatment at the sub-plot level. Treatments were control (none), +
161 P (40kg/ha), P + lime (1t/ha), + N (90kg N /ha, split applied) + lime + P (to establish the
162 need for inoculation) were applied at sub-plot level. The mineral sources were applied in
163 the form of triple superphosphate (TSP), lime and urea. TSP and lime were broadcasted
164 and incorporated before planting. At top dressing, urea was banded and incorporated near
165 the soybean lines. Before planting, all seed was surface sterilized with 96% ethanol for 30
166 sec and rinsed with sterile water, then surface sterilized with 3.3% w/v $Ca(OCl)_2$ for 3
167 min, and rinsed with sterile distilled water five times to remove excess disinfectant.
168 Disinfected seed was then drilled in rows, 45 cm apart, and thinned to 5 cm distance
169 between plants after emergence. Main plot sizes were 2.5 m x 7.2 m while sub-plot sizes

170 were 2.5 m x 1.8 m consisting 4 rows of 2.5 m long and 0.45 m wide. The variety Nyala,
171 a specific variety bred in Zimbabwe was used as a specific nodulating control while TGx
172 genotypes, crosses between non promiscuous North American soybean genotypes and
173 promiscuous Asian soybean genotypes (Kueneman et al. 1984) were used as test
174 varieties. These were SB 4 (TGx 1871-12E), SB 8 (TGx 1895-33F), SB 9 (TGx1895-
175 49F), SB 15 (TGx 1889-12F), SB 17 (TGx 1893-10F), SB 19 (TGx 1740-2F) and SB 20
176 (TGx 1448-2E).

177

178 **Nodule sampling and storage.**

179 At full podding (R3) (Fehr et al. 1971), plants from a 0.5 m row long section were
180 randomly sampled leaving at least 0.5m from each end of any of the two net plot rows.
181 The plants were excavated and the entire roots mass including nodules were carefully
182 collected. Nodules were thoroughly cleaned to remove traces of soil then immersed in
183 95% ethanol and kept in glycerol at 30°C. Before analysis each nodule was surface
184 sterilized with 96% ethanol for 30 sec and rinsed with sterile water, then surface
185 sterilized with 3.3% w/v $Ca(OCl)_2$ for 3 min, and three times rinsed with sterile distilled
186 water. From this stage the nodules were manipulated aseptically. Each nodule was
187 crushed in 300 µl of sterile water with plastic micro pestles sterilized in 96% ethanol in a
188 1.5 ml Eppendorf tube.

189

190 **DNA extraction**

191 Direct DNA-extraction from nodules as defined by Rouvier et al. (1996) and optimized
192 by Thiao et al. (2004) with few modifications was used. Total genomic DNA from ten

193 nodules per treatment was extracted as described by Krasova-Wade et al. (2003). The
194 resulting DNA pellet was washed with 70% v/v ethanol by centrifugation for 10 min at
195 13000 rpm at room temperature, air dried and re-suspended in 50µl of sterile double
196 distilled water. Ten µl of RNase (40 µg/ml) were added to DNA extract and incubated at
197 37°C for 30 min. DNA was also extracted from the reference strain USDA 110 using the
198 same procedure. Two methods were used to determine genetic diversity in this study,
199 PCR amplification and restriction of the 16S-23S rDNA intergenic region and 16S rRNA
200 gene sequencing.
201

202 **PCR amplification of the 16S-23S rDNA spacer region.**

203 A 930-1100 bp intergenic region between the 16S and 23S rDNA from 289 nodules was
204 amplified by PCR with primers derived from the 3' end of the 16S rDNA (FGPS 1490-
205 72; 5'-TGCGGCTGGATCCCCTCCTT-3') corresponding to positions 1521-1541 of *E.*
206 *coli* (Navarro et al. 1992) and from the 5' end of the 23S rDNA (FGPL 132-38; 5'-
207 CCGGGTTTCCCCATTCGG-3') corresponding to positions 114-132 of *E. coli*
208 (Ponsonnet and Nesme 1994). PCR amplification was carried out in a 25µl reaction
209 volume containing 2µl of pure total DNA extract, freeze dried beads (Ready-to-Go PCR
210 beads, Pharmacia Biotech) containing 1.5 U of Taq DNA polymerase, 10mM Tris-HCL,
211 (pH 9 at RT), 50 mM KCL, 1.5 mM $MgCl_2$, 200 µM of each dNTP and 1.0 µM of each
212 primer. PCR amplification was performed in a Primus 96 ^{plus} thermal cycler (MWG AG
213 BIOTECH) adjusted to the following program: initial denaturation for 5 min at 94 ^{o}C, 35
214 cycles of denaturation (30 s at 94 ^{o}C), annealing (30 sec at 58 ^{o}C) and extension (30 sec
215 at 72 ^{o}C) and a final extension (7 min at 72°C). PCR amplified DNA was visualized by

216 electrophoresis of 3µl of the amplified DNA on 1% (w/v) horizontal agarose gel
217 (SIGMA®) in TBE buffer (1.1 w/v Tris-HCL; 0.1% w/v Na_2EDTA $2H_2O$; 0.55% w/v
218 Boric acid), pre-stained with 3.5µl of ethidium bromide. The gel was photographed under
219 UV illumination with Gel Doc (BIO-RAD) Software (USA).
220
221 **Restriction fragment analysis of 16S-23S r DNA intergenic spacer region**
222 Aliquots (10 µl) of PCR products were digested with the restriction endonuclease *Msp*I
223 as specified by the manufacture (Roche, Germany) in a total volume of 15 µl for 3 hours
224 at 37°C. The restriction fragments were separated by horizontal electrophoresis in 1 X
225 TBE buffer with 3% (w/v) agarose Sigma® (Sigma-Aldrich Chemie Gmbh, Steinheim,
226 Germany) pre-stained with 3.5 µl of ethidium bromide. The gels were run at 100 V for 3
227 hours and photographed under UV illumination with Gel Doc (BIO-RAD, USA)
228 software. Restriction fragment data was scored twice as band presence (1) and absence
229 (0) and configured as an input file and analyzed using the NTSYS package. Un-weighted
230 Pair Group Method with Arithmetic Averaging (UPGMA) algorithm was used to perform
231 cluster analysis and to construct a dendogram. Strains that had identical restriction
232 fragment profiles were classified into the same genotypic/intergenic spacer (IGS) group.
233 Shannon's index of diversity Ho, (King and Schaal 1989) was estimated based on the
234 number of isolates belonging to each IGS group.
235
236 **Sequence analysis of the 16S rRNA gene**
237 A sample of twenty nine bradyrhizobial isolates from different varieties grown at
238 different sites and treatments were selected from the 18 IGS groups for 16S rRNA gene

239 sequencing. The forward primer 27F (5'-AGA GTT TGA TCC TGG CTC AG-3')
240 corresponding to positions 27-46 of the *E. coli* of the 16S rRNA gene sequence and the
241 reverse primer 1492R (5'-GGTT TAC CTT GTT ACG ACT T -3') corresponding to
242 positions 1525-1506 of *E. coli* (Lane, 1991) were used to amplify the 16S rRNA gene.
243 The 1500 bp PCR products were sequenced for the DNA region coding for the 16S rRNA
244 gene in an ABI 377 (PE-Applied Biosystems sequence analyzer. The generated
245 sequences were submitted to the GenBank database through BLAST to search for
246 significant 16S rRNA alignments. A phylogenetic tree was constructed based on the
247 partial 16S rRNA gene sequences of the TGx soybean nodule isolates and rhizobial
248 reference strains from the GenBank. The sequences of the rhizobial strains were aligned
249 pairwise and compared to type strains in the GenBank database. A dendogram was
250 inferred with Neigbour-Joining Algorithm (Saitou and Nei, 1987) using ClustalX
251 software (Thompson et al. 1997) and the phylogenetic tree reconstructed with PHYLIP
252 (Felsenstein 1993), package and a bootstrap analysis using 100 replications. Shannon's
253 index of diversity (Ho) was estimated based on the number of strains belonging to each
254 genus / species (King and Schaal 1989).
255
256 **RESULTS**
257 **Soil Properties**
258 The Bungoma had a pH of 6.1 and contained 0.07 g N kg $^{-1}$ while the Mitunguu soil had a
259 pH of 6.9 and contained 0.25 g N kg $^{-1}$ (Table 1). Extractable P content was 2.39 mg kg $^{-1}$
260 and 9.25 mg kg $^{-1}$ at Bungoma and Mitunguu, respectively (Table 1).
261

262 **Nodulation of promiscuous varieties with indigenous bradyrhizobia across**
263 **treatments and sites**

264 Nodules were observed on all varieties at both sites and for all treatments except the +N
265 treatment (Table 2 and 3). There were more nodules per 0.5 m row and nodule fresh
266 weights at Mitunguu than Bungoma (Table 2 and 3). When averaged over treatments,
267 nodule numbers ranged from 5.0 on Nyala to 136 on SB 15, while nodule fresh weight
268 ranged from 0.26g for Nyala to 9.71g for SB 8 at Bungoma (Table 2). At Mitunguu,
269 nodule numbers per 0.5 m row ranged from 124 on SB 17 to 275 on SB 4 while nodule
270 fresh weight ranged from 9.66 g for SB 17 to 22.40 g for SB 4. There was a significant
271 interaction ($P<0.05$) in nodulation response to the application of P and lime at both
272 Bungoma and Mitunguu (Table 2 and 3). In general, the application of phosphorus
273 resulted in a positive response in nodule numbers and nodule fresh weights in all but
274 Nyala, SB-17 and SB-8 for Mitunguu (Table 3) and in all but SB 17 and SB 4 (Table 2)
275 in Bungoma. Of all the nodules, 87 % were active in presence of P compared to 64 % in
276 the absence of P (data not shown). There was a significant growth and biomass yield
277 response to the application of +N treatment in Bungoma but not at Mitunguu (data not
278 shown).
279

280 **PCR-RFLP of 16S-23S rDNA IGS Region**

281 Single IGS PCR products ranging from 930-1100 bp were obtained from the 289 nodules
282 and one reference strain (USDA110). Digestion with the restriction enzyme *Msp* I
283 produced 18 different RFLP profiles (IGS groups) (Figure 1). Each nodule presented a
284 single profile. The five most predominant IGS groups were I, III, II, IV and VI which

285 constituted, 43.9%, 24.6%, 8.3% 7.6% and 6.9% respectively of all the analyzed nodules
286 from the two sites, while IGS groups VII, IX, X, XI, XII, XIV, XVI, XVII, XVIII each
287 constituted 1 % or less (Table 4). Both sites had relatively similar numbers of different
288 indigenous bradyrhizobia strains and IGS groups. Mitunguu had 141 strains
289 disaggregated into 12 IGS groups while Bungoma had 148 strains disaggregated into 13
290 IGS groups. Some IGS groups were specific to sites and treatments but not varieties
291 (Table 4). While five IGS groups (IX, XIII, XVI, XVII, and XVIII) were specific to
292 Bungoma, six groups (V, VII, VIII, X, XII, XIV) were detected only in Mitunguu. The
293 Shannon-Weaver (Ho) indices were similar for Bungoma (Ho = 1.9) and Mitunguu (Ho =
294 1.7).
295
296 **Sequence Analysis of the 16S rRNA gene**
297 All the selected 29 isolates produced a single PCR product with approximately 1500 bp.
298 The partial sequences of the 16S rRNA gene of these selected isolates of indigenous
299 bradyrhizobia were deposited in the GenBank and given accession numbers EU625518 to
300 EU625546 (Table 5). Alignments of partial sequences of the TGx isolates with related
301 16S rRNA gene sequences in GenBank database revealed that the 29 strains were all
302 closely related to the *Bradyrhizobium* genus (Figure 2). *Bradyrhizobium elkanii,*
303 *Bradyrhizobium* spp and *Bradyrhizobium japonicum* related strains were the most
304 predominant and accounted for 37.9%, 34.5% and 20.7%, respectively, while
305 *Bradyrhizobium yuanmigense* related strains accounted for 6.9% of all nodules analyzed.
306 Eleven strains (TSBF-161, TSBF-402, TSBF-344, TSBF-444, TSBF-404, TSBF-442,
307 TSBF-260A, TSBF-336A, TSBF-488, TSBF-137 and TSBF-530) were closely related to

308 *Bradyrhizobium elkanii,* ten strains (TSBF-531, TSBF-523, TSBF-534, TSBF-331,
309 TSBF-341, TSBF-333 TSBF-381, TSBF-504, TSBF-438 and TSBF-101A) to
310 *Bradyrhizobium* spp*,* six strains (TSBF-345, TSBF-336, TSBF-131, TSBF-216, TSBF-
311 101 and TSBF-102 to *Bradyrhizobium japonicum* while two strains *(*TSBF-441 and
312 TSBF-160) to *Bradyrhizobium yuanmingense* (Table 5). A phylogenetic tree derived
313 from the partial sequences of the 16S rRNA gene by neighbor-joining analysis (Fig. 2)
314 confirmed the greater relationship of indigenous strains of *Bradyrhizobium* to reference
315 strains of *B. elkanii, B. japonicum* and *Bradyrhizobium* spp. The *Rhizobium* and
316 S*inorhizobium* reference strains constituted an outside group in the phylogenetic tree.
317

318 **Discussion**

319 The TGx soybean varieties developed by **the** breeding program at IITA for promiscuity
320 nodulated with indigenous rhizobia in both sites where they have been introduced for the
321 first time. The presence of these indigenous *Bradyrhizobium* in both Nigeria and Kenya
322 soils separated by a tropical forest in central Africa would suggest a common
323 evolutionary path of bacteria influenced by comparable biotic and abiotic conditions in
324 both countries, Nigeria and Kenya. It could also be due to genetic exchange of *nif* genes
325 through a variety and combination of events such as strain dispersion, genomic
326 combination and horizontal gene transfer among indigenous *Bradyrhizobium*
327 communities along the Nigerian and Kenya contiguous path through the Congo forest.
328 Previous studies (Mulongoy and Ayanaba 1986) have reported the presence of
329 *Bradyrhizobium japonicum* in some African soils even though soybean was not
330 commonly grown. Kasasa (1999) and Musiyiwa et al. (2005) reported the presence of

331 indigenous rhizobia nodulating promiscuous soybean varieties in many soils in
332 Zimbabwe. Some of the isolates were as good or superior in N_2 fixation effectiveness to
333 commercial inoculant strains under greenhouse conditions.
334 The present study shows that P improved nodulation across tested varieties at both sites
335 although the magnitude of this response was higher at Bungoma which had a low
336 inherent soil P status. In the presence of P, nearly all improved varieties had more
337 nodules than the local variety at both sites and most of the nodules contained
338 leghaemoglobin indicating active N_2 fixation. Application of phosphorus has previously
339 been shown to increase soybean nodular traits (nodule number, nodule mass and size).
340 The significant interaction between varieties and treatments in nodulation response at
341 both sites suggests that some promiscuous soybean varieties may be less pH sensitive and
342 may require relatively less phosphorus than others for optimal nodulation (Munns et al.
343 1981). P requirement has previously been shown to vary among soybean genotypes
344 (Gunawardena et al. 1993) while the degree of nodulation has also been reported to
345 depend on plant genotype and field site (Sanginga et al 2000). Promiscuous varieties
346 showed improved growth (data not shown) and biomass yield in response to N fertilizer
347 application in Bungoma as compared to Mitunguu. This could be attributable to the low
348 population of background indigenous bradyrhizobia (Thies et al. 1991a). As a result, N_2
349 fixation induced by the indigenous bradyrhizobial community supplied less than optimal
350 amounts of N. This indicates that there is necessity to apply a rhizobial inoculum on the
351 varieties poorly nodulated by indigenous bradyrhizobia.
352 The phylogenetic tree clearly shows that the Kenyan isolates form a distinct group. Thus
353 the indigenous strains of *Bradyrhizobium* nodulating TGx varieties are distinct from

354 *Bradyrhizobium* that nodulate North American soybeans varieties. In Nigeria, similar
355 results were obtained by Abaidoo et al. (2000) with the TGx varieties. This is not
356 unexpected because the indigenous *Bradyrhizobium* from the two sites, with no previous
357 history of soybean cultivation and hence no introduction of exotic *Bradyrhizobium*
358 strains, have been genetically isolated and consequently have evolved independently. The
359 phylogenetic tree nevertheless shows that there is adequate genetic variation among the
360 indigenous strains of *Bradyrhizobium*. This study showed that *Bradyrhizobium* strains
361 nodulating promiscuous soybean varieties grown under lime and phosphorus application
362 in two contrasting sites in Kenya were highly diverse. This diversity could be linked to
363 the fact that *Bradyrhizobium* strains may have different capacities to utilize P in their
364 metabolic activities which influence nodule initiation and effectiveness (Mullen et al.
365 1988). There was a positive relationship between diversity assessed as number of IGS
366 groups and abundance of bradyrhizobia strain population at the two sites. Bungoma had
367 13 IGS groups comprising 148 strains while Mitunguu had 12 IGS groups comprising
368 141 strains. The relatively higher diversity in Bungoma (Ho=1.9) compared to Mitunguu
369 (Ho=1.7) could be attributed to a combination of factors such as the overall improved
370 environmental soybean growing conditions in Bungoma (humid) as compared to semi-
371 humid conditions at Mitunguu. Elsewhere, several authors have reported similar genetic
372 diversity indices of rhizobia nodulating soybean (Giongo et al. 2008; Sikora and
373 Redzepovic 2003; Chen et al. 2004; Hungria et al. 2006) and *Phaseolus vulgaris*
374 (Andrade et al. 2002) using molecular markers.
375 In our study, IGS groups were specific to sites and treatments but not varieties. This
376 finding is in accordance with results described by Wei Tao Zang et al. (2007) who

377 showed that geographical location affects composition and biodiversity of indigenous
378 rhizobia. Lime application has previously been reported to increase diversity of IGS
379 groups in *Phaseolus* nodulating rhizobia in Brazil (Andrade et al. 2002). Strains restricted
380 to a geographical location generally develop special phenotypic and genotypic
381 characteristics (Xu et al. 1995). In contrast to our findings, other researchers (Chen et al.
382 2004, Thiao et al. 2004) found no relationship between IGS groups and geographical
383 location.
384 In Kenya, few studies have investigated the genetic diversity of indigenous rhizobia
385 nodulating legumes (Anyango et al. 1995; Odee et al. 2002). The preponderance of
386 *Bradyrhizobium* spp related strains in Mitunguu and *B. elkanii* related strains in Bunguma
387 sites is attributed to their saprophytic competence at the respective sites (Anyango et al
388 1995; Batista et al. 2006). Our results corroborate those of Abaidoo et al. (2002) who
389 showed that TGx varieties in Nigeria were nodulated by *Bradyrhizobium* spp. It also
390 suggests that *Bradyrhizobium* spp, *Bradyrhizobium elkanii* and *Bradyrhizobium*
391 *japonicum* required for effective nodulation and cultivation of soybean in Africa are
392 endemic in the eastern (Mitunguu) and western (Bungoma) Kenya.
393
394 **Conclusion.**
395 This study has revealed considerable genetic diversity among *Bradyrhizobium* nodulating
396 seven promiscuous soybean varieties grown at two contrasting sites. Our results show
397 that the tested promiscuous soybean varieties in the two sites are nodulated by a
398 population of *Bradyrhizobium* strains which are genetically diverse and are closely
399 related to *B. japonicum, B. elkanii, Bradyrhizobium* spp and *B. yuanmingense.* The results

also show that to realize the full potential of promiscuous soybeans in enhancing soil fertility status of soils in the two sites requires the application of P. However, these results need to be confirmed by analysis of a larger sample of strains from more sites in order to fully assess the biodiversity in inherent in Kenyan soils and to select more competitive and efficient adapted strain(s) at each site for potential use as inoculants in order to optimize biological nitrogen fixation and thus increase soybean yields at low cost.

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598

599

600 **Table 1. Top soil (0-15cm depth) chemical characteristics of the experimental sites**

601

602

Site	pH (water)	Soil Organic C (g kg $^{-1}$)	Total soil N (g kg $^{-1}$)	Extractable P (mg kg $^{-1}$)	Exchangeable Ca (cmol $_{(+)}$ kg $^{-1}$)	Exchangeable Mg (cmol $_{(+)}$ kg $^{-1}$)	Exchangeable K (cmol $_{(+)}$ kg^{-1})
Bungoma	6.1	1.01	0.07	2.39	6.2	1.8	0.2
Mitunguu	6.9	2.46	0.25	9.25	17.2	4.6	1.0

603

604 **Table 2. Mean of number of nodules and fresh nodule weight of plants in 0.5 m row length of seven**
605 **soybean varieties grown in Bungoma site of Western Kenya in 2006.**
606

607

Varieties	Control	P	P+Lime	Control	P	P+Lime
	Number of nodules on plants in a 0.5 m row length			**Nodule fresh weight (g)**		
Nyala	3 (2)a	7 (7)a	5 (5)a	0.10 (0.05)a	0.09 (0.09)a	0.58 (0.58)a
SB 15	13 (9)b	344 (7)a	50 (23)b	1.15 (0.77)b	15.80 (0.35)a	4.24 (1.72)b
SB 17	41 (11)a	12 (5)a	39 (17)a	2.37 (0.39)a	1.62 (0.48)a	4.05 (1.96)a
SB 19	18 (10)a	36 (18)a	77 (9)a	1.90 (1.09)b	2.64 (1.50)b	6.87 (0.62)a
SB 20	80 (41)a	123 (23)a	99 (23)a	3.23 (1.59)a	5.83 (1.06)a	6.36 (1.97)a
SB 4	34 (15)ab	8 (4)b	84 (21)a	3.32 (1.09)	0.96 (0.58)	5.82 (1.68)
SB 8	46 (7)a	103 (38)a	80 (7)a	5.93 (2.06)b	10.72 (1.20)a	12.47 (2.10)a
SB 9	53 (11)b	88 (29)b	150 (25)a	4.79 (0.73)b	9.03 (2.64)a	12.33 (0.49)a

608 Values indicate the means (SE). Means followed by the same letter in a row are not significantly different from each other at $P<0.05$
609 according to Duncan's Multiple Range Test.
610

611
612 **Table 3. Mean of number of nodules and fresh nodule weight of plants in 0.5 m row length of seven soybean varieties grown in**
613 **Mitunguu site of Eastern Kenya in 2006.**
614

	Control	**P**	**P+Lime**	**Control**	**P**	**P+Lime**
Varieties	**Number of nodules on plants in a 0.5 m row length**			**Nodule fresh wt (g)**		
Nyala	167 (26)ab	96 (36)b	241 (11)a	11.95(2.3)a	14.02(2.6)a	17.27(0.77)a
SB 15	177 (18)b	278 (22)a	247 (53)ab	12.74(1.30)b	20.03(1.58)a	17.82(3.78)ab
SB 17	170 (19)a	171 (9)a	30 (7)b	13.43(1.69)a	13.23(0.69)a	2.31(0.53)b
SB 19	136 (29)b	274 (53)a	276 (9)a	9.32(1.97)b	18.71(3.63)a	16.89(2.56)a
SB 20	162 (12)c	336 (36)a	249 (47)b	12.95(0.97)c	26.43(2.83)a	19.59(3.66)b
SB 4	205 (29)b	338 (62)a	261 (17)ab	17.14(2.44)b	28.23(5.17)a	21.82(1.43)b
SB 8	168 (13)a	228 (54)a	211 (40)a	9.17(0.69)a	12.49(2.98)a	11.56(2.20)a
SB 9	162 (17)b	338 (22)a	324 (11)a	8.03(1.24)b	24.12(1.59)a	23.09(0.80)a

615 Values indicate the means (SE). Means followed by the same letter in a row are not significantly different from each other at $P<0.05$ according to Duncan's
616 Multiple Range Test.

617 618 **Table 4. Distribution of *Bradyrhizobium* strains among different IGS groups, treatments and sites.**

Sites	Bungoma				Mitunguu					
Treatments	Control	P	P+Lime	Sub-total	Control	P	P+Lime	Sub-total	Total	% of total
IGS group										
I	18	17	16	51	28	17	31	76	127	43.9
II	6	8	4	18	1	2	3	6	24	8.3.
III	8	12	9	29	10	24	8	42	71	24.6
IV	6	6	6	18	3	1	0	4	22	7.6
V	0	1	2	3	0	0	0	0	3	1.0
VI	6	4	8	18	2	0	0	2	20	6.9
VII	0	0	1	1	0	0	0	0	1	0.3
VIII	1	2	0	3	0	0	0	0	3	1.0
IX	0	0	0	0	0	1	0	1	1	0.3
X	0	1	0	1	0	0	0	0	1	0.3
XI	1	0	0	1	0	0	1	1	2	0.7
XII	1	0	0	1	0	0	0	0	1	0.3
XIII	0	0	0	0	2	1	0	3	3	1.0
XIV	0	0	1	1	0	0	0	0	1	0.3
XV	2	0	1	3	1	2	0	3	6	2.1
XVI	0	0	0	0	0	1	0	1	1	0.3
XVII	0	0	0	0	1	0	0	1	1	0.3
XVIII	0	0	0	0	0	1	0	1	1	0.3
Total	**49**	**51**	**48**	**148**	**48**	**50**	**43**	**141**	**289**	

619 Values indicate the number of strains in each IGS groups for each treatment, n= 289. Treatments: Treatments were control (none), + P (40 kg P/ha), + lime
620 (1t/ha), + N (90 kg N /ha, split applied + lime + P). No nodules formed in the + N treatment and is not reported in this table.
621
622

623 **Table 5. Genetic relatedness of Genbank to indigenous strains isolated from promiscuous soybean varieties grown at two sites**
624 **under phosphorus and lime using partial 16S rRNA gene sequences analysis.**
625

Isolate ID.	Genbank Acc. No.	Site*	Variety	Treatment	Sequence Length (bp)	Species affiliation	IGS group	% Similarity
TSBF-531	EU625518	1	SB 9	P+Lime	924	*Bradyrhizobium* spp	I	92
TSBF-523	EU625519	1	SB 9	P	898	*Bradyrhizobium* spp	I	91
TSBF-345	EU625520	1	SB 15	P	850	*B. japonicum isolate TSBF-607*	I	88
TSBF-441	EU625521	1	SB 20	P+Lime	592	*B. yuanmingense isolate TSBF-627*	I	85
TSBF-534	EU625522	1	SB 9	P+Lime	917	*Bradyrhizobium* spp	I	91
TSBF-331	EU625523	2	SB 15	Control	1024	*Bradyrhizobium* spp	I	91
TSBF-336	EU625524	1	SB 15	Control	837	*B. japonicum strain TSBF734*	I	88
TSBF-341	EU625525	2	SB 15	P	867	*Bradyrhizobium.* spp SjCL5 (MS 867)	I	88
TSBF 333	EU625526	1	SB 15	Control	996	*Bradyrhizobium* spp *PAC 41*	I	91
TSBF-131	EU625527	2	SB 20	P	725	*B. japonicum isolate 734*	I	88
TSBF 216	EU625538	2	SB 8	Control	942	*B. japonicum isolate JZ 1*	I	86
TSBF-381	EU625528	1	SB 17	P+Lime	676	*Bradyrhizobium* spp *MAF 210190*	II	86
TSBF 161	EU625539	2	SB 4	P	183	*B. elkanii isolate TSBF 717*	II	69
TSBF-402	EU625529	1	SB 19	P	963	*B. elkanii strain USDA61*	III	89
TSBF-344	EU625530	1	SB 15	P	680	*B. elkanii*	III	84
TSBF-444	EU625531	2	SB 20	P+Lime	1125	*B. elkanii*	III	93
TSBF-404	EU625532	1	SB 19	P	295	*B. elkanii SEMIA 6425*	III	80
TSBF-442	EU625533	2	SB 20	P+Lime	870	*B. elkanii*	III	88
TSBF-101A	EU625534	2	SB 19	P	289	*Bradyrhizobium* spp. *MAF 210190*	III	78
TSBF-260A	EU625535	2	SB 9	P	1044	*B. elkanii isolate TSBF 694*	III	93
TSBF-336A	EU625536	1	SB 15	Control	902	*B. elkanii isolate TSBF 694*	III	91
TSBF-488	EU625540	1	SB 8	Control	1083	*B. elkanii strain USDA 61*	III	91
TSBF-101	EU625541	2	SB 19	P	863	*B. japonicum isolate TSBF 734*	IV	87
TSBF-504	EU625537	1	SB 8	P+Lime	996	*Bradyrhizobium* spp	V	90
TSBF-60	EU625542	2	SB 15	P+Lime	809	*B. yuanmingense isolate TSBF 627*	VI	88
TSBF 438	EU625543	1	SB 20	P	1373	*Bradyrhizobium* spp *KO3G*	VIII	99
TSBF-137	EU625544	2	SB 20	P	284	*B. elkanii isolate TSBF 717*	IX	73
TSBF-530	EU625545	1	SB 9	P	355	*B. elkanii isolate TSBF-734*	X	77

TSBF 102	EU625546	2	SB 19	P	898	*B. japonicum isolate TSBF 734*	XIII	92

626 *1= Mitunguu, 2 = Bungoma

627

628 **Figure 1. IGS groups obtained from *Msp* I restricted products of indigenous *Bradyrhizobia* isolated from promiscuous soybean**
629 **varieties grown in Bungoma and Mitunguu sites in Kenya**
630
631
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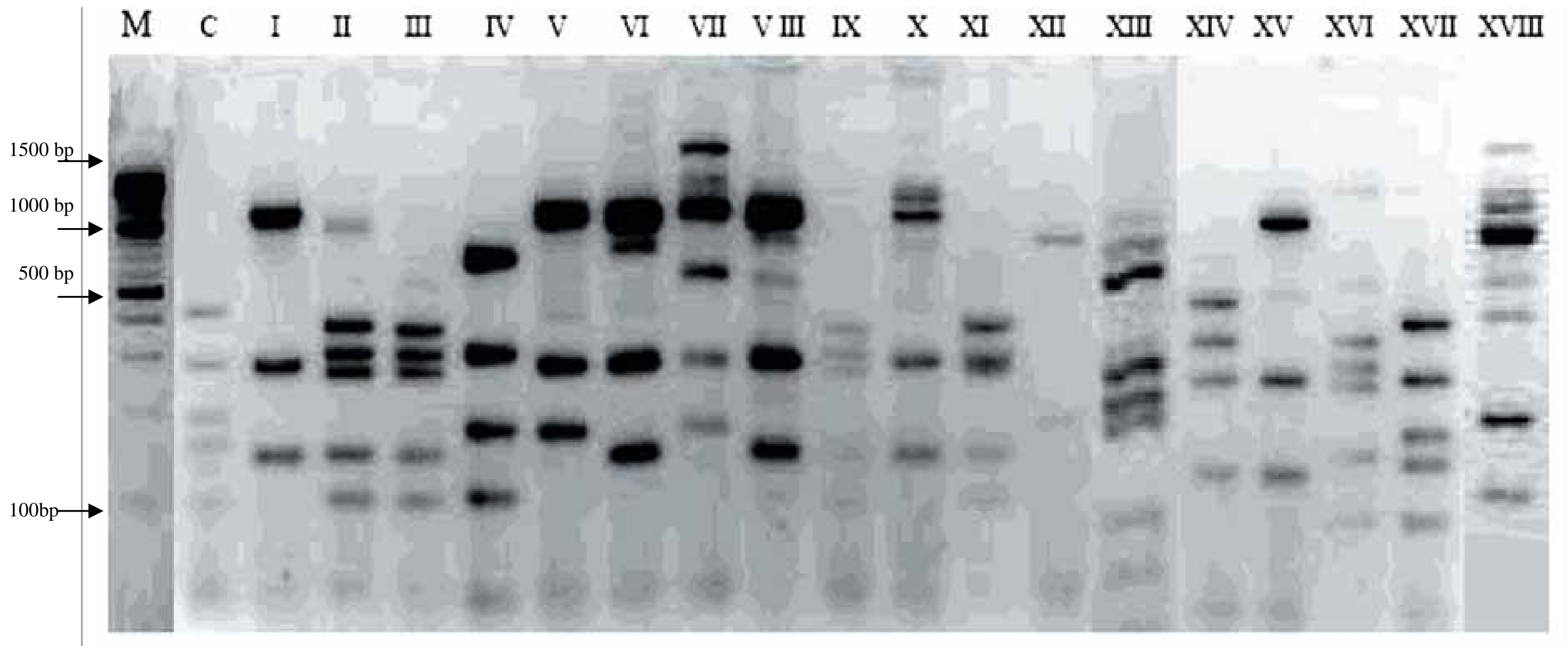
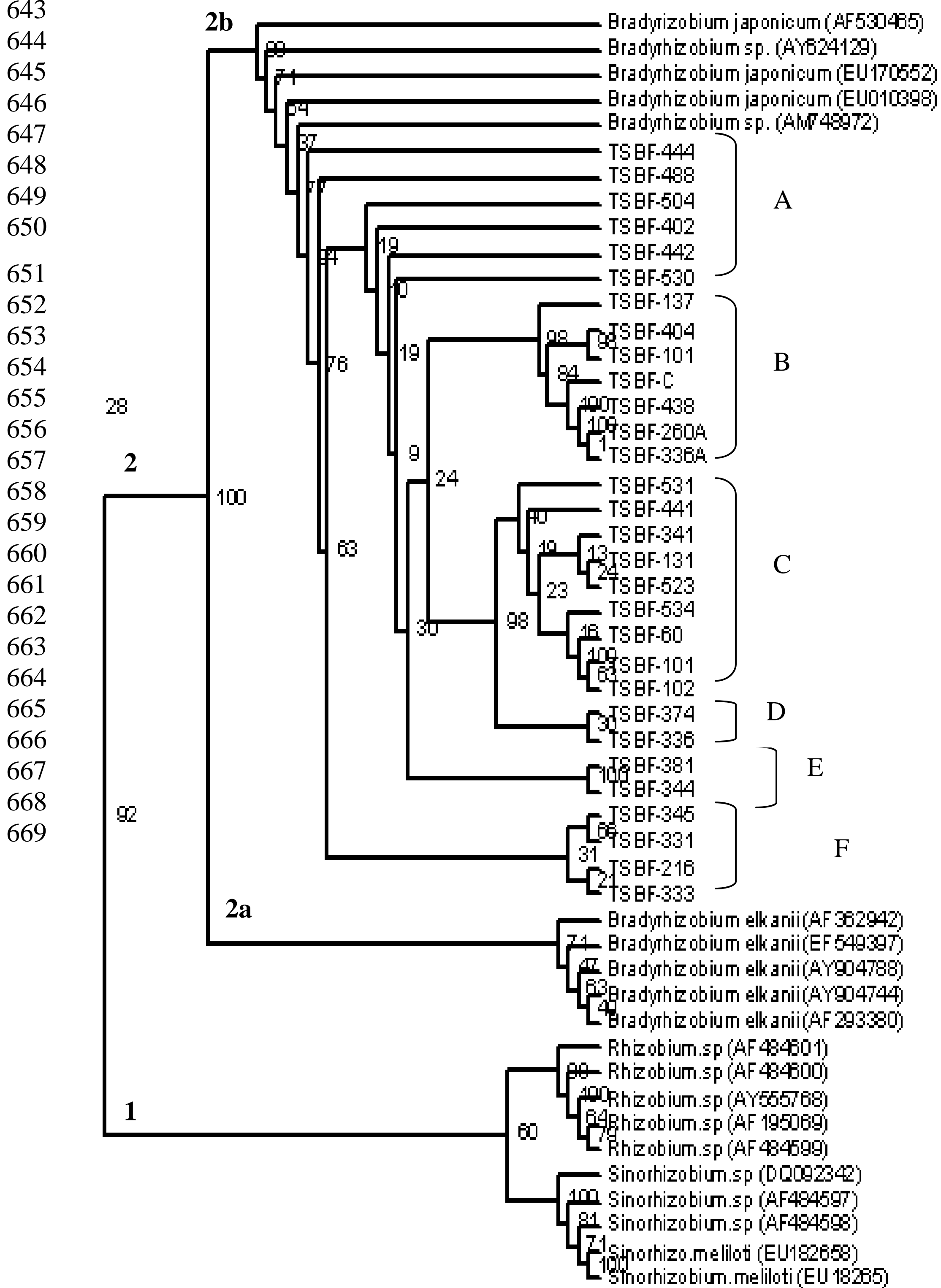
633



634
635
636 **Key: M- 100bp marker; C – Control strain (USDA 110)**
637
638

Figure 2. Phylogenetic relationship between experimental and GenBank reference strains based on aligned sequences of 16S rRNA gene, constructed as unrooted tree using the nearest neighbor-joining method. Bootstrap confidence levels at 100 percent are indicated at the nodes.



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