SSR ANALYSIS OF GENETIC POLYMORPHISM IN A *NICOTIANA* GERMPLASM COLLECTION

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INTRODUCTION

The genus *Nicotiana* is a member of the Solanaceae family and is subdivided into 13 sections based on morphological, cytological, and molecular data (Goodspeed, 1954; Chase et al., 2003; Knapp et al., 2004). *Nicotiana tabacum* L. (tobacco), the most well-known *Nicotiana* species, is a natural amphidiploid (2n=48) that probably evolved by interspecific hybridization between *N. sylvestris* Spegazzini et Comes (Goodspeed, 1954; Bland et al., 1985; Olmstead and Palmer, 1991) and a member of section Tomentosae, likely *N. tomentosiformis* Goodspeed with possible genomic contributions from *N. otophora* Grisebach, (Goodspeed, 1954; Kenton et al., 1993; Gerstel and Sisson, 1995; Kitamura et al. 2000; Aoki and Ito, 2000; Lim et al., 2000; Murad et al., 2002; Chase et al., 2003). Tobacco is an important agricultural crop plant that plays a significant role in the economies of many countries. Tobacco breeding aims to develop varieties with wide adaptability, higher potential and suitable chemical constituents for cigarette industry. To explore the genetic potential and select suitable parents it is necessary to assess genetic diversity of tobacco germplasm resources.
The aim of this study was to investigate on the genetic variability in CRA-CAT germplasm collection of *Nicotiana* genus, as revealed by SSR molecular markers.

MATERIALS AND METHODS

In this study 64 tobacco accessions and 11 wild *Nicotiana* species including the presumed progenitors of tobacco, belonging to CRA-CAT germplasm collection of *Nicotiana* genus were examined. *Nicotiana* species were grown in greenhouse under natural light conditions and above 15°C. Tobacco lines were grown from seeds in greenhouse and successively transplanted in field. For each genotype, DNA was extracted from leaf tissue as reported in Fulton et al. (1995) with slight modification.
DNA amplifications were performed utilizing 35 SSR primer pairs chosen from those previously described by Bindler et al. (2007) and Moon (2008). SSR-PCR mixture (25 µl) consisted of 20 ng DNA, 0.4 µM primer, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 2 mM MgCl2, 200 µM of each dNTP, and 0.04 U/ml of Taq polymerase (Applied Biosystem). The amplification conditions were: 94°C, 5 min followed by 35 cycles at 94°C, 30 sec; 55°C, 45 sec; 72°C, 1 min; then a final step at 72°C, 10 min. The amplified fragments obtained from SSR markers were examined for polymorphisms by electrophoresis in 3% metaphor agarose gels in TAE, containing GelRed nucleic acid gel stain (Sichim). Electrophoretic banding patterns were recordered by a molecular image system (BioRad Gel Doc 2000).
SSR profiles generated by the different primer pairs were scored in terms of presence (1) or absence (0) of the bands and the data were assembled in a matrix. To summarize data and detect relationship among traits ordination and clustering methods were tried, with hierarchical clustering (Euclidean metric and Ward's method) on principal components, using the R environment (R Development Core Team, 2012) with functions of the contributed packages FactoMinerR (Husson et al., 2008) and ggplot2 packages (Wickham, 2009).

MOLECULAR MARKERS

Genomic DNA of 65 accessions of *N. tabacum* including unrelated and related lines and 11 wild *Nicotiana* species was extracted and amplified utilizing 35 SSR primer pairs. Amplification profiles of tobacco genotypes showed from 1 to 7 bands with sizes ranging from 70 to 450 bp. Among the tobacco genotypes a total of 113 bands were generated of which 46 (40.7%) were polymorphic. Among the tobacco genotypes and the wild *Nicotiana* species, the 35 SSR primer pairs produced from 2 to 17 bands each with sizes ranging from 70 to 1300 bp. A total of 236 bands were generated of which all were polymorphic .

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INTRASPECIFIC ANALYSIS

Ordination and clustering of the SSR data separated tobacco lines in five groups. In figure 1 the dendrogram obtained is shown and in figure 2 the projections on the plane of the first two principal components.

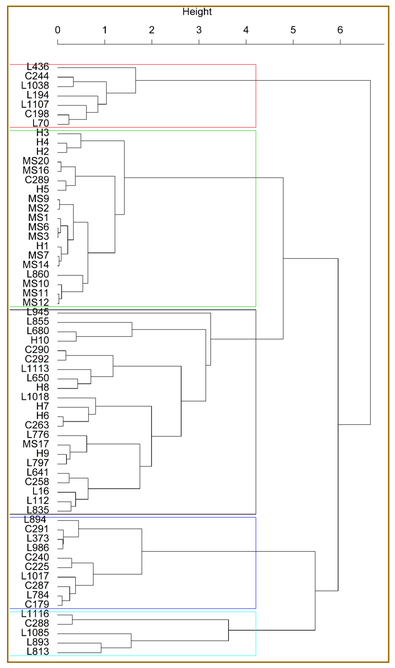

Figure 1. Dendrogram based on SSR data.
L= tobacco line; C= tobacco cultivar; H= hybrid tobacco line; MS= tobacco male sterile line

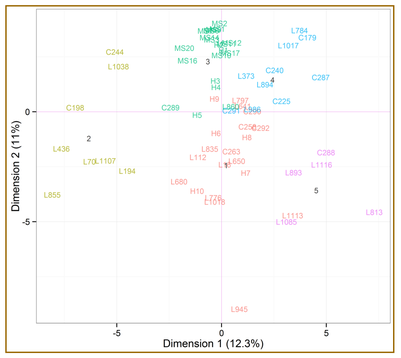

Figure 2. Map of first factor plan for SSR markers, with projected clusters

The identified groups show partial overlapping of known groups and can be characterized as:

1. traditional lines, hybrids and some cultivar, higher than average frequency of 14 markers (highest for P1607) and low frequency of 14 (lowest for P1606), best represented by H6 and C263, with L945 and L1113 most specific;
2. traditional lines and cultivars, higher than average frequency of 5 markers (highest for P3307) and low frequency of 6 (lowest for P3301), best represented by C198 and L70, with L436 and L194 most specific;
3. mainly male sterile hybrids, higher than average frequency of 14 markers (highest for P1606) and low frequency of 12 (lowest for P1607), best represented by MS14 and MS6, with H3 and H2 most specific;
4. traditional lines and cultivars, higher than average frequency of 11 markers (highest for P1609) and low frequency of 8 (lowest for P1601), best represented by C240 and L373,with C287 and C225 most specific;
5. four traditional lines and one cultivar, higher than average frequency of 11 markers (highest for P106) and low frequency of 7 (lowest for P102), best represented by L893 and C288, with L813 and L1085 most specific.

Hybrid H6 (Cluster 1)

Cultivar C198 (Cluster 2)

Male sterile hybrid MS14 (Cluster 3)

Line L373 (Cluster 4)

Line L893 (Cluster 5)

INTERSPECIFIC ANALYSIS

Ordination and clustering of the SSR for *Nicotiana* species, including tabacum as an average of all genotypes, separated six groups (Figure 3). The SSR analysis performed, although preliminary, indicates that these molecular markers are a valuable tool for genetic diversity studies within the collection of *Nicotiana* germplasm.

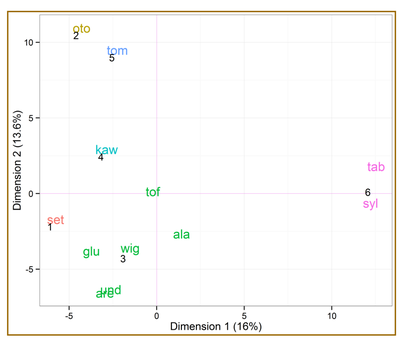

Figure 3. Map of first factor plan for SSR markers, with projected clusters

One group includes *tabacum* (tab) and *sylvestris* (syl), already recognized close in early studies of the genus, four groups separate the species *othophora* (oto), *tomentosa* (tom) , *kawakami* (kaw) and *setchellii* (set) and one group includes *tomentosiformis* (tof), *glutinosa* (glu), *alata* (ala), *wigandioides* (wig), *arentsii* (are) and *undulata* (und). Group characterization is as follows:
1. *N. setchellii*, higher than average frequency of 18 markers (highest for P3202);
2. *N. otophora*, higher than average frequency of 25 markers (highest for P3201);
3. six species, lower than average frequency of 4 markers (lowest for P1601), with *N. arentsii* and *N.undulata,* its maternal parent, most specific;
4. *N. kawakamii*, higher than average frequency of 20 markers (highest for P3007);
5. *N. tomentosa*, higher than average frequency of 22 markers (highest for P1605) and low frequency of P1202);
6. *N. sylvestris* and *N. tabacum*, higher than average frequency of 34 markers (highest for P2702) and low frequency of 3 (lowest for P2703).

CONCLUSIONS

The SSR analysis performed, although preliminary, indicates that these molecular markers are a valuable tool for genetic diversity studies within the collection of *Nicotiana* germplasm.

2013 CORESTA Joint Study Groups Meeting - Brufa di Torgiano, Italy- October 13-17