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RESEARCH

Analysis of genetic polymorphism in wild *Nicotiana* species and Cuban cultivated tobacco (Solanaceae) through AFLP

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ABSTRACT

Wild *Nicotiana* species are commonly used in tobacco's breeding programs to obtain cultivars of enhanced productivity, and to improve its tolerance or resistance to diseases or different types of stress. In Cuba, tobacco production is one of the main sources of economic income and during the last decades several new tobacco varieties have been generated, being essential to study their genetic background for better crop management. In this work, the genetic polymorphism of four Cuban varieties of *Nicotiana tabacum* L. and six wild species used in Cuban tobacco breeding programs were assessed through AFLP analysis. Polymorphic profiles were obtained with four selective primers combinations (*Eco*R I/*Mse* I) among the studied accessions. A total of 203 polymorphic bands (57.79 %) were used for cluster analysis (UPGMA) based on genetic similarity and genetic distance matrices. A common group was detected, comprising all the cultivated varieties that showed high genetic similarity (0.87446-0.93920) according to the Nei and Li's distance measure, whereas wild species showed the highest genetic diversity. It was also possible to identify some bands shared among cultivated accession as specific markers for the analyzed Cuban cultivated tobacco. Our results indicate that AFLP analysis effectively detects the genetic diversity at levels enough to differentiate wild species of *Nicotiana* from Cuban varieties of *N. tabacum*, even though a narrow genetic diversity was present in Cuban varieties. All the accessions were distinguished through AFLP analysis.

Keywords: Genetic diversity, molecular markers, *Nicotiana*, polymorphism, tobacco

RESUMEN

Análisis de polimorfismo genético en especies salvajes de *Nicotiana* y tabaco cultivado en Cuba mediante AFLP. Las especies salvajes de *Nicotiana* se utilizan comúnmente en los programas de mejoramiento genético, para obtener cultivares con nuevas características que lleven al incremento de la productividad, y a la tolerancia o resistencia a enfermedades o a diferentes tipos de estrés. En Cuba, la producción de tabaco es una de las principales fuentes de ingreso económico, de ahí que en la pasada década se obtuvieran nuevas variedades del cultivo. En este estudio se analizó el polimorfismo genético de cuatro variedades cubanas de *Nicotiana tabacum* L. y seis especies salvajes mediante AFLP, dichas variedades empleadas en los programas de mejoramiento genético. Se utilizaron cuatro combinaciones selectivas de cebadores (*Eco*R I/*Mse* I) (*Eco*R I: E-ACT combinado con *Mse*I: M-CAC; M-CAG, M-CTC y M-CAT) para generar los perfiles polimórficos entre los genotipos estudiados. Se obtuvo un total de 203 bandas polimórficas (57.79 %), suficientes para el análisis de clúster (UPGMA) basado en similitudes genéticas y matrices de distancias genéticas. Esto permitió la distinción de un grupo común que comprende todas las variedades analizadas, los que mostraron una alta similitud genética (0.87446-0.93920) mediante la medida de distancia de Nei y Li, mientras las especies salvajes mostraron la más alta diversidad genética. También fue posible identificar algunas bandas similares como marcadores específicos para las variedades cultivadas de tabaco cubano analizadas. Nuestros estudios indican que existe suficiente diversidad genética entre las variedades cubanas de *N. tabacum* estudiadas y las especies salvajes como para diferenciarlas, y que, a su vez, la diversidad genética entre las variedades cubanas es estrecha. El análisis mediante AFLP permitió distinguir a todos los genotipos estudiados.

Palabras clave: Diversidad genética, marcadores moleculares, *Nicotiana*, polimorfismo, tabaco

How to cite (Vancouver style):
Domínguez Y, Peréz-Álvarez S, Magallanes-Tapia MA, Chávez-Medina JA, Héctor-Ardisana EF. Analysis of genetic polymorphism in wild *Nicotiana* species and Cuban cultivated tobacco (Solanaceae) through AFLP. Biotecnol Apl. 2018;35(2):2201-5.

Introduction

The genus *Nicotiana* L. is the fifth largest genus within Solanaceae, including up to 75 species [1], but only two of them are cultivated (*Nicotiana tabacum* L. and *Nicotiana rustica* L.) which are commonly known as tobacco [2]. It is known that in the early evolution of a genus, related species share close genetic affinity and gene exchanges are frequent. Chromosome number in *Nicotiana* is very variable; according to Goodspeed [3], the basic chromosome number is $n = 6$, but further studies have revealed variations from $n = 9$ to n = 32, with some species regarded as amphidiploids or polyploids ($n = 24$) which arose from interspecific hybridization (allopolyploidy). And this is the case of *N. tabacum* and its varieties. In fact, genetic relationships

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have been found for *Nicotiana* species [4, 5], demonstrating that genetic processes and geographic isolation have driven important changes in genome organization and divergence in nuclear DNA sequences, which resulted in the evolution and diversification of the genus.

Particularly, Cuban tobacco is famous worldwide and almost the whole production is exported as cigars [6], with large plantations in the country and being an economically relevant crop. This has fostered the development of new varieties and hybrids in an attempt to minimize the damage caused by diseases, environmental stress and to increase production yields [7, 8]. The main varieties of tobacco grown in Cuba are Corojo and Criollo, although some new hybrids have been introduced due to its resistance to some typical tobacco diseases as the blue mold, with Habana-92, Habana 2000 and Burley Habana 13 (BH-13) among them [9, 10].

Due to the importance of tobacco for Cuban economy, some studies had focused on the analysis of genetic diversity and relationships among *Nicotiana* germplasm based on isozymes variation [4] and randomly amplified polymorphic DNA (RAPD) analysis [5]. However, Cuban cultivated varieties remain to be studied. According to Julio *et al*. [11], the few cultivars resistant to tobacco's diseases are consistent with the low genetic diversity found within *N. tabacum*. On the other hand, several studies have described genetic diversity among wild and cultivated *Nicotiana* [12] or just among cultivars [13, 14] using amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD), but just a poor sampling of Cuban varieties have been included in such studies.

It is of general knowledge that *N. tabacum* varieties have low genetic diversity [12-14]. Therefore, a better knowing of the available germplasm of Cuban tobacco will contribute to its preservation. This will also aid for better planning of Cuban tobacco breeding programs, especially to select the most appropriate parental genetic background able to provide the most advantageous genetic properties of cultivated varieties and wild species. Hence, this work was aimed to analyze the genetic polymorphism in plants of six wild *Nicotiana* species and in four cultivated varieties of *N. tabacum* germplasm used for tobacco breeding in Cuba. The cultivated varieties are among the most cultivated ones. Their germplasms were screened through amplified fragment length polymorphism (AFLP) analysis.

Materials and methods

Plant material and DNA extraction

Six wild species of *Nicotiana* and four Cuban varieties of *N. tabacum* (Habana 2000, Corojo 99, Criollo 98, Burley Habana 13) were studied (Table 1). Plant material was provided by the Tobacco Research Institute (IIT; Havana, Cuba). Samples of 5 g of fresh leaves from each species and variety were processed and the respective total genomic DNA was extracted by the CTAB method [15], being further purified as described by Ausubel *et al*. [16]. The integrity and quality of the DNA obtained was verified by electrophoresis in 1.8 % agarose gels.

Table 1. Species and varieties included in the analyses through AFLP of wild *Nicotiana* species and Cuban cultivated tobacco (Solanaceae) genotypes

No.	Species / variety	*n*	Section	Geographical distribution
1	*N. glutinosa* L.*	12	Tomentosae Goodsp.	Peru, Ecuador
2	*N. tomentosiformis*	12	Tomentosae Goodsp.	Bolivia
3	*N. debneyi* Domin*	24	Suaveolentes Goodsp.	Australia, New Caledonia
4	*N. exigua* H.-M.Wheeler*	16	Suaveolentes Goodsp.	Australia
5	*N. excelsior* (J.M.Black)	19	Suaveolentes Goodsp.	Australia
6	*N. megalosiphon* van Heurck & Müll.	20	Suaveolentes Goodsp.	Australia
7	*N. tabacum* L. var. Habana 2000	24	Genuinae Goodsp.	Cultivated (Cuba)
8	*N. tabacum* L. var. Corojo 99	24	Genuinae Goodsp.	Cultivated (Cuba)
9	*N. tabacum* L. var. Criollo 98	24	Genuinae Goodsp.	Cultivated (Cuba)
10	*N. tabacum* L. var. Burley Habana 13	24	Genuinae Goodsp.	Cultivated (Cuba)

* Haploid chromosome number (*n*) and geographical distributions according to Goodspeed [3].

AFLP analysis

AFLP analysis was done following the standard procedure described by Vos [17] and using the IRDye® Fluorescent AFLP Kit® for large plant genome analysis (LI-COR® Biosciences, USA) as recommended by the manufacturer. High-quality DNA was digested with a pair of restriction enzymes (*Eco*R I/*Mse* I) and then ligated to adapters. The assembled fragments were pre-amplified with non-selective primers *Eco*R I (5´-GACTGCGTACCAATTCA-3´) and *Mse* I (5´-GATGAGTCCTGAGTAAC-3´). Subsequently, four selective primer sets chosen to generate the AFLP profiles: *Eco*RI + ACT/*Mse* I + CAC (E-ACT/M-CAC); *Eco*R I + ACT/*Mse* I + CAG (E-ACT/M-CAG); *Eco*R I + ACT/*Mse* I + CTC (E-ACT/M-CTC); *Eco*RI + ACT/*Mse* I + CAT (E-ACT/M-CAT) with *Eco*R I fluorescent labeled primers. Amplification products were separated in 6.5 % polyacrylamide gels using IRDye® 800-labeled molecular weight marker (LI-COR®) as a size standard (50-700 bp). Band patterns were detected using a LI-COR® DNA Analyzer model 4200 and photographed with SAGA^{MX} (LI-COR®).

Data analysis

The patterns of the amplified fragments were analyzed and scored as discrete characters states (presence/absence of bands: +/–) with the software SAGA^{MX} (LI-COR®) to produce a binary matrix from primary data. A matrix of genetic similarity was calculated using the metric of Nei and Li [18]. Additionally, a matrix of genetic distances [19] was generated by the Simple Matching coefficient [20] between genotypes based on the number of shared bands, and further subjected to cluster analysis in FreeTree [21]. A dendrogram was constructed based on the unweighted pair-group method with arithmetic averages (UPGMA). For the validation of particular branches, bootstrap analysis [22] was carried out using 1000 replicates.

Results

AFLP profiles revealed a large number of bands per primer combination which gave fragments of different size within the range of 200-700 bp. A total of 347 amplification bands were obtained for the whole set of primers, of which 57.79 % were polymorphic (203). High levels of polymorphisms were also detected for each primer pair separately (Table 2). The maximum number of polymorphic fragments (68 bands, 64.76 %) was amplified by the primer pair E-ACT/M-CAG (Figure 1) whereas the minimum (36, 52.17 %) was

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obtained with the pair E-ACT/M-CTC. Since all primer pair's amplifications resulted in more than 50 % polymorphisms, these results demonstrated the usefulness of these sets for diversity analysis in *Nicotiana* spp.

Several fragments obtained by each primer combination were shared exclusively among *N. tabacum* accessions (Figure 1); therefore, they were identified as species' specific markers.

Based on the 203 polymorphic fragments, pairwise comparison among the ten accessions studied revealed genetic similarities from 0.42809 to 0.94222; whereas genetic distance ranged from 0.16548 to 0.82320 (Table 3).

The results of cluster analyses varied according to the measure used. Based on genetic similarity, all accessions were grouped into four main groups (Figure 2 A). Attending to genetic distance analysis, accessions were separated into two main groups (Figure 2 B). Nevertheless, all cultivars of *N. tabacum* remained separated from wild species since they were clearly grouped together with maximum bootstrap support in both analyses, regardless the measure used for cluster analysis (Figure 2). On the other hand, the greatest differences were observed among wild species, their grouping patterns are in disagreement with their geographic origin or phylogenetic relationships previously described.

Discussion

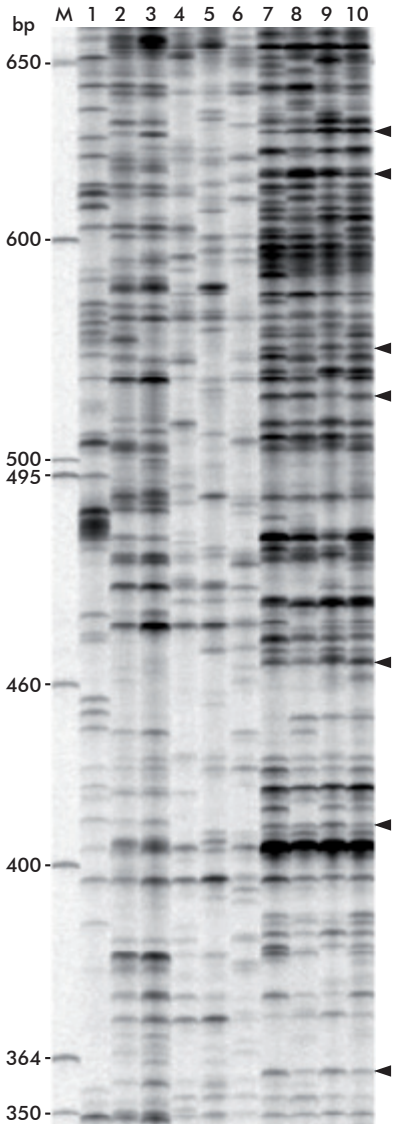
The results concern the reliability of the four primer combinations used for assessing genetic diversity in the genus *Nicotiana* since all sets provided sufficient number of polymorphic fragments (> 50 %) to differentiate among studied accessions (Table 2). Similar results were previously obtained with other combinations of *Eco*R I/*Mse* I primers, which found higher levels of polymorphism in *Nicotiana* than those obtained with other molecular markers such as Sequence-Specific Amplification Polymorphism (SSAP) [23] and RAPD [24].

The highest diversity rates were found among wild species of *Nicotiana* (Table 3) which is consistent with previous interspecific AFLP variation, as reported by Ren *et al*. [12]. As suggested by Mehrotra and Goyal [25], nuclear DNA in *Nicotiana* has experienced several reorganizations during the evolution and diversification of the genus, supporting the high levels of polymorphism observed among wild species. Phylogenetic reconstructions based on plastid and nuclear DNA data have established that the genus *Nicotiana* is monophyletic and supports the understanding of species relationships and patterns of divergence according to geographical distribution [1, 26, 27]. Besides, in this study it was not possible to recognize a congruent pattern between genetic diversity as revealed by AFLP and geographic origin of the analyzed species. *N. megalosiphon* showed genetic affinity with *N. glutinosa* meanwhile *N. tomentosiformis* showed affinity with other Australian species (Figure 2). Most of the presumed relationships between diploid and polyploid *Nicotiana* species have been confirmed using molecular cytogenetics and mapping of repetitive DNA [28, 29]. The basic chromosome number of the genus is $n = 6$, but more than 30 species (including the entire Australian section *Suaveolentes*) are allopolyploids, although some species in section *Suaveolentes* show secondary chromosome number reductions in the range of 32 to 48 [3; 27], such as some of the species from this section analyzed (Table 1).

Regarding *N. tabacum*, it is known that about 77 % of the genome is made up of repetitive DNA

Table 2. *Eco*R I/*Mse* I primer sets with selective nucleotides (+3) used to generate AFLP profiles and polymorphisms detected by each combination

Set	*Eco*R I primers / selective nucleotides	*Mse* I primers / selective nucleotides	Total bands	Polymorphic bands	Polymorphism (%)
A	E-ACT	M-CAC	89	54	60.67
B	E-ACT	M-CAG	105	68	64.76
C	E-ACT	M-CTC	69	36	52.17
D	E-ACT	M-CAT	84	45	53.57
Total	–	–	347	203	57.79



Lanes	Species / variety
1	*N. glutinosa* L.
2	*N. tomentosiformis*
3	*N. debneyi* Domin
4	*N. exigua* H.-M.Wheeler
5	*N. excelsior* (J.M.Black)
6	*N. megalosiphon* van Heurck & Müll.
7	*N. tabacum* L. var. Habana 2000
8	*N. tabacum* L. var. Corojo 99
9	*N. tabacum* L. var. Criollo 98
10	*N. tabacum* L. var. Burley Habana 13

Figure 1. Section of a typical gel of AFLP fragments generated by *Eco*R I-adapter primers E-ACT and *Mse* I-adapter primer M-CAG of wild *Nicotiana* species and Cuban cultivated tobacco (Solanaceae) genotypes. Arrowheads indicate polymorphic bands exclusively shared among *N. tabacum* varieties. M: Molecular size standard IRDye® 800-labeled molecular weight marker (LI-COR®).

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Table 3. Matrix of genetic similarity (above the diagonal) and matrix of genetic distance (below the diagonal) between genotypes based on the number of shared AFLP bands of wild *Nicotiana* species and Cuban cultivated tobacco (Solanaceae) genotypes

Species / variety	No.	1	2	3	4	5	6	7	8	9	10
N. glutinosa L.*	1	–	0.60504	0.57851	0.45570	0.48649	0.42809	0.51491	0.52381	0.50674	0.50282
N. tomentosiformis	2	0.42673	–	0.94222	0.68486	0.70000	0.62176	0.66667	0.67527	0.65502	0.63039
N. debneyi Domin*	3	0.36674	0.80848	–	0.69438	0.72300	0.60714	0.66234	0.67091	0.64655	0.63087
N. exigua H.-M.Wheeler*	4	0.22386	0.41764	0.45011	–	0.72296	0.59710	0.59277	0.58962	0.58034	0.57000
N. excelsior (J.M.Black)	5	0.24499	0.34516	0.39772	0.47826	–	0.62983	0.62963	0.63492	0.60829	0.61391
N. megalosiphon van Heurck & Müll.	6	0.21063	0.28989	0.30293	0.36671	0.41642	–	0.57286	0.58477	0.57000	0.56397
N. tabacum L. var. Habana 2000	7	0.24878	0.20425	0.18267	0.21240	0.22505	0.24808	–	0.93920	0.91915	0.89183
N. tabacum L. var. Corojo 99	8	0.26798	0.19341	0.17193	0.17223	0.21658	0.24751	0.82320	–	0.93111	0.87446
N. tabacum L. var. Criollo 98	9	0.26155	0.19333	0.16548	0.18910	0.17686	0.26798	0.81241	0.79857	–	0.89231
N. tabacum L. var. Burley Habana 13	10	0.25794	0.18740	0.18740	0.18336	0.21748	0.26715	0.75601	0.70805	0.72130	–

sequences [30] which are regarded as responsible for most variations among cultivars. The high genetic similarity observed among cultivated accessions (0.87446-0.93920, Table 3) may be related to inbreeding in Cuban tobacco germplasm, due to the aim of maintaining the desired agronomic and quality traits during breeding programs. Some studies have pointed out low levels of genetic diversity among *Nicotiana* species [31], mainly on cultivated plants. Zhang *et al*. [24] selected 28 accessions with similar agronomic characteristics for an AFLP study. They used 14 selective primers to screen against all 28 accessions and it resulted in 154 polymorphic fragments with an average of 15.4 (27.45 %) per primer pair. They also found that high similarity measures revealed by AFLP in cultivars, which shares gene pools, were linked to morphological similarities. Such low genetic diversity detected by AFLP is common among cultivated plants as reported for acid lime [32], soybean [33] and others.

Regarding the cultivated varieties, the presence of amplification fragments of similar size shared exclusively among the four cultivated accessions (Figure 1) may be useful as species' specific markers. They also might be related to inbreeding processes induced by the use of a limited gene pool in Cuban tobacco germplasm.

Specifically for the AFLP analysis, it is highly efficiently compared to morphological analyses and other molecular markers since it covers the entire genome [34]. Other groups had used it to study genetic polymorphism in tobacco as we implemented it. For instance, Ren and Timko [12] recommended five combinations, because they were suitable to detect genetic polymorphism between cultivars (46 lines of *N. tabacum*) and seven wild species (*N. sylvestris*, *N. tomentosiformis*, *N. otophora*, *N. glutinosa*, *N. suaveolens*, *N. rustica*, and *N. longiflora*). These authors recommended the use of the combinations E-ACT + M-CAG and E-ACT + M-CAC which were able to detect the highest percentage of polymorphism in the genotypes evaluated. Denduangboripant *et al*. [35] reported a similar outcome, the highest percentage of polymorphism (87 %) found among 19 tobacco cultivars by using the combination E-ACT + M-CAG. This precedent, together with these results showing the maximum number of polymorphic fragments with the E-ACT + M-CAG combination, lead us to propose that these AFLP markers could be converted into Sequence Characterized Amplified Region (SCAR), specific locus markers, prior confirmation that they can be used for the identification of cultivars and the studied species. SCAR markers are simpler and less expensive to use for cultivar identification studies than AFLP markers.

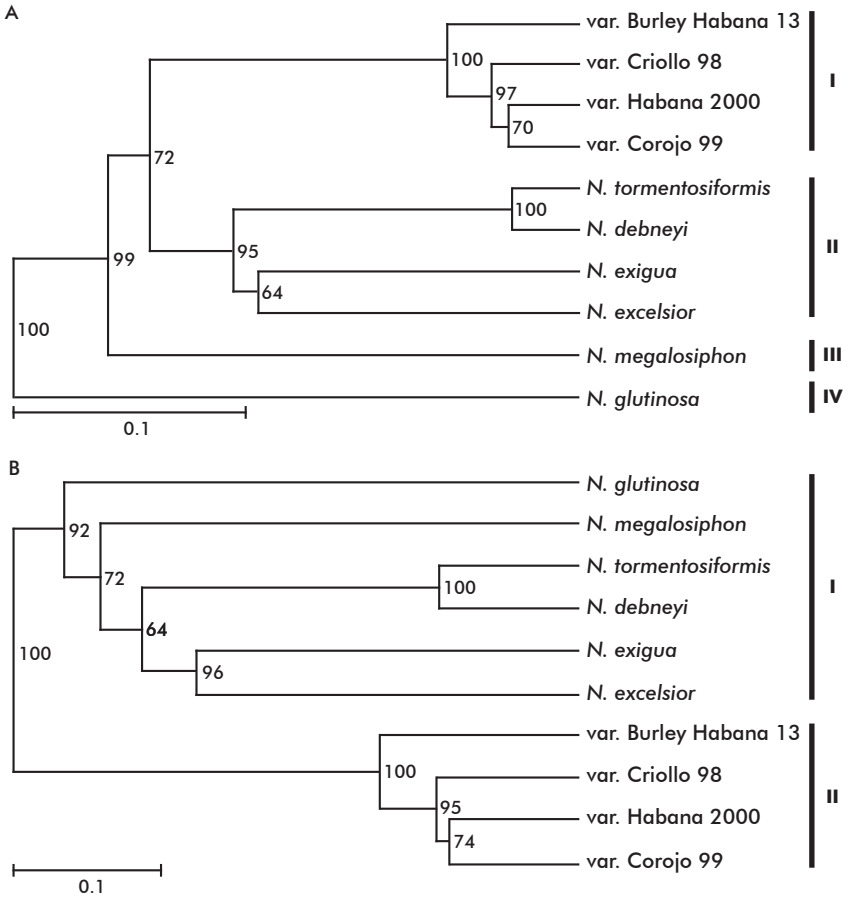

Figure 2. Dendrograms showing genetic relationships among the 10 *Nicotiana* accessions corresponding to wild *Nicotiana* species and Cuban cultivated tobacco (Solanaceae) genotypes. A) Cluster analyses performed using UPGMA from genetic similarity (I-IV: groups defined by genetic similarity). B) Genetic distance based on AFLP data (I-II: groups defined by genetic distance). Bootstrap values are given at each node.

On the other hand, the lowest percentage of polymorphism was obtained with the E-ACT + M-CTC combination, in agreement with reports by Zhang *et al*. [24] on the obtention of just monomorphic amplification products using this combination. Dadras *et al*. [36] also used AFLP markers to analyze 50 *N. tabacum* genotypes with 21 primer combinations, and high polymorphic rates varying from 52.63 to

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92.59 % were found, demonstrating that the AFLP technique can be a powerful and valuable tool in the breeding program of *N. tabacum*. Overall, these findings indicate that AFLP analysis effectively detects the genetic diversity enough to differentiate wild species of *Nicotiana* from Cuban varieties of *N. tabacum*. Moreover, they also remark the usefulness of AFLP for studying the polymorphism of tobacco genome.

Apart from the narrow genetic diversity present in Cuban varieties, all accessions were identified by the AFLP analysis. The reduced genetic diversity of the Cuban varieties represents an important knowledge for further research on cultivar identification through the utilization of molecular markers. It also emphasizes on the need to focus on breeding programs improvement by introducing more diverse cultivated tobacco or using desirable traits found in wild species for cultivated plants.

Acknowledgements

We thank Yosvanis Acanda Artiga and Humberto García Cruz for providing samples of Cuban tobacco germplasm bank. We are also grateful to Katia Gil-Vega from CINVESTAV (Mexico) and Miriam Isidrón Pérez from the Agrarian University of Havana (Cuba) for their assistance with the analyses, and Vitor Fernandes Oliveira de Miranda (Brazil) for comments on the early manuscript.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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Received in March, 2018.
Accepted in June, 2018.