

DNA Barcodes in Fig Cultivars (*Ficus carica* L.) Using ITS Regions of Ribosomal DNA, the *psbA-trnH* Spacer and the *matK* Coding Sequence

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Abstract

Molecular markers provide a useful method for genotype characterization and allow a high precision determination of the genetic relationship between cultivars and varieties. A system based on DNA sequences—which is known as DNA barcoding—will choose one or several standard loci which can be sequenced and compared to differentiate between species. In this research, the ITS, *matK*, and *trnH-psbA* sequences were evaluated for the molecular identification of seven *F. carica* genotypes, generating complete sequences for the first two loci, but unable to produce bidirectional sequences by using the *trnH-psbA* sequence. The ITS sequence presented the highest variation rates, while the phylogeny constructed with the *matK* sequence obtained the highest percentage of solved monophyletic groups. Through Pearson's correlation analysis, it was possible to determine the existence of a significant correlation between the ITS region and *psbA-trnH*, and the *matK* and *psbA-trnH* sequences, but not between ITS and *matK*. The phylogenies constructed with the ITS + *matK* barcodes and ITS + *matK* + *psbA-trnH* presented the highest percentage for resolution. However, considering the cost efficiency and the facilitated recovery by using PCR, the *matK* + ITS combination is recommended.

Keywords

Ficus carica, DNA Barcodes, ITS, *psbA-trnH*, *matK*

1. Introduction

Fig (*Ficus carica* L.) has been an emblematic fruit tree since ancient times, associated to the beginning of horti-

culture in the Mediterranean basin. It is a known fact that fig was domesticated from a group of various species from the South and East regions of the Mediterranean, possibly in the same time span as cereals were domesticated, hence suggesting its cultivation since early Neolithic times [1].

Morphological, physiological, and agronomical performance parameters are frequently used in fig trees in order to establish the descriptors required for the identification of existing genotypes, with the parameters related to leaves and fruits being the most selective. However, these traits are very sensitive and are dependent upon environmental conditions; hence the number of descriptors is limited and in some cases; it does not allow separating the phenotypes in different groups [2] [3].

Presently, different DNA analysis methods have allowed a precise identification of the different genotypes, and they can be classified into three basic types [4]. One of these categories corresponds to methods which are not based on polymerase chain reaction (PCR), where it is possible to find, for example, restriction fragment length polymorphism analysis (RFLP) and the variable number tandem repeats (VNTRs). The second classification includes techniques that employ arbitrary or semi-arbitrary primers; for instance, multiple arbitrary PCR primers such as (MAAP), RAPD and RAMPO. The third category is the one where a PCR with a specific objective is used, enumerating the microsatellite markers (SSR) and the inter-single sequence repeats (ISSR).

Recently, due to the development of fast, efficient, and accessible technologies for DNA sequencing, a new system based on DNA sequences and used for the identification of living organisms has been developed. Known as “DNA barcoding”, it consists of choosing one or several standard *loci* that can be sequenced routinely and reliably, resulting in easily comparable data that allows distinguishing species from one another. The defendants of this initiative establish that DNA barcoding facilitates the identification of species and contributes to the renewal of biological collections, while it also allows accelerating biodiversity inventory [5].

In the *Ficus* genus, different molecular markers have been used to characterize the germplasm; however, the results are limited in comparison to other plant species [6]. In *F. carica*, studies report the use nuclear ITS, the *trnH-psbA* intergenic spacer or the *matK* sequence as DNA barcodes, for the phylogeny reconstruction in different population groups [7] [8].

The aim of this research was to evaluate the use of ITS regions of ribosomal DNA, the *psbA-trnH* spacer, and the *matK* sequence as markers for molecular identification through DNA barcoding of different *Ficus carica* L. accessions present at the Centro de Investigación en Biotecnología of Instituto Tecnológico de Costa Rica.

2. Materials and Procedures

2.1. DNA Extraction, Amplification and DNA Sequencing

Total DNA extraction was obtained from young leaf tissue of seven different accessions of *F. carica* (Table 1), using the “DNeasy plant extraction mini kit” (Qiagen Inc.), according to the protocol suggested by the manufacturer. The concentration and purity of the DNA was quantified through spectrophotometric analysis, by determining the absorbance at 260 nm and 280 nm.

The PCR reactions were performed in a final volume of 50 μ L, using DreamTaq™ Master Mix 2X (Fermentas), 1.5 ng of the primers and 0.5 μ L of genomic DNA. Amplification of the ITS region was achieved using the primers 5'-AAGGTTTCCGTAGGTGAAC-3' and 5'-TATGCTTAAACTCAGCGGG-3', KIM3F and KIM1R primers for *matK*, and finally, *psbA3'*f and *trnH* primers for the *psbA-trnH* spacer.

Table 1. *F. carica* accessions found at the Centro de Investigación en Biotecnología of Instituto Tecnológico de Costa Rica.

Accession	Variety or origin
Fc 1	Guarinta
Fc 2	Brown Turkey
Fc 3	Brogiotto Bianco
Fc 4	Colombia
Fc 5	El Salvador
Fc 6	Negro San Juan
Fc 7	Pacayas, Costa Rica

PCR chain reaction was optimized through a temperature gradient trial (ΔT) between 50°C and 57.5°C, with a gradual temperature increase of 2.5°C for ITS and *matK* sequences, and between 62°C and 65°C with a 1°C increase for *psbA-trnH*; using, in all cases, the following thermal profile: [94°C 2 min, (94°C for 30 s, ΔT for 30 s, 72°C for 50 s) \times 30 cycles. Furthermore, the effect of adding dimethyl sulfoxide (DMSO) in a final concentration of 5% (v/v), as an adjunct in the PCR reactions, was also assessed.

The sequencing process of the amplified products was obtained by sending 20 μ L of each sample to Macro-gen Inc. (USA).

2.2. Editing, Sequence Alignment and Phylogenetic Reconstruction

The DNA sequences were edited using BioEdit program [9], assembled through the CAP3 tool [10], and aligned to their homologous sequences using the EMBL-EBI MUSCLE tool [11]. The multiple alignment file was analyzed with the MEGA v 5.0 software [12] in order to calculate the genetic distance per *loci* between the accessions; according to the number of base pair substitutions between the sequences, eliminating all the positions with missing data, and using Kimura's 2-parameter model. Additionally, a Pearson's correlation analysis was performed between the distance matrices from the three *loci*. The development of a phylogenetic tree was achieved through the maximum likelihood (ML) method with a 500 replicate *bootstrap*. To estimate the resolution of each DNA barcode, the percentage of monophyletic groups generated was calculated using a bootstrap higher than 50% as a parameter to define the nodes, according to the recommendation provided by Tripathi *et al.* (2013) [13].

3. Results

3.1. DNA Extraction, Amplification and Sequencing

The Qiagen D Neasyplant extraction mini kit obtained total DNA from the seven fig accessions at an average concentration of 31.70 μ g/mL with an average A_{260}/A_{280} ratio of 1.24. In the optimization of the PCR chain reaction, the 55°C temperature allowed the generation of more intense bands for the ITS region and *matK* sequence, while for the *psbA-trnH* sequence, the best results were achieved at 64°C. Moreover, it was determined that adding 5% (v/v) of DMSO benefited the PCR chain reaction for the ITS region sequence, however, in *matK* and *psbA-trnH* it presented an inhibitory effect (Figure 1).

From the analyzed *loci*, *matK* presented a longer sequence and *psbA-trnH* has the shortest one; meanwhile, the highest G-C content was obtained with the ITS and the smallest content with *psbA-trnH*. In the case of *psbA-trnH*, there were no bidirectional sequences produced; hence the longest amplified sequence was the one reported (Table 2).

3.2. Editing, Sequence Alignment and Phylogenetic Reconstruction

The sequences obtained from each locus per accession were compared to the NCBI database, through a BLAST search, producing a 99% match with the *Ficus carica* sequence for all cases. However, this comparison did not allow separating the samples into different variety or cultivar groups. The intraspecific distance analysis evidenced that the ITS sequences presented the highest variation rate, with 0.0188, followed by the *multilocus* ITS + *psbA-trnH* and ITS + *matK* combinations, with values of 0.0143 and 0.0121, respectively (Table 3). Pearson's correlation analysis determined the positive and statistically significant correlation ($\alpha = 0.01$) between the intraspecific distance of the ITS and *psbA-trnH* locus (0.895, $p = 0.000$) as well as with *matK* and *psbA-trnH* (0.675; $p = 0.001$), e correlation was not present between ITS and *matK* (0.434; $p = 0.056$). When reconstructing the phylogeny employing individual locus, it was observed that the ITS (Accession Numbers: Fc1 = KJ579170, Fc2 = KJ579166, Fc3 = KJ579167, Fc4 = KJ579168, Fc5 = KJ579169, Fc6 = KJ579171, Fc7 = KJ579172) and *psbA-trnH* (Accession Numbers: Fc1 = KM456207, Fc2 = KM456203, Fc3 = KM456204, Fc4 = KM456205, Fc5 = KM456206, Fc6 = KM456208, Fc7 = KM456209) sequences did not allow the separation of all of the accessions, while this did occur with *matK* (Accession Numbers: Fc1 = KM456200, Fc3 = KM456197, Fc4 = KM456198, Fc5 = KM456199, Fc6 = KM456201, Fc7 = KM456202). Furthermore, the phylogenetic trees developed with the *matK*, ITS + *matK*, and the ITS + *matK* + *psbA-trnH* combination barcodes, presented the highest percentage of monophyletic groups, while the phylogenies ITS + *matK*, ITS + *psbA-trnH*, and ITS + *matK* + *psbA-trnH* combinations grouped the genotypes similarly.

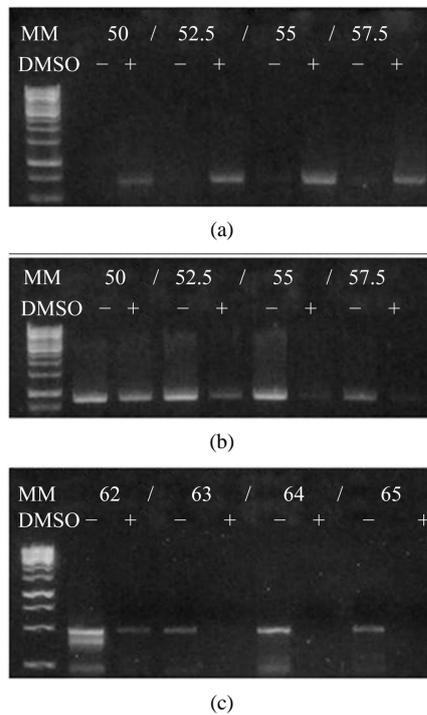


Figure 1. Electrophoretic bands generated during the optimization of the PCR. ITS (a), *matK* (b) and *psbA-trnH* (c).

Table 2. Size and percentage of G-C content for the ITS region, *matK* and *psbA-trnH* sequences.

Locus	Size (pb)	GC content (%)
ITS	613 - 618	63.72
<i>matK</i>	892 - 895	33.09
<i>psbA-trnH</i>	267	16.70

Table 3. Efficiency of the DNA barcodes of individual locus and their combinations.

Locus or multilocus	Alignment length (pb)	Percentage of variable points	Average intraspecific distance (K2P)	Percentage of monophyletic groups
ITS	613	6.20	0.0188	28.57
<i>matK</i>	892	2.24	0.0109	85.71
<i>psbA-trnH</i>	244	2.87	0.0108	50.00
ITS + <i>matK</i>	1504	3.52	0.0121	100
ITS + <i>psbA-trnH</i>	880	4.43	0.0143	57.14
<i>matK</i> + <i>psbA-trnH</i>	1159	2.50	0.0109	57.14
ITS + <i>matK</i> + <i>psbA-trnH</i>	1700	3.53	0.0096	100

4. Discussion

4.1. DNA Extraction, Amplification and Sequencing

In this research, the absorbance ratio of 260/280 in the DNA extractions was always less than 1.57, demonstrating the presence of impurities with strong absorbance in, or close to, 280 nm [14]. There is a probability that la-

tex and endogenous phenols of the fig's leaf tissue were not totally eliminated with the kit. Regarding this matter, Weiblen (2000) [15] mentions that modifications in extraction procedures have been required in order to obtain DNA of increased purity, due to the presence of latex in *F. carica* tissue.

The amplified ITS region, with an average of 614 pb, was similar to the one reported by Baraket *et al.* (2009) [7], who found ITS regions of 697 pb in 31 fig cultivars. The G-C content in the ITS sequence of the evaluated accessions was of 63.72%, which is comparable to the ones obtained by Baraket *et al.* (2009) [7], which ranged between 53.1% to 64.1%. Due to the high percentage of G + C content present in the ITS region, its amplification through PCR was inhibited, since the considerable intermolecular forces formed by the triple hydrogen bonds in the DNA generate structures which are difficult to denature [16]. DMSO interrupts the formation of a secondary structure in the DNA, resulting in a destabilization of the double helix structure, and hence aiding the PCR chain reaction [17]. These results are consistent with those obtained by Razafimandimbison *et al.* (2004) [18], who recommended the use of DMSO or BSA along with TMACI in the final PCR chain reaction. Similarly, Kress *et al.* (2009) [8] mentioned adding a final concentration of 5% DMSO in all the PCR reactions.

The amplified *matK* sequence of 894 pb was analogous to the one reported by Kress *et al.* (2009) [8], with an average of 850 pb in a plant community from Panamá, which included samples from the *Ficus* genus. In contrast to the ITS region, the percentage of the G + C content of this *locus* was below 40%; which explains the reason why adding DMSO inhibited the PCR chain reaction instead of optimizing it. Even though low recovery percentages were reported for the sequence, the amplification problems for *matK* were not due to the sequence composition; but they were rather caused by the efficiency and specificity of the primers used [19] [20]. However, the *KIM3F* and *KIM1R* primers were effective for the locus amplification in the varieties of *F. carica* that were analyzed.

In this study, it was not possible to generate a complete bidirectional sequence for the *psbA-trnH* sequence, and only 267 pb segment was obtained, which is smaller than the 386 pb sequence amplified by Roy *et al.* (2010) [21] for the *Ficus* genus, and the 488 pb reported for *F. carica* by Kirin *et al.* (gb|KC584953.1|; unpublished). The complications in generating bidirectional sequences in this *locus* have been previously documented by several research groups. Li *et al.* (2011) [22] explained that the sequence interruption occurs because of the mononucleotide repetitions; meanwhile Fazekas *et al.* (2008) [23] argue that the problems in producing bidirectional sequences is caused mainly by the presence of homopolymers in the electrophoresis runs.

4.2. Editing, Sequence Alignment and Phylogenetic Reconstruction

The percentage of variable sites and the intraspecific distances of the *loci* evidenced the presence of polymorphisms between the analyzed *F. carica* cultivars, with the ITS region being the locus that presented the most variability out of the three *loci* used. Fu *et al.* (2011) [24] found similar results when comparing the ITS, *rbcL*, *matK*, and *trnH-psbA* sequences, since the researchers determined that the ITS region showed the highest average genetic distance as well as the number of variable sites. On the other hand, Tripathi *et al.* (2013) [13] mention that, in an intraspecific level, *rbcL* and *trnH-psbA* are the least divergent *loci*, matching the present research in which *psbA-trnH* had the lowest average K2P distance value.

In regards to the resolution of the monophyletic groups, the ITS region by its own was the *locus* with the lowest resolution (25.57%), contrary to the findings of Roy *et al.* (2010) [21] who reached the best species resolution of the *Ficus* genus with this molecular marker. The existence of a strong, common genetic basis among the accession could be the possible explanation for these results [25]. On the other hand, the *matK* sequence presented the highest individual resolution percentage (85.71%), followed by *psbA-trnH* (50%). Even though, in many cases, *matK* has been considered efficient in differentiating species, there are opposing reports on using *trnH-psbA* as a *locus* for DNA barcoding [13].

The individual phylogenies of the three *loci* presented two main inconsistencies. In the first place, some individuals were grouped together with the ITS sequence, but were then divided in two or more groups when using the plastome sequences; and secondly, the species which were grouped together using the *psbA-trnH* sequence, were then divided when grouping them with *matK*, both belonging to the plastid DNA. These scenarios suggest hybridization and introgression between closely related varieties, the existence of shared ancestral polymorphisms, and/or a different mutation rate between some of the sequences [22].

These results also indicate that the analyzed sequences contribute in different ways to distinguishing the diverse fig genotypes. This hypothesis is supported in the fact that the polymorphisms generated in each of the

sequences have different origins from a molecular level viewpoint, and can differ in their application when searching for genetic diversity and establishing the relationship between the genotypes.

The strong and statistically significant correlation observed between ITS and *psbA-trnH*, as well as with *matK* and *psbA-trnH* suggest that the phylogenetic information between these *locus* pairs is closely related, while no significant correlation between ITS and *matK* indicate the presence of different evolutionary events between the two regions, allowing each other to complement the distinction between genotypes [7].

The *multilocus* DNA codes allowed a more clear phylogenetic separation of the fig varieties, and in general, they provided a similar grouping pattern for the accessions, especially when the ITS region was complemented with the information from any of the plastome sequences.

While the greatest K2P distance was observed in the *multilocus*ITS + *psbA-trnH*, the highest percentage of monophyletic groups was achieved only when the ITS + *matK* combination was present. Li *et al.* (2011) [22] presented similar results when establishing that *matK* allows greater species differentiation. On the other hand, Fu *et al.* (2011) [24] reported high monophyly percentages when combining ITS with the *matK*+ *rcbL*barcode.

Additionally the *multilocus* phylogenetic analysis evidenced the speciation process that the cultivars from Colombia, El Salvador, Negro San Juan and Pacayas have experienced from the traditional varieties of Brown Turkey and Brogiotto Bianco; which is a connection that is not fully evident from the analysis based only on the plastome sequences (Figure 2).

When identifying varieties of *F. carica*, the most efficient DNA barcodes were the *matK* + ITS and the *matK* + *psbA-trnH* + ITS combinations, since they allowed a 100% monophyly differentiation of the species. However, considering cost efficiency and the difficulty in obtaining bidirectional *psbA-trnH* sequences, the recommendation would be to use a two-sequence *multilocus* rather than one with three sequences. The reported results are consistent to those gathered by Li *et al.* (2011) [22], who found a higher species differentiation percentage with the *matK* + *psbA-trnH* + ITS *multilocus*, but recommended the *matK* + ITS combination based on cost efficiency.

Table 4 shows the genetic distance matrix of the analyzed accessions, using the proposed barcode, while the ITS + *matK* multiple sequence alignment is found in Appendix 6. The genetic distance of the cultivars ranged between 0.0020 and 0.0285, being the Guarinta accession the one presenting the highest divergence when compared to the rest. Related results were obtained by Baraket *et al.* (2009) [7] who reported that the genetic dis-

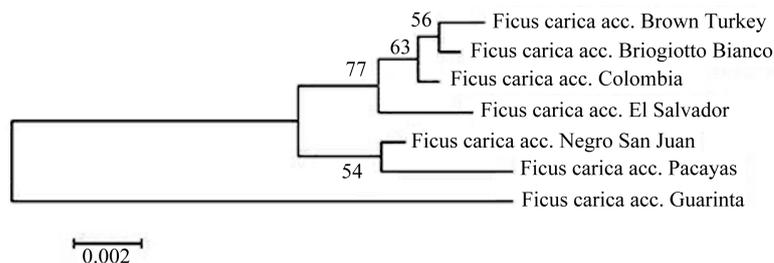


Figure 2. Phylogeny reconstruction based on the nrITS + *matK* sequences and using the maximum likelihood (ML).

Table 4. Genetic distance matrix between the *F. carica* accessions, calculated using Kimura's 2-parameter model with the ITS + *matK* barcode*.

Accession	1	2	3	4	5	6	7
1 Guarinta		0.0042	0.0040	0.0040	0.0041	0.0039	0.0040
2 Brown Turkey	0.0264		0.0011	0.0013	0.0019	0.0023	0.0026
3 Brogiotto Bianco	0.0257	0.0020		0.0011	0.0017	0.0020	0.0022
4 Colombia	0.0264	0.0027	0.0020		0.0017	0.0022	0.0025
5 El Salvador	0.0285	0.0060	0.0053	0.0047		0.0021	0.0025
6 Negro San Juan	0.0264	0.0087	0.0067	0.0073	0.0067		0.0017
7 Pacayas	0.0257	0.0100	0.0080	0.0100	0.0100	0.0047	

*The bottom diagonal corresponds to the genetic distance values while the upper diagonal is the standard deviation with a 500 bootstrap. The values in bold show the highest and the lowest genetic distances.

tances calculated when comparing the ITS sequences of nine *F. carica* cultivars from Tunisia ranged from 0.027 to 0.386. Similarly, Baraket *et al.* (2011) [25] found a genetic distance ranging from 0.004 to 0.170 in 31 *F. carica* cultivars with the ITS + *trnL-trnF* multilocus.

5. Conclusion

The gathered data demonstrated that the local fig germplasm was characterized by its genetic diversity. Despite the low genetic variation levels in the *F. carica* accessions, the multilocus ITS + *matK* barcode was not only the most reliable and cost efficient alternative for the identification of the cultivars but it also was useful for explaining the phylogenetic relationships in this taxonomic level. Furthermore, the three analyzed locus presented their particular strengths and weaknesses for genotype identification in *F. carica* at intraspecific taxonomic level, which should be accounted before designating them as universal DNA barcodes for plants.

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References

- [1] Zohary, D. and Hopf, M. (1993) Domestication of Plants in the Old World. Clarendon Press, Oxford.
- [2] Saddoud, O., Salhi-Hannachi, A., Chatti, K., Mars, M., Rhouma, A., Marrakchi, M. and Trifi, M. (2005) Tunisian fig (*Ficus carica* L.) Genetic Diversity and Cultivar Characterization Using Microsatellite Markers. *Fruits*, **60**, 143-153. <http://dx.doi.org/10.1051/fruits:2005018>
- [3] Guasmi, F., Ferchichi, A., Farés, K. and Touil, L. (2006) Identification and Differentiation of *Ficus carica* L. Cultivars Using Inter Simple Sequence Repeat Markers. *African Journal of Biotechnology*, **5**, 1370-1374.
- [4] Karp, A., Kresovich, S., Bhat, K., Ayad, W. and Hodgkin, T. (1997) Molecular Tools in Plant Genetic Resources Conservation: A Guide to the Technologies. International Plant Genetic Resources Institute, Roma.
- [5] Hollingsworth, P.M., Graham, S.W. and Little, D.P. (2011) Choosing and Using a Plant DNA Barcode. *PLoS ONE*, **6**. <http://dx.doi.org/10.1371/journal.pone.0019254>
- [6] Achtak, H., Oukabli, A., Ater, M., Santoni, S., Kjellberg, F. and Khadari, B. (2009) Microsatellite Markers as Reliable Tools for Fig Cultivar Identification. *Journal of the American Society for Horticultural Science*, **134**, 624-631.
- [7] Baraket, G., Saddoud, O., Chatti, K., Mars, M., Marrakchi, M., Trifi, M. and Salhi-Hannachi, A. (2009) Sequence Analysis of the Internal Transcribed Spacers (ITSs) Region of the Nuclear Ribosomal DNA (nrDNA) in Fig Cultivars (*Ficus carica* L.). *Scientia Horticulturae*, **120**, 34-40. <http://dx.doi.org/10.1016/j.scienta.2008.09.013>
- [8] Kress, W.J., Erickson, D.L., Jones, A.F., Swenson, N.G., Perez, R., Sanjur, O. and Bermingham, E. (2009) Plant DNA Barcodes and a Community Phylogeny of a Tropical Forest Dynamics Plot in Panama. *Proceedings of the National Academy of Sciences of the United States of America*, **106**, 18621-18626. <http://dx.doi.org/10.1073/pnas.0909820106>
- [9] Hall, T.A. (1999) BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symposium Series*, **41**, 95-98.
- [10] Huang, X. and Madan, A. (1999) CAP3: A DNA Sequence Assembly Program. *Genome Research*, **9**, 868-877. <http://dx.doi.org/10.1101/gr.9.9.868>
- [11] Edgar, R.C. (2004) MUSCLE: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Research*, **32**, 1792-1797. <http://dx.doi.org/10.1093/nar/gkh340>
- [12] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. and Kumar, S. (2011) MEGA5: Molecular Evolutionary Genetics Analysis Using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution*, **28**, 2731-2739. <http://dx.doi.org/10.1093/molbev/msr121>
- [13] Tripathi, A.M., Tyagi, A., Kumar, A., Singh, A., Singh, S., Chaudhary, L.B. and Roy, S. (2013) The Internal Transcribed Spacer (ITS) Region and *trnhH-psbA* Are Suitable Candidate Loci for DNA Barcoding of Tropical Tree Species of India. *PLoS ONE*, **8**, e57934. <http://dx.doi.org/10.1371/journal.pone.0057934>
- [14] Glasel, J.A. (1995) Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios. *Biotechniques*, **18**, 62-63.
- [15] Weiblen, G.D. (2000) Phylogenetic Relationships of Functionally Dioecious *Ficus* (Moraceae) Based on Ribosomal DNA Sequences and Morphology. *American Journal of Botany*, **87**, 1342-1357. <http://dx.doi.org/10.2307/2656726>
- [16] Chakrabarti, R. and Schutt, C.E. (2001) The Enhancement of PCR Amplification by Low Molecular-Weight Sulfones.

- Gene*, **274**, 293-298. [http://dx.doi.org/10.1016/S0378-1119\(01\)00621-7](http://dx.doi.org/10.1016/S0378-1119(01)00621-7)
- [17] Simon, L.S., Grierson, L.M., Naseer, Z., Bookman, A.A. and Zev-Shainhouse, J. (2009) Efficacy and Safety of Topical Diclofenac Containing Dimethyl Sulfoxide (DMSO) Compared with Those of Topical Placebo, DMSO Vehicle and Oral Diclofenac for Knee Osteoarthritis. *Pain*, **143**, 238-245. <http://dx.doi.org/10.1016/j.pain.2009.03.008>
- [18] Razafimandimbison, S.G., Kellogg, E.A. and Bremer, B. (2004) Recent Origin and Phylogenetic Utility of Divergent ITS Putative Pseudogenes: A Case Study from Naucleaeae (Rubiaceae). *Systematic Biology*, **53**, 177-192. <http://dx.doi.org/10.1080/10635150490423278>
- [19] Kress, W.J. and Erickson, D.L. (2008) A Two-Locus Global DNA Barcode for Land Plants: The Coding *rbcL* Gene Complements the Non-Coding *trnH-psbA* Spacer Region. *PLoS ONE*, **2**, e508. <http://dx.doi.org/10.1371/journal.pone.0000508>
- [20] Lahaye, R., Van der Bank, M., Bogarin, D., Warner, J., Pupulin, F., Gigot, G. and Savolainen, V. (2008) DNA Barcoding the Floras of Biodiversity Hotspots. *Proceedings of the National Academy of Sciences of the United States of America*, **105**, 2923-2928. <http://dx.doi.org/10.1073/pnas.0709936105>
- [21] Roy, S., Tyagi, A., Shukla, V., Kumar, A., Singh, U.M., Chaudhary, L.B. and Tuli, R. (2010) Universal Plant DNA Barcode Loci May Not Work in Complex Groups: A Case Study with Indian Berberis Species. *PLoS ONE*, **5**, e13674. <http://dx.doi.org/10.1371/journal.pone.0013674>
- [22] Li, D.Z., Gao, L.M., Li, H.T., Wang, H., Ge, X.J., Liu, J.Q. and Duan, G.W. (2011) Comparative Analysis of a Large Dataset Indicates That Internal Transcribed Spacer (ITS) Should Be Incorporated into the Core Barcode for Seed Plants. *Proceedings of the National Academy of Sciences of the United States of America*, **108**, 19641-19646. <http://dx.doi.org/10.1073/pnas.1104551108>
- [23] Fazekas, A.J., Burgess, K.S., Kesanakurti, P.R., Graham, S.W., Newmaster, S.G., Husband, B.C. and Barrett, S.C. (2008) Multiple Multilocus DNA Barcodes from the Plastid Genome Discriminate Plant Species Equally Well. *PLoS ONE*, **3**, e2802. <http://dx.doi.org/10.1371/journal.pone.0002802>
- [24] Fu, Y.M., Jiang, W.M. and Fu, C.X. (2011) Identification of Species within *Tetrastigma* (Miq.) Planch. (Vitaceae) Based on DNA Barcoding Techniques. *Journal of Systematics and Evolution*, **49**, 237-245. <http://dx.doi.org/10.1111/j.1759-6831.2011.00126.x>
- [25] Baraket, G., Abdelkrim, A.B., Mars, M. and Salhi-Hannachi, A. (2011) Cyto-Nuclear Discordance in the Genetic Relationships among Tunisian Fig Cultivars (*Ficus carica* L.): Evidence from Non Coding *trnL-trnF* and ITS Regions of Chloroplast and Ribosomal DNAs. *Scientia Horticulturae*, **130**, 203-210. <http://dx.doi.org/10.1016/j.scienta.2011.06.038>