

1 **Stem nodulation: diversity and occurrence in *Aeschynomene***
2 **and *Sesbania* legumes from wetlands of Madagascar**

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34 Abstract

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36 As an adaptation to flooding, few legume species have the original ability to develop nitrogen-fixing
37 nodules on the stem. By surveying wetlands of Madagascar, we found a large occurrence and
38 diversity of stem nodulation in *Aeschynomene* and *Sesbania* legumes. They represent opportunities
39 to investigate different modalities of the nitrogen-fixing symbiosis in legumes.

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42 Introduction

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44 The symbiosis between legume plants and soil rhizobia results in the formation of nitrogen-fixing
45 nodules, generally exclusively appearing on the roots. However, in a handful of tropical legumes
46 growing in wetlands, nodulation with rhizobia can also occur at stem-located dormant root
47 primordia, a process that is referred as stem nodulation. Seen the semi-aquatic lifestyle of these
48 legumes, it has been hypothesized that the stem nodulation trait is an evolutionary adaptation to
49 flooding (Ladha *et al.*, 1992). Stem nodulation was first described for the African *Aeschynomene*
50 *afraspera* (not to be confounded with *Aeschynomene aspera* found in Asia) (Hagerup, 1928). Stem
51 nodulation gained agricultural interest after the discovery that the profuse stem nodulation as found
52 in *A. afraspera* and *Sesbania rostrata* results in a high nitrogen fixation activity (Dreyfus and
53 Dommergues, 1981; Alazard and Becker, 1987). To date, stem nodulation has been reported for
54 species belonging to four legume genera: *Aeschynomene*, *Discolobium*, *Neptunia* and *Sesbania*
55 (Boivin *et al.*, 1997). While, in the three latter genera, stem-nodulation has been described for one or
56 very few species, more than 20 *Aeschynomene* species have been shown to form stem nodules
57 (Chaintreuil *et al.*, 2013; 2016). These legume species can differ in their stem nodulation ability and
58 intensity, and actually *S. rostrata* and *A. afraspera* are the ones for which stem-nodulation is the
59 most profuse (Boivin *et al.* 1997a).

60 Strikingly, *S. rostrata* is stem-nodulated only by *Azorhizobium caulinodans* while other
61 *Sesbania* species root-nodulate with rhizobia of the *Ensifer* genus (Boivin *et al.*, 1997a,b). Similarly,
62 different types of *Bradyrhizobium* strains have been identified as nodulating *Aeschynomene* species,
63 (Boivin *et al.*, 1997a; Alazard, 1985). The most important difference between the strains were the
64 presence or absence of photosynthetic activity and *nod* genes to produce Nod factors (Molouba *et*
65 *al.*, 1999; Giraud *et al.*, 2007; Miché *et al.*, 2011). So far, photosynthetic *Bradyrhizobium* strains have
66 been exclusively found in nodules of stem-nodulating *Aeschynomene* species (Boivin *et al.*, 1997a)
67 and strains lacking *nod* genes have been isolated from nodules of *Aeschynomene* species that cluster

68 in a single clade (Chaintreuil *et al.*, 2013, 2016). The *Bradyrhizobium* ORS278-*A. evenia* interaction
69 serves as model for the deciphering of the latter very specific interaction called Nod-independent
70 symbiosis (Quilbé *et al.*, 2021). In contrast, strains having *nod* genes, such as *Bradyrhizobium*
71 ORS285, have been isolated from nodules of *A. afraspera* that is one of the *Aeschynomene* species
72 using a Nod-dependent interaction (Arrighi *et al.*, 2014; Brottier *et al.*, 2018). *S. rostrata* stem-
73 nodulating *A. caulinodans* is not photosynthetic and has *nod* genes, but in both cases, the symbiotic
74 interaction is very specific.

75 Although research has shed some light on the genetics of both partners in the
76 *Bradyrhizobium*-*Aeschynomene* and *A. caulinodans*-*S. rostrata* symbiotic systems, our understanding
77 of the molecular mechanism that causes stem nodulation is still in its infancy. Furthermore, our
78 knowledge of the diversity and occurrence of stem nodulation *in natura* is relatively limited as it has
79 been investigated in only a few geographical regions (e.g., James *et al.*, 2001; Molouba *et al.*, 1999;
80 Miché *et al.*, 2011). To fill this gap, we conducted a field study in Madagascar that contains a variety
81 of wetlands with an important plant biodiversity. A series of expeditions were organized to explore
82 wetland-rich regions in the Central (RN1-Itasy Lake), Northern (Nosy Be), Western (RN4-Majunga)
83 and Eastern (RN2-Alaotra Lake) parts of Madagascar (Fig. 1a). In these regions, we found stem-
84 nodulated *Aeschynomene*, *Sesbania* and *Neptunia* spp. Omitting these latter from this analysis, as
85 nodules were only found on floating stems forming adventitious roots, a total of 69 *Aeschynomene*
86 and *Sesbania* samples was collected (Table S1). Field observations were completed with molecular
87 and flow cytometry analyses for accurate specimen identification (Tables S2 and S3). Here, we report
88 on the stem-nodulated *Aeschynomene* and *Sesbania* species from Malagasy wetlands and discuss the
89 opportunity of these resources to fuel research on the nitrogen-fixing symbiosis in legumes.

90

91 **Stem nodulation in Nod-independent *Aeschynomene* species**

92 Three stem-nodulated *Aeschynomene* species belonging to the Nod-independent clade were found in
93 Malagasy wetlands (Fig. 1b). *A. evenia* was by far the most widespread one, being seen in all parts of
94 Madagascar and in all wetland types: river banks, marshes and ricefields. *A. evenia* has a transatlantic
95 distribution and a well-defined geographically-structured genetic diversity (Chaintreuil *et al.*, 2018).
96 The form present in Madagascar was previously classified as the Eastern African genotype. As a
97 result, it is closely related to the reference line from Malawi that was selected for the genetic
98 dissection of the Nod-independent symbiosis (Quilbé *et al.*, 2021). Stem-nodulated *A. evenia* plants
99 were observed in 24 out of the 25 sampling sites. Nodules were green, indicative of the presence of
100 chloroplasts, and often located in the lower part of the stem, but their distribution could extend to

101 the upper branch-containing part and they were usually present in a profuse fashion. These stem
102 nodules were hemispherical and with a broad attachment to the stem (Fig. 2a).

103 The second typical species found in different wetlands is *A. sensitiva*. Similarly to *A. evenia*,
104 this species has also a transatlantic distribution and the genotype occurring in Madagascar is
105 concomitantly present in Africa and Eastern Brazil (Chaintreuil *et al.*, 2018). *A. sensitiva* is well-
106 known because the model strain ORS278, a photosynthetic *nod* gene-lacking *Bradyrhizobium*, was
107 isolated from stem nodules in Senegal (Giraud *et al.*, 2000, 2007). The photosynthetic activity of this
108 strain was demonstrated to be important for the efficiency of stem nodulation. In addition, *A.*
109 *sensitiva* has the particularity to develop unique ‘collar’ nodules on the stem (Fig. 2b). These were
110 green and readily observed in all 14 sampling sites. Although *A. sensitiva* was globally less frequently
111 found as compared to *A. evenia*, at the collection sites the two species were frequently growing
112 adjacent to each other (Fig. 2c). The two species are known to have overlapping distributions in
113 Madagascar but *A. sensitiva* is absent in the drier regions whereas *A. evenia* does (Dupuy *et al.*,
114 2002).

115 Unexpected was the discovery of *A. indica* in marshes from Majunga and in ricefields in Nosy
116 Be because this species is not native of Madagascar. *A. evenia* and *A. indica* are morphologically
117 similar but they can be distinguished from each other via the flowers that on *A. indica* plants are in
118 general larger than those on *A. evenia* (Fig. 2d). This high resemblance is due to their belonging to a
119 same polyploid species complex where *A. evenia* 2x, *A. indica* 4x and *A. indica* 6x forms are present
120 (Arrighi *et al.*, 2014; Chaintreuil *et al.*, 2018). To confirm the visual species identification, we
121 sequenced the nuclear *ITS* and chloroplastic *matK* gene and determined the genome size of the
122 specimens JFA29 and JFA109. The obtained data were typical for *A. indica* 4x plants, confirming the
123 visual identification (Fig. S1, Table S2) (Arrighi *et al.*, 2014). In the four collection sites, *A. indica*
124 plants were stem-nodulated, the nodules varying in shape and size but always with an enlarged base
125 and with a green color (Fig. 2d). Similar to other *Aeschynomene* species, pink root nodules could be
126 observed in unflooded conditions, (Fig. 2d).

127

128 **Stem nodulation in Nod-dependent *Aeschynomene* species**

129 Among *Aeschynomene* species falling outside of the Nod-independent clade, the pantropical *A.*
130 *americana* and the endemic *A. patula* were frequently present in explored sites. Both species are of
131 interest because they have been proposed as model plants complementary to *A. evenia* to develop a
132 comparative genetic system to study the Nod-independent and Nod-dependent symbioses in
133 *Aeschynomene* (Brottier *et al.*, 2018). However, for both species no stem-nodulated plants were
134 found at the collection sites (Fig. 1b).

135 Two *Aeschynomene* species, *A. elaphroxylon* and *A. schimperi*, which are believed to be
136 introduced in Madagascar, were found in the Central Plateaux (Fig. 1b) (Du Puy *et al.*, 2002). *A.*
137 *elaphroxylon* was specially present around the Aloatra lake. It is a distinctive *Aeschynomene* species
138 as it can form large shrubs to small trees with very showy yellow flowers and spiny stems (Fig. 3a). In
139 the two sampled sites, plants were scarcely nodulated and, if so, only on the submerged parts of the
140 stems. In that case, nodules were green and had a flattened hemispherical shape with a broad
141 attachment to the stem (Fig. 3a). In contrast, *A. schimperi* was frequently found in ricefields. For this
142 species, previous genetic analysis uncovered the presence of 4x and 8x cytotypes, and Malagasy
143 specimens correspond to the 8x cytotype (Chaintreuil *et al.*, 2016). In non-waterlogged conditions,
144 numerous pink nodules could be observed on the main root, while under flooded conditions (9 of the
145 12 sampled sites) green nodules were present in the lower part of the stem (Fig. 3b). These nodules
146 were spherical with a narrow attachment to the stem.

147 In regions at lower altitude, in the North and West of Madagascar, two other Nod-dependent
148 *Aeschynomene* species were found. *A. uniflora* was present in the region of Majunga and in Nosy Be.
149 Here it grew either at the edge of marshes or in ricefields where often also *A. evenia* or *A. sensitiva*
150 species were present. Strikingly, two plant morphotypes were observed: one corresponding to
151 erected plants with well-visible flowers (3 sampled sites), and a second represented by shrubby
152 plants bearing small flowers (two sampled sites) (Fig. 3c). It is likely that the two morphotypes
153 correspond to the botanical varieties *A. uniflora* var. *grandiflora* and var. *uniflora* (Gillet *et al.*, 1971).
154 *ITS* and *matK* sequencing for the samples JFA_51 and JFA_71 (examples of plants with well-visible
155 and small flowers, respectively) revealed consistent differences while flow cytometry measurements
156 were relatively homogenous (Fig. 1b, Fig. S2, Table S2) (Chaintreuil *et al.*, 2016). In both
157 morphotypes, numerous green nodules with a spherical shape and a narrow neck, running on the
158 stem were visible (Fig. 3c).

159 *A. cristata* was found in pristine marshes and slow-flowing streams between Majunga and
160 Mitsinjo where they often formed important stands of flooded plants. At sites where *A. cristata*
161 plants were found *A. evenia* plants could be also present (Fig. 3d). *A. cristata* has retained little
162 attention until it was shown to be one of the genome donors of *A. afraspera* and being a sister
163 species of the Asian *A. aspera*, both species being profusely stem nodulated (Chaintreuil *et al.*, 2013,
164 2016; Devi., 2013a,b). Whereas the *A. cristata* specimen characterized by Chaintreuil *et al.* (2016)
165 had densely-hairy stems, those found and collected in Madagascar were glabrous. Based on this
166 characteristic, they may be tentatively associated to *A. cristata* var. *cristata* (hairy stems) and var.
167 *pubescens* (glabrous stems) as described by Gillet *et al.* (1971). However, sequence and flow
168 cytometry data additional to those obtained for sample JFA_34 are required to assess any genetic
169 differentiation (Fig. 1b, Fig. S3, Table S2). In all sampling sites, *A. cristata* specimens caught attention

170 very quickly due to the profuse nodulation all over the stem (Fig. 3d). These stem nodules were
171 green and hemispherical with a broad base as described for the related *A. afraspera* species (Alazard
172 and Duhoux, 1987).

173

174 **Stem nodulation in *Sesbania rostrata***

175 *S. rostrata* was observed in only two sampling sites in the region of Majunga. *S. rostrata* has been
176 used a research model for nodulation due to its profuse stem nodulation and its ability to switch
177 from classical nodulation to Lateral Root Base nodulation in flooded conditions (Capoen *et al.*, 2009;
178 Dreyfus and Dommergues, 1981). *S. rostrata* is the single species of its lineage in the *Sesbania*
179 phylogeny (Furruggia *et al.*, 2020). However, *S. rostrata* specimens of Senegal and Madagascar were
180 shown to be morphologically different and both hybridization and grafting experiments were less
181 successful when interspecific (Ndiaye, 2005). For this reason, it has been proposed they could
182 represent different subspecies of *S. rostrata*. *ITS* and *matK* sequencing of *S. rostrata* samples JFA_21
183 and JFA_45 also point to genetic differentiation when compared to an accession from Senegal, while
184 flow cytometry analysis revealed similar genome sizes for the same accessions (Fig. 1c, Fig. S4, Table
185 S2). In plants of both sampling sites, nodulation all along the stem was remarkable for its profusion
186 and nodulation sites were typically distributed in vertical rows (Fig. 3e). These stem nodules were
187 green, prominent, and had a constricted base.

188

189 **Conclusions and perspectives**

190 In 2001, James *et al.* identified several stem-nodulated legumes in the Brazilian Pantanal wetlands.
191 To broaden our knowledge on stem nodulating legumes, this research inspired us to explore
192 wetlands in a geographically distinct tropical region, Madagascar. In Malagasy wetlands, a large
193 occurrence and diversity of stem nodulation in *Aeschynomene* and *Sesbania* legumes was detected.
194 While some of the species found are already well-known to form stem nodules (*A. elaphroxylon*, *A.*
195 *evenia*, *A. indica* 4x, *A. schimperi*, *A. sensitiva* and *S. rostrata*), for two other identified species, *A.*
196 *cristata* and *A. uniflora*, this has been once furtively mentioned in a review article and subject to very
197 limited experimentations (Ladha *et al.*, 1992; Chaintreuil *et al.*, 2016). Here, we report on their stem
198 nodulation in the field. Following the traditional classification of stem-nodulating legumes, *A. cristata*
199 was convincingly a *bona fide* profusely stem-nodulated species, equaling the stem nodulation level
200 found for *A. afraspera* and *S. rostrata* (Boivin *et al.*, 1997a; Ladha *et al.*, 1992). *A. uniflora* stem
201 nodulation did not reach such level in the field. However, in greenhouse conditions *A. uniflora* stem
202 nodulation has been shown to be exceptionally dense and to occur all along the stem (Chaintreuil *et*

203 *al.*, 2016), indicating a profuse stem-nodulation capacity. The discovery of all these species and the
204 demonstration of genetic diversity in several of them (*A. cristata*, *A. uniflora*, and *S. rostrata*) point
205 out that more systematic studies including the collection of plants to evaluate species and ecotypes
206 endowed with stem-nodulation are required.

207 What lessons could we learn from these studies ? On the plant side, profuse stem nodulation
208 may be indicative of high nitrogen fixation activity, the presence of two stem-nodule morphologies
209 (either hemispherical with a broad attachment to the stem or spherical with a narrow base) supports
210 the existence of two developmental programs, and in both cases the photosynthetic activity in stem
211 nodules (inferred from their green color) points to a physiology that likely differs from those of root
212 nodules (discussed in Ladha *et al.*, 1992; Legocki and Szalay, 1984). On the bacterial side, while *A.*
213 *caulinodans* has been isolated from *S. rostrata* stem nodules in both Senegal and Madagascar (Boivin
214 *et al.*, 1997b), a great variety of *Aeschynomene*-nodulating *Bradyrhizobium* strains do exist but their
215 genetics are insufficiently understood (Molouba *et al.*, 1999; Miché *et al.*, 2011; Okazaki *et al.*, 2016).
216 New photosynthetic strains having or lacking *nod* genes are expected to be present in stem nodules
217 of *A. cristata* (because it is a parent species of *A. afraspera*) and of the Nod-independent
218 *Aeschynomene* species (e.g., *A. evenia*, *A. indica* 4x, and *A. sensitiva*) respectively. Intriguingly,
219 previously only non-photosynthetic strains were isolated from *A. uniflora* root nodules (Molouba *et*
220 *al.*, 1999). It would thus be very interesting to investigate the nature of those present in its stem
221 nodules. Given all the valuable information that can be gained from stem-nodulating legumes, we
222 advocate reviving their study as this would significantly increase our understanding on the diversity
223 of mechanisms underlying the nitrogen-fixing symbiosis in legumes.

224

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233

234 **Competing interests**

235 None declared.

236

237 **Author contributions**

238 JFA and NN wrote the manuscript. FFM, MFR, CC, ATER, MDA, H Randriambanona, H
239 Ramanankierana and JFA carried out specimen collection, photography, identification and herbarium
240 conservation. FF, MP and MB performed sequence and flow cytometry analyses.

241

242 **Data availability**

243 Data obtained in this study are listed in Tables S1 to S3, and the methods are given in Methods S1.
244 The DNA sequences generated in this study were deposited in GenBank under accession numbers
245 OR448903-OR448909 (nuclear *ITS*) and OR463925-OR463932 (chloroplastic *matK*).

246

247 **References**

248 **Alazard D. 1985.** Stem and root nodulation in *Aeschynomene* spp. *Appl. And Environ. Microbiol.*
249 **50:**732-734.

250 **Alazard, D., and M. Becker. 1987.** *Aeschynomene* as green manure for rice. *Plant Soil* **101:**141-143.

251 **Alazard, D., Duhoux, E. 1987.** Nitrogen-fixing stem nodules on *Aeschynomene afraspera*. *Biological*
252 *fertility Soils* **4:** 61-66.

253 **Arrighi JF, Cartieaux F, Brown SC, Rodier-Goud M, Boursot M, Fardoux J, Patrel D, Gully D, Fabre S,**
254 **Chaintreuil C, Giraud E. 2012.** *Aeschynomene evenia*, a model plant for studying the molecular
255 genetics of the Nod-independent rhizobium-legume symbiosis. *Molecular Plant-Microbe*
256 *Interactions* **25(7):** 851-861.

257 **Arrighi JF, Chaintreuil C, Cartieaux F, Cardi C, Rodier-Goud M, Brown SC, Boursot M, d'Hont A,**
258 **Dreyfus B, Giraud E. 2014.** Radiation of the Nod-independent *Aeschynomene* relies on multiple
259 allopolyploid speciation events. *New Phytologist* **201:** 1457-68.

260 **Becker M, JK Ladha, Je Ottow. 1988.** Stem-nodulating legumes as green manure for lowland rice.
261 *Phil. J. Crop Sci.* **13(3):**121-127.

262 **Boivin C, Ndoye I, Molouba F, De Lajudie P, Dupuy N, Dreyfus B. 1997a.** Stem nodulation in
263 legumes: diversity, mechanisms, and unusual characteristics. *Critical Reviews in Plant Sciences*
264 **16(1):** 1-30.

265 **Boivin C, Ndoye I, Lortet G, Ndiaye A, De Lajudie P, Dreyfus B. 1997b.** The *Sesbania* root symbionts
266 *Sinorhizobium saheli* and *S. teranga* bv. *sesbaniae* can form stem nodules on *Sesbania rostrata*,
267 although they are less adapted to stem nodulation than *Azorhizobium caulinodans*. *Appl Environ*
268 *Microbiol.* **63(3):**1040-7.

- 269 **Brottier L, Chaintreuil C, Simion P, Scornavacca C, Rivallan R, Mournet P, Moulin L, Lewis GP,**
270 **Fardoux J, Brown SC, et al. 2018.** A phylogenetic framework of the legume genus *Aeschynomene*
271 for comparative genetic analysis of the Nod-dependent and Nod-independent symbioses. *BMC*
272 *Plant Biol.* **18(1):**333.
- 273 **Capoen, W., Oldroyd, G., Goormachtig, S., and Holsters, M. (2010).** *Sesbania rostrata*: a case study
274 of natural variation in legume nodulation. *New Phytol* **186:**340-345.
- 275 **Chaintreuil C, Arrighi JF, Giraud E, Miche L, Moulin L, Dreyfus B, Munive-Hernandez J, Villegas-**
276 **Hernandez M, Béna G. 2013.** Evolution of symbiosis in the legume genus *Aeschynomene*. *New*
277 *Phytologist* **200:**1247-59.
- 278 **Chaintreuil C, Perrier X, Martin G, Fardoux J, Lewis GP, Brottier L, Rivallan R, Gomez-Pacheco M,**
279 **Bourges M, Lamy L, et al. 2018.** Naturally occurring variations in the nod-independent model
280 legume *Aeschynomene evenia* and relatives: a resource for nodulation genetics. *BMC Plant Biol.*
281 **18(1):**54.
- 282 **Chaintreuil C, Gully D, Hervouet C, Tittabutr P, Randriambanona H, Brown SC, Lewis GP, Bourge M,**
283 **Cartieaux F, Boursot, et al. 2018.** The evolutionary dynamics of ancient and recent polyploidy in
284 the African semiaquatic species of the legume genus *Aeschynomene*. *New Phytologist.*
285 **211(3):**1077-91.
- 286 **Devi WR. 2013a.** *Aeschynomene aspera* L., a nitrogen fixing stem nodulation plant from Manipur.
287 *International Journal of Scientific & technology Research* **2(6):** 232-235.
- 288 **Devi WR. 2013b.** *Aeschynomene aspera* L., a potential stem nodulated plant as green manure for rice
289 cultivation in Manipur. *Journal of Medicinal Plants* **1(4):**28-31.
- 290 **Dreyfus BL and YR Dommergues. 1981.** Nitrogen-fixing nodules induced by *Rhizobium* on the stem of
291 the tropical legume *Sesbania rostrata*. *FEMS Microbiol. Letter.* **10:**313-317.
- 292 **Du Puy DJ, Labat J-N, Rabevohitra R, Villiers J-F, Bosser J, Moat J. 2002.** The Leguminosae of
293 Madagascar. Royal Botanic Gardens, Kew.
- 294 **Farruggia FT, Lavin M, Wojciechowski MF. 2018.** Phylogenetic Systematics and Biogeography of the
295 Pantropical Genus *Sesbania* (Leguminosae). *Systematic Botany* **43(2):**414-429.
- 296 **Gillett JB, Polhill RM, Verdcourt B. 1971.** Leguminosae (part 3): subfamily Papilionoideae (part 1). In
297 Milne-Redhead E & Polhill RM (eds). *Flora of Tropical East Africa. Royal Botanic Gardens, Kew.*
- 298 **Giraud E, Hannibal L, Fardoux J, Vermeglio A, Dreyfus B. 2000.** Effect of *Bradyrhizobium*
299 photosynthesis on stem nodulation of *Aeschynomene sensitiva*. *PNAS* **97(26):** 14795-14800.
- 300 **Giraud E, Moulin L, Vallenet D, Barbe V, Cytryn E, Avarre JC, Jaubert M, Simon D, Cartieaux F, Prin**
301 **Y, et al. 2007.** Legumes symbioses: absence of Nod genes in photosynthetic bradyrhizobia.
302 *Science.* **316(5829):**1307-12.

- 303 **Hagerup O. 1928.** En hygrophil baelgplante (*Aeschynomene aspera* L.) med bakterieknolde paa
304 staenglen. *Dansk Bot. Arkiv* **14**: 1-9.
- 305 **James EK, Loureiro FM, Pott A, Pott VJ, Martins CM, Franco AA, Sprent JI. 2001.** Flooding-tolerant
306 legume symbioses from the Brazilian Pantanal. *New Phytologist* **150**: 723-738.
- 307 **Ladha JK, Pareek RP, Becker M. 1992.** Stem-nodulating Legume-Rhizobium symbiosis and its
308 agronomic use in lowland rice. *Advances in Soil Science*. pp 147–192.
- 309 **Legocki RP and Szalay AA. 1983.** Molecular biology of stem nodulation. In DPS Verma *et al.* (eds).
310 Genes involved in Microbe-Plant Interactions. *Springer Verlag, Berlin, Heidelberg*. pp. 210-219..
- 311 **Loureiro MF, Defaria SM, James EK, Pott A, Franco AA. 1994.** Nitrogen-fixing stem nodules of the
312 legume *Discolobium pulchellum* Benth. *New Phytologist* **128(2)**: 283-295.
- 313 **Loureiro MF, James EK, Sprent JI, Franco AA. 1995.** Stem and root nodules on the tropical wetland
314 legume *Aeschynomene fluminensis*. *New Phytologist* **130**: 531-544.
- 315 **Miché L, Moulin L, Chaintreuil C, Contreras-Jimenez JL, Munive-Hernández JA, Del Carmen Villegas-
316 Hernandez M, Crozier F, Béna G. 2011.** Diversity analyses of *Aeschynomene* symbionts in Tropical
317 Africa and Central America reveal that nod-independent stem nodulation is not restricted to
318 photosynthetic bradyrhizobia. *Environ Microbiol.* **12(8)**:2152-64.
- 319 **Molouba F, Lorquin J, Willems A, Hoste B, Giraud E, Dreyfus B, Gillis M, de Lajudie P, Masson-
320 Boivin C. 1999.** Photosynthetic bradyrhizobia from *Aeschynomene* spp. are specific to stem-
321 nodulated species and form a separate 16S ribosomal DNA restriction fragment length
322 polymorphism group. *Appl Environ Microbiol.* **65(7)**:3084-94.
- 323 **Ndiaye M. 2005.** Caractérisation taxonomique de deux provenances de *Sesbania rostrata* Brem :
324 *Sesbania rostrata* du Sénégal, *Sesbania rostrata* de Madagascar. PhD thesis. *Université Cheikh*
325 *Anta Diop (UCAD)*, Dakar, Senegal.
- 326 **Okazaki S, Tittabur P, Teulet A, Thouin J, Fardoux J, Chaintreuil C, Gully D, Arrighi JF, Furuta N,
327 Miwa H et al. 2016.** Rhizobium-legume symbiosis in the absence of Nod factors: two possible
328 scenarios with or without the T3SS. *ISME J.* **10(1)**: 64-74.
- 329 **Quilbé J, Lamy L, Brottier L, Leleux P, Fardoux J, Rivallan R, Benichou T, Guyonnet R, Becana M,
330 Villar I, et al. 2021.** Genetics of nodulation in *Aeschynomene evenia* uncovers mechanisms of the
331 rhizobium-legume symbiosis. *Nat Commun.* **12(1)**:829.

332
333

334 **Supporting Information**

335 **Fig. S1** Alignments of the nuclear *ITS* and chloroplastic *matK* gene sequences for *A. indica* 4x.

336

337 **Fig. S2** Alignments of the (a) nuclear *ITS* and (b) chloroplastic *matK* gene sequences for *A. uniflora*.

338

339 **Fig. S3** Alignments of the (a) nuclear *ITS* and (b) chloroplastic *matK* gene sequences for *A. cristata*.

340

341 **Fig. S4** Alignments of the (a) nuclear *ITS* and (b) chloroplastic *matK* gene sequences for *S. rostrata*.

342

343 **Methods S1** Material and methods.

344

345 **Table S1** Accessions characterized in this study, origin and nodulation status.

346

347 **Table S2** Genetic and morphological characteristics of species and samples included in this study.

348

349 **Table S3** GenBank numbers for the sequences used in the phylogenetic analyses.

350

351

352 **Key words:** *Aeschynomene*, *Sesbania*, stem nodulation, nitrogen-fixation, symbiosis

353

354

355 **Methods S1** Material and methods.

356

357 **Description of the collecting areas**

358 To select collection sites of *Aeschynomene* and *Sesbania* species in Madagascar, we made use of

359 general information about their distribution as described in the compendium "The Leguminosae of

360 Madagascar" (Dupuy, 2002) and utilized precise location data of previous isolated specimens present

361 in collections through the Global Biodiversity Information Facility (GBIF - <https://www.gbif.org/>) and

362 the Tropicos database (<https://www.tropicos.org/>). This resulted in the definition of four collecting

363 areas: 1) the region of Majunga including the vicinity of the Kinkony lake where many temporary to

364 permanent marshes locally named « matsabory » are present. This area also comprises the plain of

365 Marovoay where numerous ricefields form the main rice granary of Madagascar, 2) the Itasy region

366 in the Center of Madagascar where the Itasy Lake and ricefields are present, 3) the Alaotra region in

367 the East side of Madagascar that corresponds to a large basin containing the Alaotra Lake. The

368 presence of extensive ricefields make this region the second rice granary of Madagascar, and 4) Nosy

369 Be located towards the North of Madagascar where in the whole region ricefields are present.

370 Expeditions to the four regions were made in April and May 2023, at the end of the rainy season that

371 corresponds to the flowering period for most *Aeschynomene* and *Sesbania* species. Plants were
372 collected in the above mentioned areas but also « en route » from ricefields, rivers and marshes
373 present along the RN1, RN2 and RN4 national roads and secondary roads leading to the Itasy lake,
374 the Alaotra lake and the region of Majunga, respectively.

375

376 **Plant and data collection**

377 At each sampling location, the presence or receding of water in the aquatic ecosystem was recorded.
378 The stem nodulation status of individuals for each species present in the population was examined
379 and correlated to their positions relative to the flooding area. Whenever possible, three individuals
380 of each species were chosen at random to determine their root nodulation status. Both stems and
381 roots of these individuals were photographed *in situ*. Plant material was frequently collected for
382 germplasm conservation and production of voucher specimens. The latters were deposited at the
383 Herbarium of the CNARP Institute in Antananarivo (Madagascar).

384

385 **Plant culture**

386 Seeds collected in the field were dried at 34°C for one week and used for plant cultivation when fresh
387 material production was required. Seed scarification and plant growth in the greenhouse were
388 performed as indicated in Arrighi *et al.* (2012).

389

390 **Gene sequencing and sequence analysis**

391 Genomic DNA was isolated from fresh leaves using the CTAB extraction method. The nuclear
392 ribosomal internal transcribed spacer region (ITS: ITS1-5.8S rDNA gene-ITS2) and the chloroplast
393 *matK* gene were amplified and sequenced as published in Chaintreuil *et al.* (2016). Additionnal ITS
394 and *matK* sequences were retrieved from Chaintreuil *et al.* (2016, 2018) and Brottier *et al.* (2018) for
395 *Aeschynomene* species and from Farrugia *et al.* (2018) for *Sesbania* species. To analyse sequence
396 variations, sequences were aligned using Multalin
397 (<http://multalin.toulouse.inra.fr/multalin/multalin.html>). ML phylogenetic tree reconstructions were
398 obtained by aligning nucleotide sequences with the MUSCLE program that is incorporated in the
399 MEGA X (v10.1.8) software. Aligned sequences were further processed in MEGA X using the
400 maximum likelihood approach and the Kimura 2-parameter model with a 1000x bootstrap (BS).

401

402 **Genome size estimation**

403 Flow cytometry measurements were performed using fresh leaf material as described in Arrighi *et*
404 *al.*, 2012. Genome size estimations were based on the measurements of three plants per accession
405 using *Lycopersicum esculentum* (Solanaceae) cv « Roma » ($2c = 1.99$ pg) as the internal standard.

406

407

408 **Figure legends**

409 **Fig. 1** *Aeschynomene* and *Sesbania* species collected in wetlands of Madagascar. (a) Map of
410 Madagascar with a zoom on the four collecting sites, including RN1-Itsay Lake, RN2-Alaotra Lake,
411 RN4-Majunga and Nosy Be. (b) Phylogeny of *Aeschynomene* species. (c) Phylogeny of *Sesbania*
412 species. In (b) and (c), Maximum likelihood phylogenetic reconstructions were obtained using the
413 concatenated ITS + *matK* sequences. Numbers at nodes represent bootstrap values (% of 1000
414 replicates). Dashed boxes delineate clades where stem nodulation has been reported to occur. Taxa
415 collected in Madagascar are in bold and numbers on their right correspond to the occurrence of stem
416 nodulation in the different collection sites. In orange: no stem nodulation observed, in blue: stem
417 nodulation observed in the present study.

418

419 **Fig. 2** Stem nodulation in *Aeschynomene* species of the Nod-independent clade. (a) *A. evenia*. From
420 left to right: 1- plant growing in a ricefield near Camp Bandro (Alaotra lake), 2- axillary axis bearing a
421 yellow flower and a mature pod, 3-4- stem nodules located at the base or middle portion of the
422 stem, 5- morphology of stem nodules. (b) *A. sensitiva*. From left to right: 1- plant growing in a
423 ricefield at Manakambahiny, in the direction of Alaotra lake, 2- axillary axis bearing yellow flowers
424 and developing pods, 3-4- more or less flattened stem nodules, 5- morphology of collar stem
425 nodules. (c) Co-occurrence of *A. evenia* and *A. sensitiva*. From left to right: 1- Stand of a mixed
426 population in a shallow sandy river at Marofotroboka (next to RN4), insets show stem nodules of *A.*
427 *sensitiva* and *A. evenia*, respectively, 2-4- permanent marsh at Belobaka (Majunga) where *A. sensitiva*
428 and *A. evenia* were found to grow side-by-side with entangled roots, both plants displaying stem
429 nodules. Ae: *A. evenia*, As: *A. sensitiva*. (d) *A. indica* 4x. From left to right: 1- plant growing in a
430 ricefield in Nosy Be, 2- axillary axis bearing a yellow flower and developing pods, 3-4- stem nodules
431 located near the base or at the middle portion of the stem, 5- morphology of stem nodules. Note the
432 presence of numerous pink root nodules in 3.

433

434 **Fig. 3** Stem nodulation in Nod-dependent *Aeschynomene* and *Sesbania* species. (a) *A. elaphroxylon*.
435 From left to right: 1- stand of shrubby plants growing at the edge of a marsh near the Alaotra lake, 2-
436 « showy » yellow flower with visible anthers, 3- woody stem with spines, 4- nodules developing at
437 the base of the stem, 5- morphology of stem nodules. (b) *A. schimperi*. From left to right: 1- young
438 plants in a ricefield at Maharefo, South of the Alaotra lake, 2- yellow flower, 3- pink root nodules, 4-
439 stem nodules, 5- morphology of stem nodules. *A. uniflora*. From left to right: 1- plants growing in a

440 ricefield at Bongomena next to the RN4, 2- yellow flowers with the smaller variant form shown in the
441 inset , 3 and 4- stem nodules of the of the normal and morphological variant, respectively, 5-
442 morphology of stem nodules. (d) *A. cristata*. From left to right: 1- Plant growing in a swampy palm
443 grove, 2- showy yellow flower, 3- Stand of *A. cristata* (Ac) in the center of a marsh lined with *A.*
444 *evenia* (Ae), 4- nodules present all along the stem, 5- morphology of stem nodules. (e) *S. rostrata*.
445 From left to right: 1- plant growing on the edge of a marsh at Amboromalandy next to the RN4, 2-
446 Yellow flower, 3 and 4- arrows of nodules running all along on the stem, 5- morphology of stem
447 nodules.

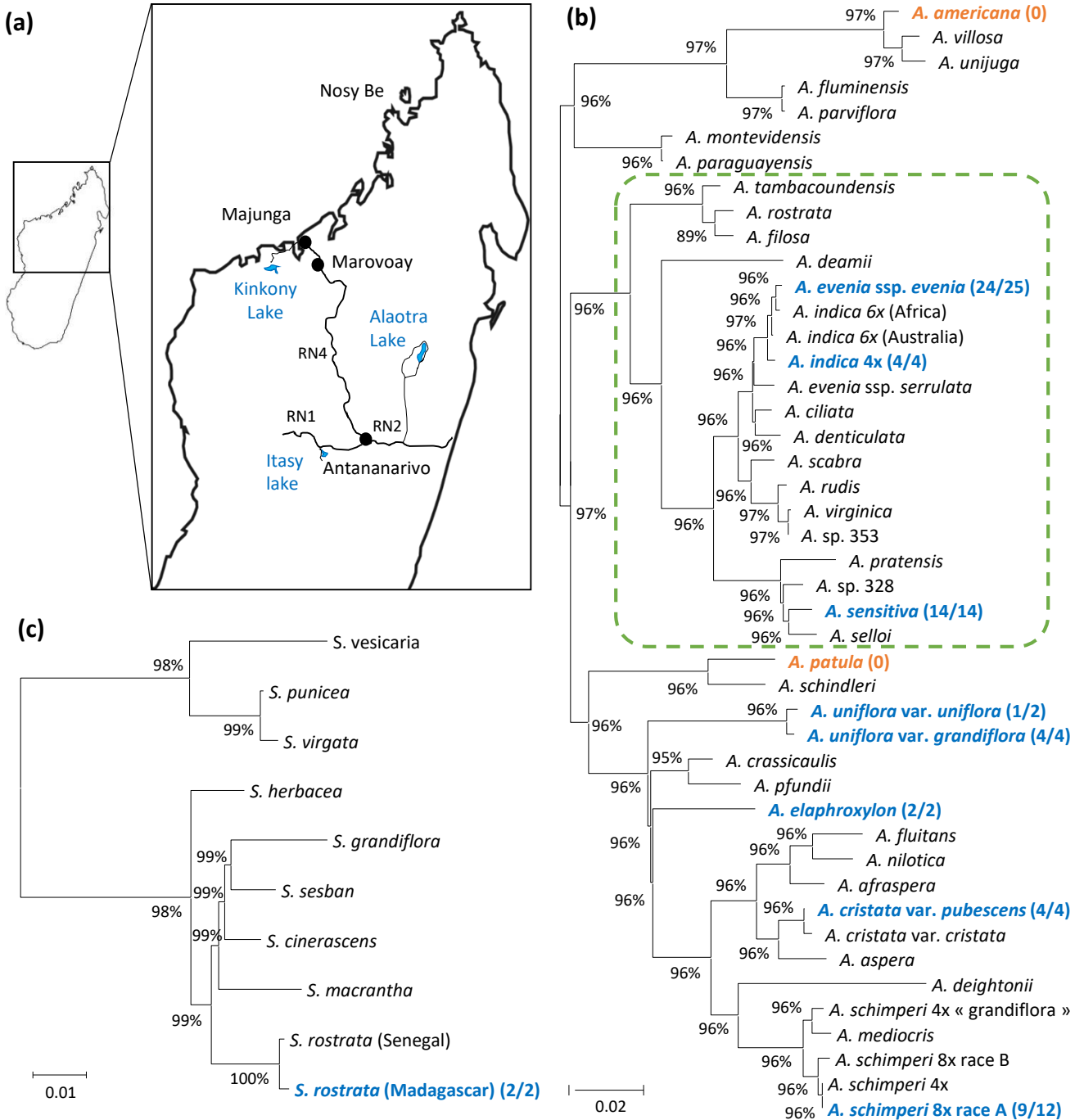


Fig. 1 *Aeschynomene* and *Sesbania* species sampled in wetlands of Madagascar. (a) Map of Madagascar with a zoom on the four collecting sites, including RN1-Itasy Lake, RN2-Alaotra Lake, RN4-Majunga and Nosy Be. (b) Phylogeny of *Aeschynomene*. (c) Phylogeny of *Sesbania*. In (b) and (c), Maximum likelihood phylogenetic reconstructions were obtained using the concatenated the ITS + *matK* sequences. Numbers at nodes represent bootstrap values (% of 1000 replicates). Dashed box delineates the Nod-independent clade. Taxa collected in Madagascar are in bold and numbers on their right correspond to the occurrence of stem nodulation in the different sampling sites. In orange: no stem nodulation observed, in blue: stem nodulation observed in the present study.

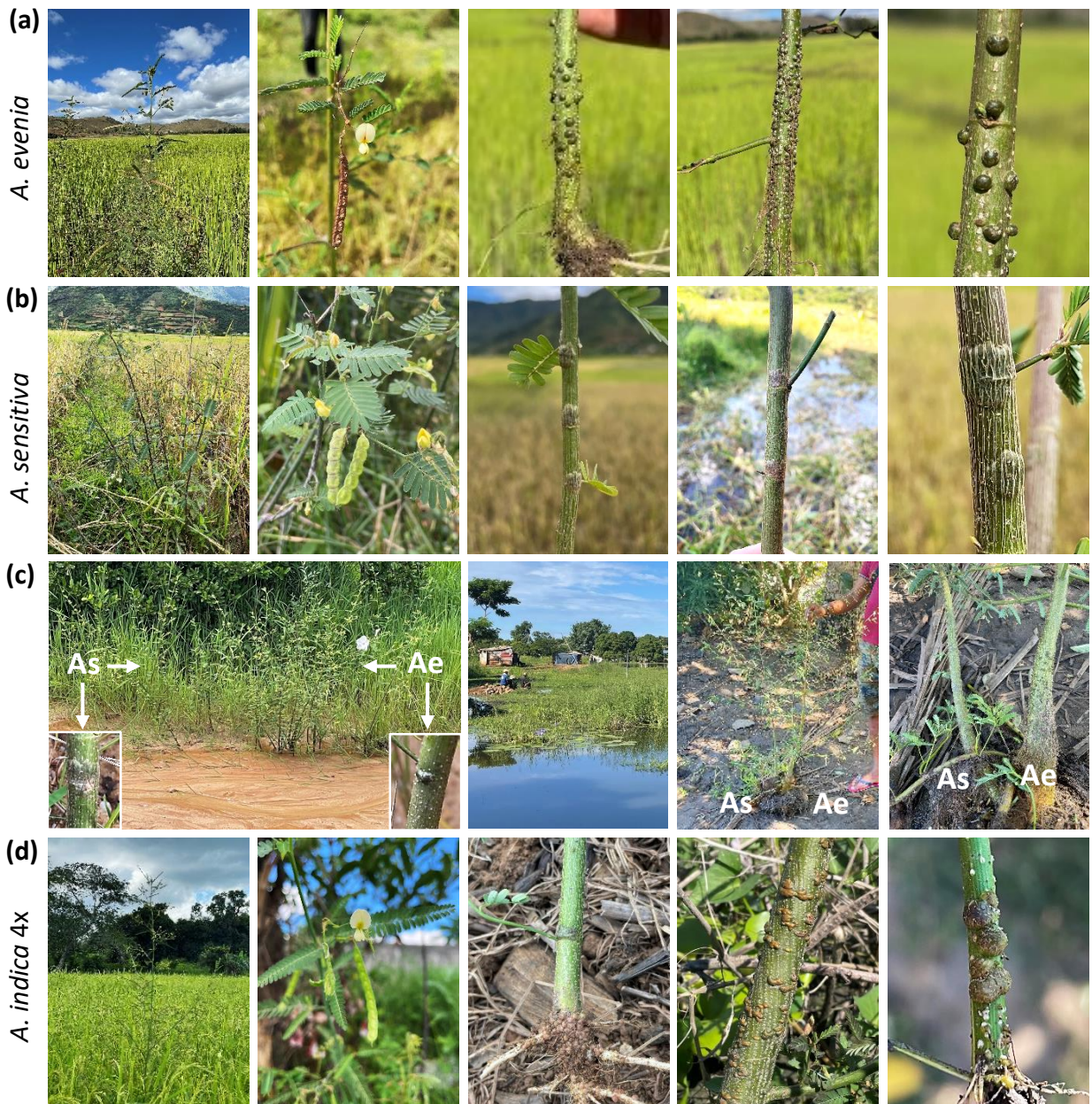


Fig. 2 Stem nodulation in *Aeschynomene* species of the Nod-independent clade. (a) *A. evenia*. From left to right: 1- plant growing in a ricefield near Camp Bandro (Alaotra lake), 2- axillary axis bearing a yellow flower and a mature pod, 3-4- stem nodules located at the base or middle portion of the stem, 5- morphology of stem nodules. (b) *A. sensitiva*. From left to right: 1- plant growing in a ricefield at Manakambahiny, in the direction of Alaotra lake, 2- axillary axis bearing yellow flowers and developing pods, 3-4- more or less flattened stem nodules, 5- morphology of collar stem nodules. (c) Co-occurrence of *A. evenia* and *A. sensitiva*. From left to right: 1- Stand of mixed population in a shallow sandy river at Marofotroboka (on RN4), insets show stem nodules of *A. sensitiva* and *A. evenia*, respectively, 2-4- permanent marsh at Belobaka (Majunga) where *A. sensitiva* and *A. evenia* were found to grow side-by-side with entangled roots, both plants displaying stem nodules. Ae: *A. evenia*, As: *A. sensitiva*. (d) *A. indica* 4x. From left to right: 1- plant growing in a ricefield in Nosy Be, 2- axillary axis bearing a yellow flower and a developing pods, 3-4- stem nodules located near the base or at the middle portion of the stem, 5- morphology of stem nodules. Note the presence of numerous pink root nodules in 3.

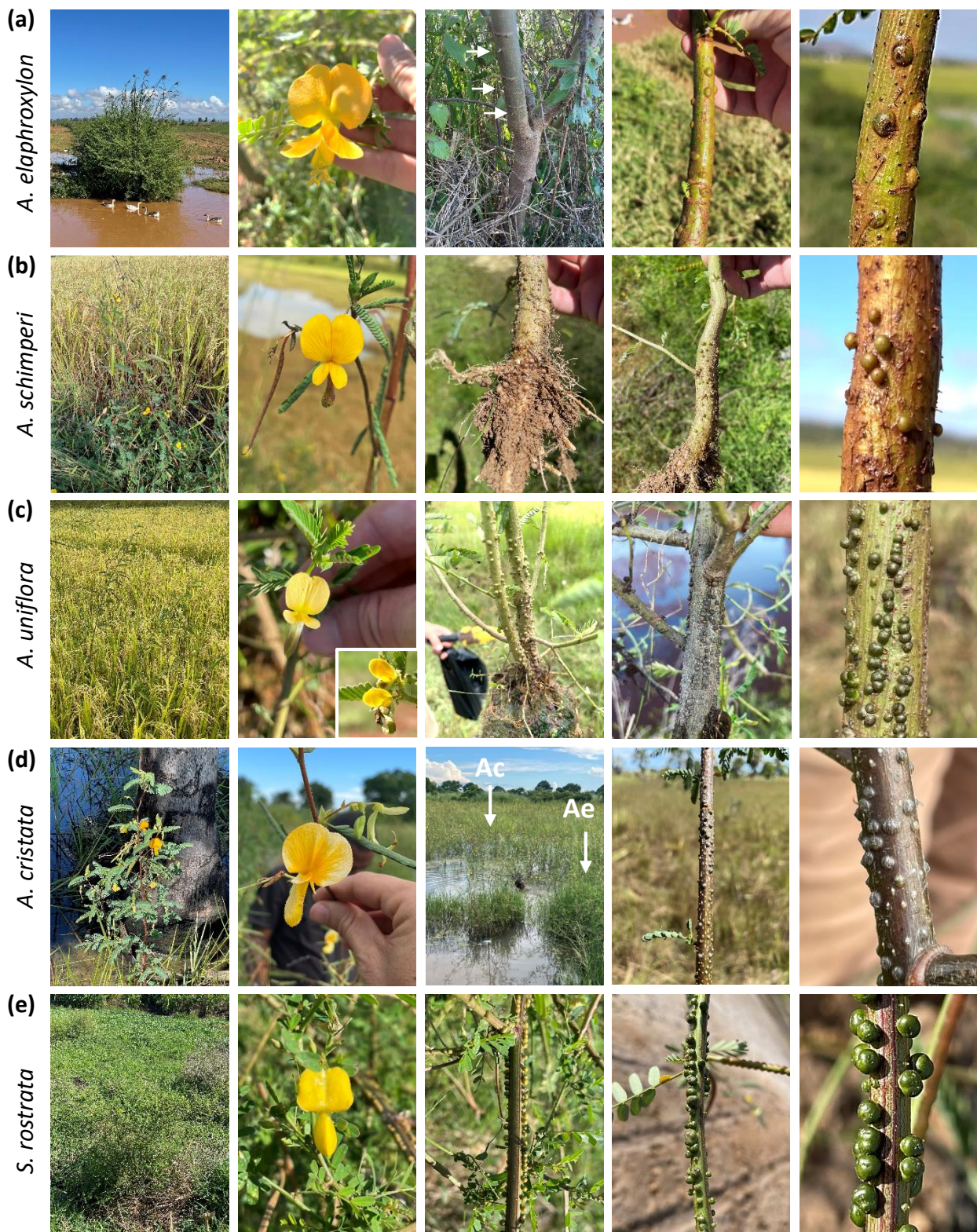


Fig. 3 Stem nodulation in Nod-dependent *Aeschynomene* and *Sesbania* species. (a) *A. elaphroxylon*. From left to right: 1- stand of shrubby plants growing at the edge of a marsh near the Alaotra lake, 2- showy yellow flower with visible anthers, 3- woody stem with spines, 4- nodules developing at the base of the stem, 5- morphology of stem nodules. (b) *A. schimperi*. From left to right: 1- young plants in a ricefield at Maharefo, South of the Alaotra lake, 2- yellow flower, 3- pink root nodules, 4- stem nodules, 5- morphology of stem nodules. *A. uniflora*. From left to right: 1- plants growing in a ricefield at Bongomena on the RN4, 2- yellow flowers with the smaller variant form shown in the inset, 3 and 4- stem nodules of the normal and morphological variant, respectively, 5- morphology of stem nodules. (d) *A. cristata*. From left to right: 1- Plant growing in a swampy palm grove, 2- showy yellow flower, 3- Stand of *A. cristata* (Ac) in the center of a marsh lined with *A. evenia* (Ae), 4- nodules present all along the stem, 5- morphology of stem nodules. (e) *S. rostrata*. From left to right: 1- plant growing on the edge of a marsh at Amboromalandy on RN4, 2- Yellow flower, 3 and 4- arrows of nodules running all along on the stem, 5- morphology of stem nodules.