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Genetic variation of *Casuarina equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* populations on the northern coast of Senegal

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ABSTRACT. The genetic variation of 70 individual samples of *Casuarina equisetifolia* (L. Johnson) subsp *equisetifolia* and *C. equisetifolia* subsp *incana* growing along the northern coast of Senegal was analyzed with RAPD markers. Of the 160 primers tested, five were chosen; they generated 1396 reproducible bands and 61 polymorphic bands that were scored. This result showed a narrow genetic variation among (4.36%) and within (5.90%) *C. equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* plantation sites. The genetic variation at each site revealed a high degree of polymorphism in Potou (5.90%) and low diversity in Retba (3.06%). In the dendrogram analyses, each sampling site was formed by two main groups. Similar results were found for the dendrograms based on the RAPD data gathered from the five different sites. These dendrograms revealed several polytomies in one of the subgroups, suggesting replication of the same specimens in different sites along the Senegalese coast. The RAPD data support the hypothesis that these populations are of the same provenance, subject to

hybridization and inbreeding depression.

Key words: *Casuarina equisetifolia* subsp *equisetifolia*; RAPD; *C. equisetifolia* subsp *incana*; Genetic diversity; Molecular marker; Northern coast of Senegal

INTRODUCTION

Casuarina equisetifolia (L. Johnson) 2n = 18 is a nitrogen-fixing tree including two subspecies, *C. equisetifolia* subsp *incana* (6-12 m) and *C. equisetifolia* subsp *equisetifolia* (7-35 m) (Midgley et al., 1983; Wilson and Johnson, 1989). They are distributed in Australia, Southern Asia, Pacific Islands, tropical and subtropical regions, where native or introduced populations are grown but the subspecies *equisetifolia* is more common outside its area of origin (Wilson and Johnson, 1989; Diouf et al., 2008). In these regions, *C. equisetifolia* is used for landscaping, timber, medicine, dye, pulp, tannin, wood fuel production, soil stabilization, reforestation of marginal ecosystems, amenity planting, and land fertilization (Pan et al., 1996). Because it is salt and drought tolerant, and fast growing, *C. equisetifolia* is also used as windbreaks and sand-shifting control in many parts of the world such as on the northern coast of Senegal (9700 ha) and Southern China (30,000 ha) (Mailly et al., 1994; Zhong et al., 2005). Due to its importance, there is a major effort underway focused on selecting and planting elite clones of *C. equisetifolia* in Australia and India, but the efficiency of this approach requires the genetic characterization of available genetic resources (Yasodha et al., 2004).

In recent years, intensive studies have been done on the genetic diversity of *C. equisetifolia* regarding the socio-economic and ecological importance of this tree. For this purpose WARD's technique based on the hierarchical methods of grouping was used in 505 phenotypically superior trees of *C. equisetifolia*. This study showed genetic variability between *C. equisetifolia* trees in different plantations and suggested hybridization among clones (Kumar and Gurumurthi, 2000). The recent inflow in applying molecular techniques in *Casuarina* chloroplast or nuclear genomes has provided a better understanding of species and subspecies delineation. The comparison of *matk* sequences, a fast evolving chloroplast gene, showed that *C. equisetifolia* from the Philippines and Malaysia are very close but genetically distant to the Australian provenances (Steane et al., 2003). Other DNA markers such as ISSR (inter-simple sequence repeat), fluorescent-ISSR or combined with morphological traits were widely used for assessing the genetic diversity of *C. equisetifolia*. They showed polymorphism between clones of *C. equisetifolia* from Australia and suggested that morphological markers can be used in combination with molecular markers in genetic variability assessment and breeding programs (Yasodha et al., 2004; Kamalakannan et al., 2006). Genetic variation at the population level was also studied in a *C. equisetifolia* plantation in Taiwan by using random amplified polymorphic DNA (RAPD) markers. The data support a wide genetic variation in native *C. equisetifolia* and introgressive hybridization among *C. equisetifolia*, *C. glauca* and *C. cunninghamiana* (Ho et al., 2002a,b). RAPD is a simple method based on DNA amplification developed by Williams et al. (1990). This technique does not require high quality or large amounts of DNA. It is useful for genetic diversity assessment at different levels such as population, species and genus but also for identifying interspecific and introgressive hybridization (Ho et al., 2002b).

In this study, our objective was to assess genetic diversity of *C. equisetifolia* popula-

tions from 5 different sites located on the northern coast of Senegal and to investigate their genetic relationship.

MATERIAL AND METHODS

Plant materials

Branchlets of *C. equisetifolia* were collected from trees growing in 5 different sites (Guédiawaye, Malika, Notto, Potou, and Retba) on the northern Senegalese coast. In each site, 15 trees including 5 female, 5 male and 5 hermaphrodite plants identified by GPS were chosen for branchlet collection (Table 1). The two subspecies *equisetifolia* and *incana* were identified by observing the branchlets and cones with binoculars (SZ-PT, Olympus, Japan). A few specimens were removed from the analysis because they did not amplify. The selection of the tree was based on three morphological criteria: circumference, height and the trunk. The branchlet samples were stored in plastic bags and dried with silica gel before DNA extraction.

DNA isolation, amplification and electrophoresis

DNA of *C. equisetifolia* was extracted according to the protocol developed by Badiane et al. (2004). After extraction, DNA was dissolved in 0.1X TE, quantified with a spectrophotometer (8500-II Spectrophotometer, Techcomp, Ltd., Hong Kong) and stored at -20°C. Amplification reactions, gel electrophoresis and staining were performed according to the protocol described by Badiane et al. (2004). Each reaction was repeated three times to ensure reproducibility of the results.

Scoring, RAPD data and statistical analyses

Bands were scored on the basis of presence (1) or absence (0) across the samples. Bands present across all the samples were excluded from the analysis because they are not informative. Data matrices generated by RAPD were submitted to principal component analysis and analysis of molecular variance (AMOVA).

Multivariate analysis

A normalized principal component analysis using the statistical package ADE-4 coupled with a hierarchical cluster analysis was performed for grouping the samples by their similar characters and the "starters were considered as variables". The *C. equisetifolia* samples were projected as individuals on a factorial plane including the first two axes. An ascending hierarchical clustering of the individuals was carried out by using the coordinates of the individuals on the factorial axes as similarity matrix, the Euclidean distance and the Ward method. The treatments were carried out with the R software (version R-2.9.0) using the ADE4 package for generating a dendrogram. The similarities shown on the dendrogram ranged from zero (high similarity) to 25 (lower similarity).

RESULTS

Table 1. List of *Casuarina equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* samples used in this study.

Code	Subsp	Sex	Location	Code	Subsp	Sex	Location
Gue1	E	Male	Guédiawaye	Not38	E	Female	Notto
Gue2	E	Male	Guédiawaye	Not39	E	Female	Notto
Gue3	E	Male	Guédiawaye	Not40	E	Female	Notto
Gue4	I	Male	Guédiawaye	Not41	E	Hermaphrodite	Notto
Gue5	E	Male	Guédiawaye	Not42	I	Hermaphrodite	Notto
Gue6	I	Female	Guédiawaye	Not43	I	Hermaphrodite	Notto
Gue7	E	Female	Guédiawaye	Not44	E	Hermaphrodite	Notto
Gue8	E	Female	Guédiawaye	Not45	E	Hermaphrodite	Notto
Gue9	I	Female	Guédiawaye	Ret46	E	Male	Retba
Gue10	I	Female	Guédiawaye	Ret47	I	Male	Retba
Gue11	I	Hermaphrodite	Guédiawaye	Ret48	E	Male	Retba
Gue12	I	Hermaphrodite	Guédiawaye	Ret51	E	Female	Retba
Gue13	E	Hermaphrodite	Guédiawaye	Ret52	E	Female	Retba
Gue14	E	Hermaphrodite	Guédiawaye	Ret54	E	Female	Retba
Gue15	E	Hermaphrodite	Guédiawaye	Ret55	E	Female	Retba
Pot16	I	Male	Potou	Ret56	E	Female	Retba
Pot17	E	Male	Potou	Ret57	I	Hermaphrodite	Retba
Pot18	E	Male	Potou	Ret58	E	Hermaphrodite	Retba
Pot19	E	Male	Potou	Ret59	I	Hermaphrodite	Retba
Pot20	I	Male	Potou	Ret60	E	Hermaphrodite	Retba
Pot21	I	Female	Potou	Mal61	E	Male	Malika
Pot23	E	Female	Potou	Mal62	E	Male	Malika
Pot24	I	Female	Potou	Mal63	E	Male	Malika
Pot25	I	Female	Potou	Mal64	E	Male	Malika
Pot26	I	Hermaphrodite	Potou	Mal65	E	Male	Malika
Pot27	I	Hermaphrodite	Potou	Mal66	E	Female	Malika
Pot28	E	Hermaphrodite	Potou	Mal67	E	Female	Malika
Pot29	E	Hermaphordite	Potou	Mal68	E	Female	Malika
Pot30	E	Hermaphordite	Potou	Mal70	I	Female	Malika
Not31	I	Male	Notto	Mal71	E	Hermaphrodite	Malika
Not32	E	Male	Notto	Mal72	I	Hermaphrodite	Malika
Not33	I	Male	Notto	Mal73	I	Hermaphrodite	Malika
Not34	E	Male	Notto	Mal74	I	Hermaphrodite	Malika
Not35	I	Male	Notto	Mal75	I	Hermaphrodite	Malika
Not36	E	Female	Notto				
Not37	E	Female	Notto				

E = subspecies *equisetifolia*; I = subspecies *incana*.

RAPD polymorphism

Among the 160 primers (Kit OPA, OPB, OPF, OPN, OPO, OPQ, OPR, and OPT; Génosphère Biotechnologies, Paris, France) tested, only 5 were chosen in this study (Table 2). Other primers were removed from the analysis because they did not show any polymorphic bands or due to the complexity of their amplification. The primers OPO15 and OPT1 did not show any polymorphic bands in the populations from Retba and Malika, respectively, compared to the OPF4 and OPT16, which generated the highest percentage of polymorphic bands in each site (data not shown). The genetic variation assessed in each site revealed a high polymorphism in Potou (5.90%) and a low diversity in Retba (3.06%) (data not shown). In total, 1396 reproducible bands were amplified, of which 61 were polymorphic (4.36%). The average number of total bands and the number of polymorphic bands per primer were 279.2 ± 65.404 and 12.2 ± 4.5, respectively. The primer OPF4 showed the highest percentage of polymorphic bands (7.01%).

Table 2. Nucleotide sequences and name, total number of bands, number of polymorphic bands, and percentage of polymorphic bands for each primer.

Name and sequence of primers		Total No. of bands	No. of polymorphic bands	Percentage of polymorphic bands
OPA7	5'GAAACGGGTG3'	308	9	2.92
OPF4	5'GGTGATCAGG3'	228	16	7.01
OPO15	5'TGGCGTCCTT3'	192	6	3.125
OPT1	5'GGGCCACTCA3'	329	14	4.25
OPT16	5'GGTGAACGCT3'	339	16	4.71
Average		279.2 ± 65.404	12.2 ± 4.5	
Total		1396	61	4.36

Genetic variation within sites

The samples from Guédiawaye were clustering in two main groups in the dendrogram (Figure 1A). The first group was formed by two main subgroups. Subgroup 1 includes only the subspecies *equisetifolia* (the males Gue5, Gue1, and the female Gue7), but the female Gue8 and the male Gue3 were isolated from the rest of the subgroup forming a polytomy. The second subgroup encompasses small subgroups such as the *incana* specimens (hermaphrodite Gue12 and the female Gue9), which are distant from the *equisetifolia* hermaphrodite Gue13. The *equisetifolia* hermaphrodite Gue14 and the *incana* female Gue10 were clustered together. In contrast, the subspecies *equisetifolia* (hermaphrodite Gue15 and the male Gue2) are located at the base of this subgroup. The second group includes only the subspecies *incana* (the female Gue6 and the male Gue4), but the *incana* hermaphrodite Gue11 showed a high coefficient of dissimilarity with the other specimens.

The dendrogram carried out for the population from Potou showed two groups (Figure 1B). The *incana* female Pot25 and the *equisetifolia* hermaphrodite Pot30, isolated at the base of the first group, seem to have a high coefficient of dissimilarity with the rest of the group. In contrast, the second group showed that the *equisetifolia* female Pot23 and the *equisetifolia* hermaphrodite Pot27 were very similar to each other, but had a strong coefficient of dissimilarity with the other individuals from the same group. The *incana* female Pot21 and the *equisetifolia* hermaphrodite Pot29 were forming a polytomy. In the same group, the *equisetifolia* hermaphrodite Pot28 was isolated from the others.

The population planted in Notto can be divided into two groups; one of them in-

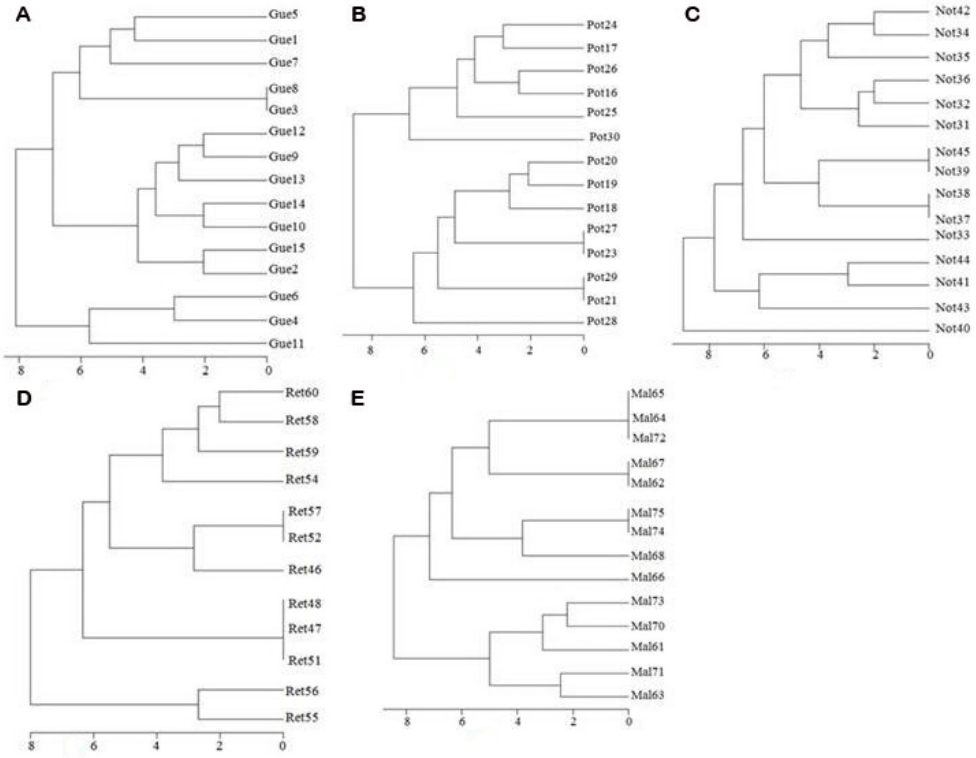

Figure 1. Dendrogram showing the similarity among *Casuarina equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* populations based on RAPD data. **A.** Site of Guédiawaye. **B.** Site of Potou. **C.** Site of Notto. **D.** Site of Retba. **E.** Site of Malika.

cludes only the *equisetifolia* female Not40, which was isolated in the dendrogram (Figure 1C). The second group included several subgroups such as the *incana* hermaphodite Not42 and the *equisetifolia* male Not34, which showed a higher coefficient of dissimilarity with the *equisetifolia* male Not35. The *equisetifolia* (male Not32 and the female Not36) are clustering together and the *incana* male Not31 is located at the base of this subgroup. However, the *equisetifolia* (the female Not39 and the hermaphrodite Not45, and the females Not37 and Not38) also formed a polytomy. But the *incana* male Not33 was isolated from the rest of the subgroup. The *equisetifolia* hermaphrodites Not41, Not43 and *incana* hermaphrodite Not44 formed the last subgroup.

The population from Retba formed two groups (Figure 1D). The *equisetifolia* female Ret52 and the hermaphrodite *incana* Ret57 clustered together to form a polytomy in our study. A second polytomy was observed between the *incana* male Ret47 and the *equisetifolia* specimen (male Ret48 and the hermaphrodite Ret51). These three samples showed a high coefficient of dissimilarity with the other specimens in this group. The *equisetifolia* (the female Ret55 and the hermaphrodite Ret56) constitute a group that is very dissimilar from the first group.

Two main groups were described for the *Casuarina* growing in Malika (Figure 1E).

The *equisetifolia* males (Mal64 and Mal65) and the *incana* hermaphrodite Mal72 formed a subgroup at the top of the dendrogram. This subgroup also includes the *equisetifolia* (the male Mal62 and the female Mal67) specimens. The second subgroup encompasses the *incana* hermaphrodites Mal74 and Mal75 and the *equisetifolia* female Mal68. The third subgroup includes only the *equisetifolia* female Mal66. In the second group, the *incana* (the female Mal70 and the hermaphrodite Mal73) are more similar than with the *equisetifolia* male Mal 61. The *equisetifolia* male Mal63 and the hermaphrodite Mal71 are clustering at the base of this subgroup.

Genetic variation between sites

The genetic relationship of *Casuarina* grown at the five sites (Guédiawaye, Malika, Notto, Potou, and Retba) is shown on the dendrogram of Figure 2 where two main groups are identified. The first group includes 80% of *Casuarina* from Guédiawaye, 64.36% from Potou, 35.7% from Malika, 33.33% from Notto, and 16.66% from Retba. This group also includes four subgroups, 21.43 and 25.71% of the collected specimens belonging to the subspecies *incana* and *equisetifolia*, respectively. The first subgroup did not have any specimens from Malika and Retba but showed similarity between *Casuarina* grown in Guédiawaye, Notto and Potou. The *C. equisetofolia* subsp *incana* (Not35, Gue9 and Pot26) were clustering at the top of the dendrogram. While in the second subgroup only two specimens from Guédiawaye (Gue12) and Malika (Mal66) were similar, the third and the fourth subgroups did not have any similar specimens. In the fourth subgroup, the *incana* (Gue11, Pot16, Pot25, Not33) are clustering together. The second group includes four subgroups, a high number of polytomies, with 14.23 and 38.57% of the collected specimens belonging to the subspecies *incana* and *equisetifolia*, respectively. In this second group, some *equisetifolia* subspecies were clustering among several subgroups. Pot18 and Mal61 formed a polytomy. The other *equisetifolia* subspecies clustered in separate subgroups like Ret58 and Ret60, Not34 and Ret46, Not37 and Not38, Mal67, Mal62, Ret56, Not36, Gue2, and Pot19.

DISCUSSION

To our knowledge, this is the first time that attention has been paid to the genetic diversity of *Casuarina* populations growing on the northern coast of Senegal, which were planted a decade ago. Unfortunately, no records have been reported concerning the origin of the specimens introduced in these areas. For this purpose it is important to understand the genetic relationship of *C. equisetifolia* growing within and among these sites before implementing a breeding program.

Genetic variation

Our data showed a low percentage of polymorphic bands (4.36%) among the amplified fragments. These results are in agreement with the conclusions of Ho et al. (2002b) who found low genetic diversity in a *C. equisetifolia* population growing in Taiwan by using RAPD. Similar results based on ISSR markers were reported between *Allocasuarina littoralis* provenances by Kamalakannan et al. (2006). In contrast, the chloroplastic gene *matk* was not efficient in differentiating *C. equisetifolia* provenances (Steane et al., 2003).

The studies carried out on international provenances showed high genetic diversity

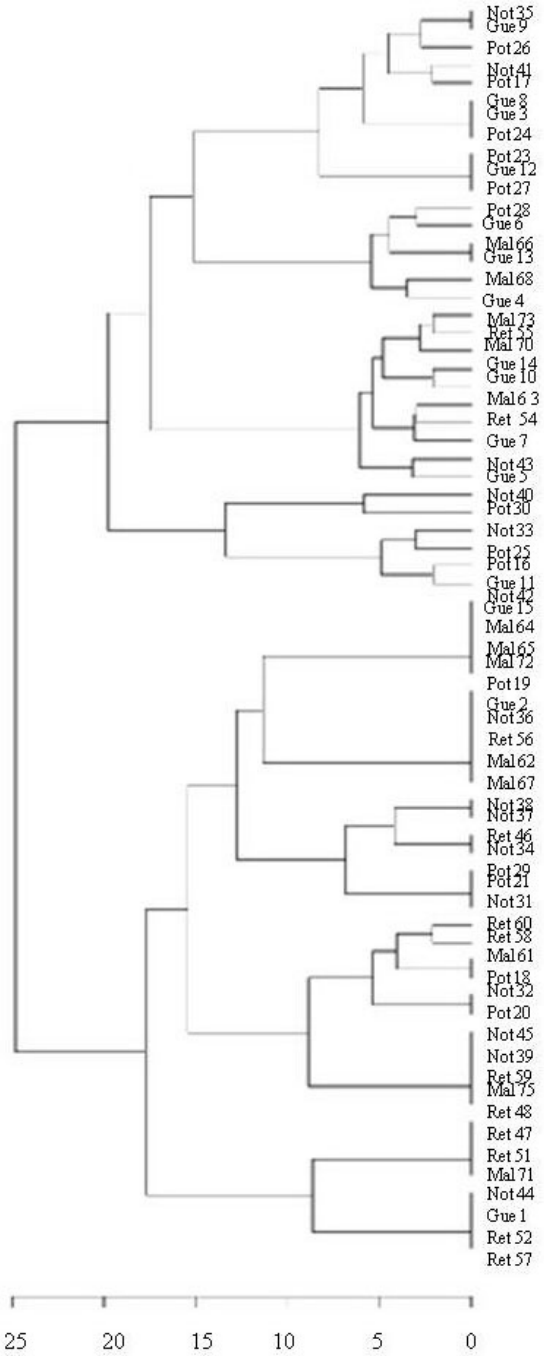

Figure 2. Dendrogram showing the dissimilarity among *Casuarina equisetifolia* subsp *equisetifolia* and *C. equisetifolia* subsp *incana* populations growing in five different sites in the Northern coast of Senegal, based on RAPD data.

among the *C. equisetifolia* (Ho et al., 2002a). Obviously, international provenances are geographically isolated and this phenomenon limits gene flow between provenances and is susceptible to inducing genetic variation due to adaptation to the environmental conditions, which supports the hypothesis of bottleneck effects. In our study, this low genetic variation suggests that the specimens grown on the northern coast of Senegal might have the same origin or could be the result of hybridization due to the cross-pollination occurring in this species. This conclusion supports the hypothesis that the plantation in this area was probably created in an effort to solve an immediate environmental problem such as windbreaks and sand-shifting control than for genetic variation. High genetic variation in *C. equisetifolia* provenances was reported by several studies by using morphological and molecular markers alone or in combination (Pinyopusarerk and Williams, 2000; Yasodha et al., 2004; Kamalakannan et al., 2006). Studies based on amplified fragment length polymorphism (AFLP) markers revealed a high genetic variation among provenances of *C. equisetifolia* (Huang et al., 2008) and other tropical trees such as *Moringa oleifera* (Muluvi et al., 1999) and *Tectona grandis* (Shrestha et al., 2005). This high variation could be attributed to the high number of fragments generated by AFLP, which increases the chance of finding polymorphic bands.

Relationship among accessions

Dendrogram analyses showed two main groups and at least one polytomy for each site but in Notto several subgroups were indentified, one of them includes only one accession (Not40) (Figure 1A-E). Similar results were described in Figure 2, which represented the samples gathered from all the sites. Interestingly, the clustering of the specimens in the dendrograms and those forming polytomies was not linked to the site and the sex of the plants. This finding suggested putative hybridization between the parents of some specimens planted in different locations along the Senegalese coastline. Hybridization was also described as occurring between *C. equisetifolia*, *C. glauca* and *C. cunninghamiana* (Ho et al., 2002b; Gaskin et al., 2009). These hybrids could be very problematic for biological control because they can be more susceptible to several diseases (virus, insects, etc.). The close relationship between the specimens would induce inbreeding depression. Inbreeding depression is known for causing embryo abortion, negatively affecting seed production, nursery performance, survival, and growth vigor in early stages in outcrossing of tropical and temperate trees (Sorensen and Cress, 1994; Williams and Savolainen, 1996; Stacy, 2001; Woods et al., 2002). This conclusion is in agreement with findings suggesting that high inbreeding depression brings about the decline of *C. equisetifolia* plantations (Chen and Li, 2002). It is likely that the lack of regeneration of the *Casuarina* population on the coastline of Senegal is the result of the combination of both inbreeding depression and the significant litter released by these trees. This litter forms a thick layer on the ground creating unfavorable conditions for seed germination. The ineffectiveness for discerning genetic relationships could be linked to RAPD technology. Sometimes RAPD amplifies different regions to the same size (co-migrating bands) in the genome that are not homologous. These bands do not share hundred percent sequence homology and represent homoplasious characters that obliviously impact negatively the analyses, leading to a lack of discrimination (Diouf and Hilu, 2005).

Our studies point to a narrow genetic variation among *C. equisetifolia* subspecies *incana* and *C. equisetifolia* subspecies *equisetifolia* populations growing on the northern coast

of Senegal, which can induce inbreeding depression with the consequence of the lack of population regeneration observed in this area. Therefore, the performance of the species in the plantation cannot be optimized unless more attention is paid to the selection and improvement of the best genetic material. Furthermore, the introduction of provenances from different geographical regions to produce inter-provenance hybrids that possess desirable traits should be explored to introduce more genetic variability.

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