Optimal Use of Random Amplified Polymorphic DNA in Estimating the Genetic Relationship of Four Major *Meloidogyne* spp.

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ABSTRACT

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The technique of random amplified polymorphic DNA (RAPD) was used to estimate the genetic relationship among four species of nematodes, *Meloidogyne incognita, M. arenaria, M. javanica,* and *M. hapla.* The clustering derived from RAPDs was fully consistent with that obtained from cytogenetic, enzymatic and restriction fragment length polymorphisms of genomic and mtDNA studies. The similarity between the species and its jackknife variance was estimated according to the number of bands scored. While similarity did not differ significantly with the number of bands scored, the variance of similarity for the less distant species diminished as the number of bands increased, and became constant beyond 200 bands. The similarities calculated from four independent replicates did not differ significantly from the estimation made from the reproducible bands in the four replicates.

Additional keywords: AMOVA, PCR, root-knot nematodes.

The random amplified polymorphic DNA–polymerase chain reaction (RAPD-PCR) technique has proved to be a powerful tool for addressing many problems concerning variation in, and taxonomy of, plant pathogens (26,27). This technique is a variant of the PCR in which a decamer oligonucleotide primer of arbitrary sequence is allowed to anneal at a relatively low temperature, priming the amplification of DNA fragments distributed at random in the genome. These highly polymorphic fragments are useful as genetic markers to identify organisms (27), and also to establish relative degrees of similarity between individuals, populations, and species. The RAPD-PCR technique has been used with plant pathogenic fungi (*Fusarium solani* and *Leptosphaeria maculans*) (6,11) and nematodes (*Heterodera* and *Meloidogyne* spp.) (2,3), among many other examples.

In spite of the great interest in, and potential of, RAPDs, there is no consensus on how to interpret the genetic information supplied by these markers. Many authors have used the percentage of shared bands or related measures between different samples as an indirect measure to infer similarities between them (2,11). Some authors have demonstrated a good agreement between the clustering obtained by RAPD data and that deduced from isozyme data (16,23). However, there was until recently no genetic model that makes it possible to infer genetic distances among individuals, populations, or species from RAPD data. This was an important pitfall because it prevented the use of RAPD data in studies of population and evolutionary genetics. Clark and Lanigan (5) developed a genetic model that allows the estimation of genetic distances from RAPD data. They demonstrate that with a set of restrictions these data can be treated mathematically in the same way as restriction fragment length polymorphism (RFLP) data. In consequence, by calculating the fraction F of shared fragments from Nei and Li (18), the genetic distance between two individuals or populations can be estimated. Lynch and Milligan (15) developed a population genetics approach to study RAPD markers in which individuals are grouped according to the different pattern of fragments obtained, and the genetic structure of the populations is analyzed considering such patterns as alleles. Another approach is the analysis of molecular variance (AMOVA) (10), which converts a phenotypic distance matrix into an equivalent analysis of variance. This procedure allows estimation of variance components for RAPD phenotypes, partitioning the variation among samples within and among populations or species. The AMOVA approach has been applied previously to the analysis of RAPD data in plants (12).

The AMOVA method can be used to optimize some aspects of the application of RAPDs for the assessment of genetic relationships. In this work, we address two aspects: 1) determining the minimum number of RAPD bands to be scored to obtain an adequate estimation of distances; and 2) estimating the minimum number of replicates needed to avoid or reduce the effect of the poor reproducibility of some bands. To test the applicability of the models mentioned, and to address those methodological aspects of RAPDs, we chose the root-knot nematodes of the genus *Meloidogyne*. The genetic relationships among its four major species, *M. incognita* (Kofoid and White) Chitwood, *M. javanica* (Traub) Chitwood, *M. arenaria* (Neal) Chitwood, and *M. hapla* Chitwood, have been extensively studied at different levels. At present, information exists on the cytogenetic (25), isoenzymatic (9), genomic DNA (20,28), mitochondrial DNA (13,21), and DNA amplification fingerprinting (DAF) (1) differences among them. In addition, RAPD-PCR has been applied to these species for taxonomic purposes (3).

MATERIALS AND METHODS

Populations used and DNA extraction. The populations of the four species of *Meloidogyne* used in this study (Table 1) came from the collection of North Carolina State University (Raleigh,

TABLE 1. *Meloidogyne* populations used in the study

Population code[a]	Species	Location	Chromosome number	Race[b]
I-68	*M. incognita*	North Carolina	41-43	Race 1
I-2152	*M. incognita*	Nigeria	45	Race 2
I-2337	*M. incognita*	Argentina	36	Race 3
I-527	*M. incognita*	Texas	42	Race 4
I-RU	*M. incognita*	Spain	42	
A-523	*M. arenaria*	Texas	53	Race 1
A-413	*M. arenaria*	Nigeria	53-54	Race 1
A-54	*M. arenaria*	Virginia	53	Race 1
J-76	*M. javanica*	Georgia	42	
J-93	*M. javanica*	Brasil	42	
H-86	*M. hapla*	Virginia	17	Race A
H-230	*M. hapla*	Chile	48	Race B

[a] Code of the nematode collection of North Carolina State University, Raleigh.

[b] Race classification according to North Carolina differential test of species and races, except races A and B, which are cytogenetic races.

NC) and have been described and characterized in previous works (4). Genomic DNA was extracted and purified as previously described (4).

PCR reactions. The primers used in the reactions were decamers of arbitrary sequence supplied by Operon Technologies (Alameda, CA). In a first experiment, one population from each species (I-68, A-413, J-93, and H-86) was selected, and these were used in reactions with the 60 primers from sets B, C, and D. Only 30 of the primers proved suitable, and with these, three replicates of the reactions were made. In a second experiment, the DNA of the 12 populations was amplified with the primers that gave good amplification previously. PCR reactions and visualization of results were as described in a previous work (3). The pictures of the gels were processed with a Scanmaster 3+ Howtek scanner and a VISAGE Electrophoresis Gel Analysis System, Ver. 4.60 (Millipore Corp. Bedford, MA). The analysis of data was made from the scanned bands.

Reproducibility experiment. In this experiment, one population from each species (I-68, A-523, J-93, and H-86) was studied. Twenty primers from sets B, C, and D were used as before. Four independent reactions were made of each primer-DNA combination, and the four amplified products were put together in the same gel. All the replicates were made with the same stock of DNA and the same batch of reagents. The data of this experiment were arranged in two different ways. In the first arrangement, the similarity F was calculated for each of the four replicates taken independently and also with the bands that appeared in the four replicates. In the second arrangement, four measures of similarity between the six pairs of species were calculated by scoring the bands that appeared one, two, three, or four times respectively in the four replicates. The number of loci scored was 296, 241, 219, and 189 for one, two, three, and four occurrences in the four replicates, respectively. In order to compare visual versus automatic reading of bands, two additional visual calculations were made by the same worker of the bands that appeared in the four replicates. One reading was made with a nonrestrictive criterion, counting as positive all the bands in the limit of visibility. The other reading was made with a restrictive criterion, by not scoring those bands.

Data analysis. The starting point of the two methods of data analysis considered was a matrix of zeros and ones, scoring the absence or presence of bands of amplified DNA. From this matrix, an estimator $F = 2n_{xy} / n_x + n_y$ of similarity (Nei and Li) (18) was computed, where n_{xy} is the number of bands shared by the populations x and y, n_x and n_y being the total number of bands of the two populations. The Clark and Lanigan model was implemented by calculating a distance D_1 by iteration as fully described in their work (5), as well as the jackknife variances of similarities and distances (8). Then the net divergence between species was calculated according to Nei (17) using the formula

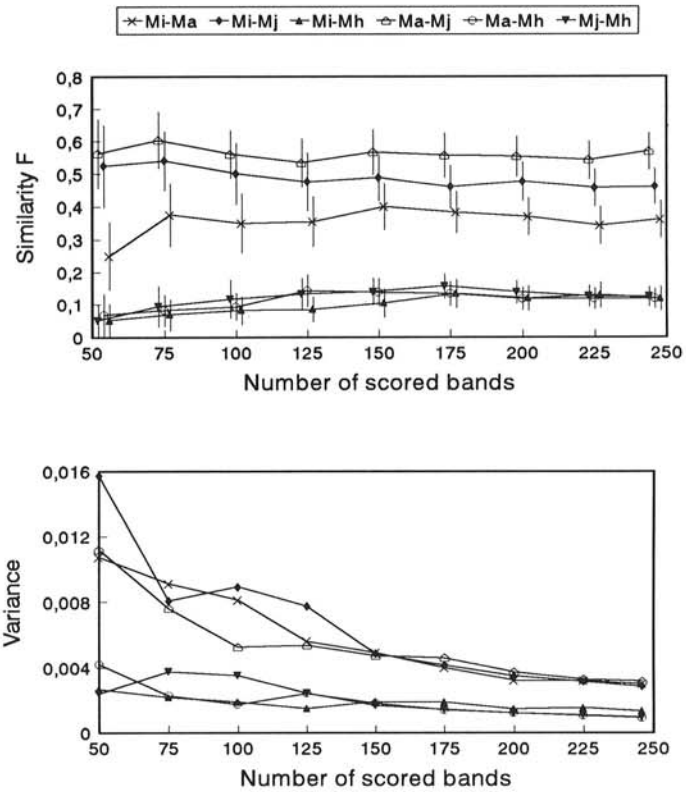

Fig. 1. Similarity F (top), and similarity jackknife variance (bottom) between the six pairs of the four species of *Meloidogyne,* as a function of the number of scored random amplified polymorphic DNA (RAPD) bands. Bars indicate standard error. (Points are slightly displaced for clarity.) Mi = *M. incognita*; Ma = *M. arenaria*; Mj = *M. javanica*; Mh = *M. hapla*.

$d_A = d_{xy} - (d_x + d_y)/2$, where d_{xy} is the distance between species x, y, and d_x, d_y the distances within the species x, y, respectively.

The AMOVA procedure was implemented after excluding markers that were monomorphic for the entire data set. Similarity F was used to compute a distance metric defined as: $D_2 = 100\ [1-F]$. Significance levels for variance component estimates were computed by nonparametrical permutational procedures (i.e., 2,000 permutations) as described (10).

The distance between species obtained following both methods was used to make a dendrogram with the UPGMA method implemented in the NTSYS ver. 1.4 package (22).

RESULTS

In the first experiment with one population from each species, 30 primers gave the best amplifications: OPB-04, -05, -06, -07, -08, -10, -11, -15, -17 and -18; OPC-01, -02, -09, -10, -11, -12, -13, -14, and -15; OPD-02, -03, -05, -07, -08, -11, -12, -13, -15, -18, and -20. The total number of reproducible bands in the three replicates was 250. The F value and the jackknife variance were calculated and grouped in sets of different number of bands. The F values and the variance between species as a function of the number of bands analyzed appear in Figure 1. Similarities between the six pairs of species were not different for any number of bands scored. However, the value of the variance for the less distant species diminished as the number of bands increased up to 200 bands. For the more distant species, the variance reduction with an increase in the number of bands was not so marked.

In the second experiment with the 12 populations of the four species, a total of 274 bands were scored after reactions with the primers mentioned above. The data of similarity and distance D_2 for the 12 populations appear in Table 2. The AMOVA analysis of distance D_2 showed that the most significant clustering of the four

TABLE 2. Matrix with the similarity F (×100) (upper half) and genetic distance (1-F) ×100 (bottom half) among the 12 populations of four species of *Meloidogyne*

...	I-68	I-2152	I-2337	I-527	I-RU	A-523	A-413	A-54	J-93	J-76	H-86	H-230
I-68	...	94,55	91,57	90,00	87,01	30,57	27,67	27,67	41,11	38,71	12,64	11,70
I-2152	5,45	...	89,44	92,90	87,21	31,58	28,57	28,57	41,14	40,00	11,83	12,05
I-2337	8,43	10,56	...	93,59	86,71	31,37	28,39	28,39	40,91	38,41	10,59	10,78
I-527	10,00	7,10	6,41	...	85,03	32,65	29,53	29,53	41,18	40,00	10,98	11,18
I-RU	12,99	12,79	13,29	14,97	...	26,83	26,51	26,51	38,50	35,80	13,26	12,36
A-523	69,43	68,42	68,63	67,35	73,17	...	78,08	79,45	62,28	57,75	7,45	10,13
A-413	72,33	71,43	71,61	70,47	73,49	21,92	...	93,24	57,99	55,56	7,36	10,00
A-54	72,33	71,43	71,61	70,47	73,49	20,55	6,76	...	60,36	59,72	7,36	10,00
J-93	58,89	58,86	59,09	58,82	61,50	37,72	42,01	39,64	...	80,00	9,78	11,05
J-76	61,29	60,00	61,59	60,00	64,20	42,25	44,44	40,28	20,00	...	10,06	10,26
H-86	87,36	88,17	89,41	89,02	86,74	92,55	92,64	92,64	90,22	89,94	...	77,71
H-230	88,30	87,95	89,22	88,82	87,64	89,87	90,00	90,00	88,95	89,74	22,29	...

species was Mh(Mi(Ma,Mj)), as can be appreciated in Table 3. *Meloidogyne hapla* is the most distant species from the other three, *M. javanica* and *M. arenaria* being the closest. This is graphically illustrated by the dendrogram made with the data of net divergence among species (Fig. 2). The corresponding dendrogram using the net divergence derived from the distance D_1 between pairs of species showed identical topology (Fig. 2). As several of the restrictions imposed by the Clark and Lanigan model were not met, it is important to notice that the numerical values of the distances obtained with it are not completely accurate. Apart from remedying the lack of correction for dominance, complete accuracy would require verification that the polymorphic bands behave as Mendelian factors. This would require performing crosses that are not possible with parthenogenetic species like the ones studied here. So it is necessary to be cautious with the distances reflected in the scale numbers of the dendrogram and with the numerical data shown in column 5 of Table 4, which is a summary of the similarities and distances previously obtained using different approaches.

Reproducibility experiment. The results of the reproducibility experiment appear in Figure 3. As can be observed, each pair of species shows a similarity that is not identical according to replication, but the differences with the similarities calculated only with the reproducible bands are not relevant (Fig. 3, top). When scoring the bands by the number of times they appear in the four replicates, the similarity differences of the six pairs of species were not significant (Fig. 3, bottom). The results obtained through a nonrestrictive visual scoring of bands did not differ significantly from that obtained through their automatic scoring. However, when the visual scoring was made with a restrictive criterion (i.e., not scoring faint bands) the similarity values of two pairs of species differed significantly compared with automatic reading of bands and with the nonrestrictive visual scoring.

DISCUSSION

The results of the present work show that the RAPD markers produce results that are consistent with other approaches for estimating genetic relationships. In the dendrogram deduced from the RAPD data, *M. arenaria* and *M. javanica* are seen to be the more closely related species, and this cluster is close to *M. incognita.* By contrast, *M. hapla* appears in a different branch. This topology is fully consistent with cytogenetic, enzymatic, and RFLPs of genomic DNA data, including data from DAF, a method very similar to RAPD that also supports the clustering Mh (Mi(Ma, Mj) (1).

With respect to cytogenetic evidence, *M. arenaria, M. incognita,* and *M. javanica* share the feature of their obligatory (mitotic) parthenogenetic mode of reproduction. Within this group, *M. javanica* and *M. arenaria* have a very similar number and disposition of chromosomes in the meiosis, while *M. incognita* presents several unique cytological features such as the clumping of the chromosomes during a very prolonged prophase in maturing oocytes (24). By contrast, *M. hapla* has a very different reproduction mode, with amphimictic and facultative (meiotic) parthenogenesis.

TABLE 3. Analysis of molecular variance of random amplified polymorphic DNA data for 12 populations of *Meloidogyne* grouped according to species

Source of variation	Variance component	% Total	P Value[a]
Among species	28.2507	80.47	< 0.0005
Among populations within species	6.8563	19.53	
Ma vs. Mj			
Among	12.7397	62.82	< 0.0005
Within	7.5408	37.18	
Mi vs. (Ma, Mj)			
Among	24.4363	67.70	< 0.0005
Within	10.7024	32.30	
Mh vs. (Mi, Ma, Mj)			
Among	25.1080	53.45	<0.0005
Within	21.8681	46.55	

[a] After 2,000 permutations

Similar clustering has been obtained by Dalmasso and Bergé (7) with isoenzyme analysis. They find that acid phosphatase and isoesterase profiles of *M. arenaria* and *M. javanica* are similar, but different from those of *M. incognita* and even more different from *M. hapla* profiles. The observed similarities were confirmed later in a study of the enzymatic relationship of the genus made with 30 populations of 10 species and 27 enzymatic systems (9).

Studies carried out with RFLPs of genomic DNA also confirm the clustering of the four major species. Using probes of repeated DNA, Piotte et al. (20) found that *M. hapla* is clearly separated from the mitotic species. Xue et al. (28) used randomly cloned DNA fragments to detect RFLPs in the three mitotic species. They estimated genetic distances following the model of Nei and Li (18) and performed a phylogenetic tree, according to which *M. arenaria* and *M. javanica* are the closest species. The data of the thermal analysis of DNA homology also indicate that *M. incognita, M. arenaria*, and *M. javanica* are closely related, with 80 to 100% homology, while *M. hapla* shows a wide divergence, with 23 to 35% DNA homology (19).

The pattern of variation offered by mitochondrial DNA studies is more complex. According to Powers and Sandall, *M. arenaria* is the most genetically distinct of the four species, with a nucleotide sequence divergence of 2.1 to 3.1% (21). This discrepancy could be explained in part by an inadequate species identification, as recognized by one of the authors (14), but the reinterpretation of the results based on this fact still sustains the different grouping, showing a greater genetic diversity within *M. arenaria.* In a more detailed work based on a larger number of populations, Hugall et al. (13) found two highly divergent groups of mtDNA haplotypes, one associated with *M. hapla* and the other, more common, group associated with *M. arenaria, M. javanica,* and *M. incognita.* They also found a high degree of diversity within *M.*

arenaria, which includes lineages as distinct from each other as they are from *M. javanica*.

Although two different methods of analysis have been used in this study with the same set of data, they are based on different assumptions, and consequently no attempt was made to compare statistically the distances obtained by both. The Clark and Lanigan model allows the estimation of nucleotide divergence in the same way as RFLP data. This is useful given the amount of theory developed for using this data in evolutionary analysis. However, this model imposes important restrictions, such as the need to verify the allelism of the bands or correct the dominance, among others, which make it less applicable to parthenogenetic organisms or those not amenable to genetic analysis. By contrast, the AMOVA procedure is easier and more flexible, because it uses a great variety of distance metrics and partitions the variance into its different components. This is very suitable for hierarchical designs in studies of variation in populations of plant pathogens. Other alternative approaches to the analysis of RAPD data, not discussed here, are those proposed by Baum et al.(1), in which the DAF bands are treated as independent characters subjected to different cladistic analysis, and the already mentioned population genetics approach of Lynch and Milligan (15).

The pattern of variation of genetic similarity as a function of the number of bands scored is remarkably constant. The similar-

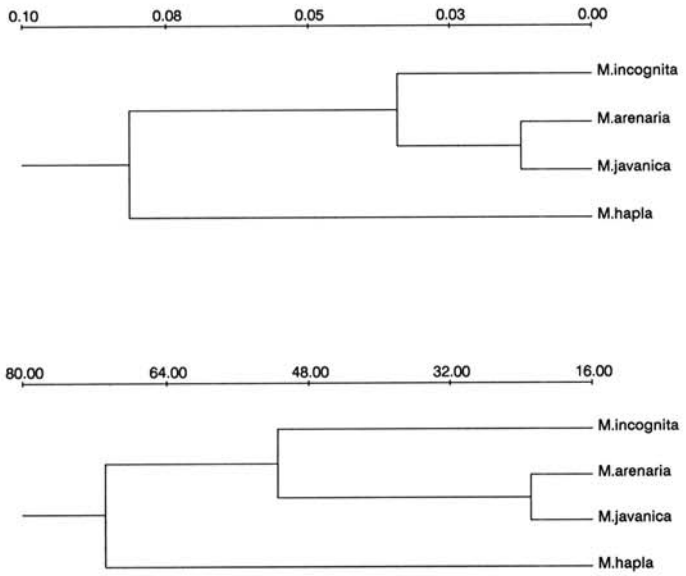

Fig. 2. UPGMA dendrograms derived from the distance matrices. Top: according to distance data calculated by the Clark and Lanigan model (5). Bottom: according to the distance data from analysis of molecular variance (AMOVA).

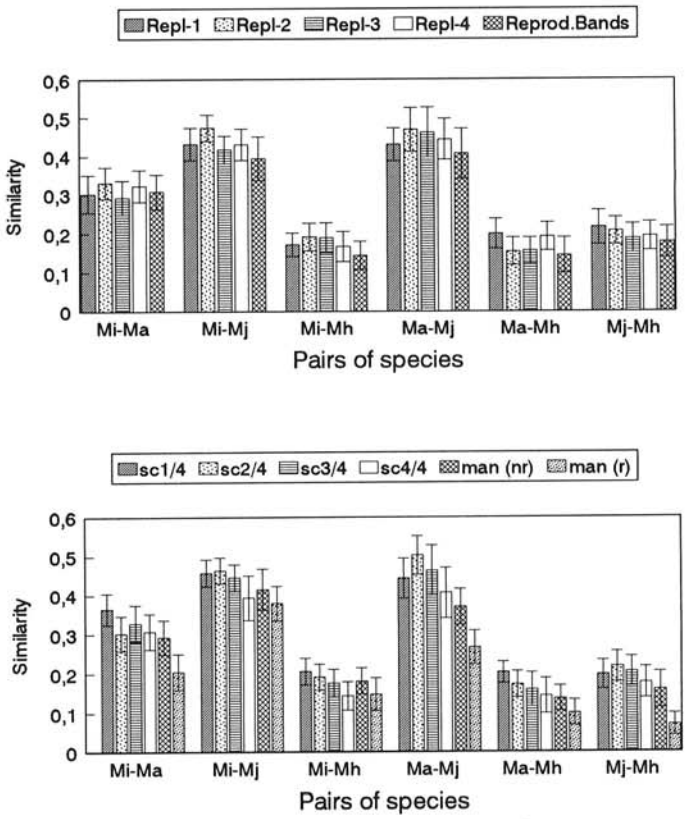

Fig. 3. Similarity values of the six pairs of species of *Meloidogyne* obtained in four different replicates. Mi = *M. incognita*; Ma = *M. arenaria*; Mj = *M. javanica*; Mh = *M. hapla*. Top: Repl-1, Repl-2, Repl-3 and Repl-4, similarity values obtained in each of four replicates; Reprod.Bands, value obtained from the reproducible bands of the four replicates. Bottom: sc1/4, sc2/4, sc3/4, and sc4/4 are similarity values calculated from the scanned bands that appear one, two, three, and four times in the four replicates; man (nr) and man (r) are similarity values obtained from the visual scoring of bands with a nonrestrictive and a restrictive criterion, respectively, and reproduced four times.

TABLE 4. Comparison of genetic distance (GD) and genetic similarity (GS) measures of four *Meloidogyne* species obtained by different authors

Species pairs[a]		Enzyme[b]	g DNA[c]	mtDNA[d]	RAPD[e]	RAPD[f]
Mi-Ma	GD:	33-40	3.49 ± 0.42	0.011-0.025	0.0402	57.73
	GS:	0.53-0.57	0.55	0.66-0.86	0.27-0.33	0.27-0.33
Mi-Mj	GD:	31-34	3.77 ± 0.45	0.008-0.021	0.0283	45.32
	GS:	0.56-0.58	0.52	0.760-0.890	0.36-0.41	0.36-0.41
Mi-Mh	GD:	78-84		0.007-0.010	0.0755	72.01
	GS:	0.13-0.15		0.87-0.91	0.11-0.13	0.11-0.13
Ma-Mj	GD:	17-28	2.12 ± 0.35	0.022-0.029	0.0124	22.85
	GS:	0.64-0.70	0.69	0.69-0.82	0.56-0.62	0.56-0.62
Ma-Mh	GD:	95-105		0.013-0.028	0.0872	71.92
	GS:	0.09-0.10		0.74-0.85	0.07-0.10	0.07-0.10
Mj-Mh	GD:	88-90		0.008-0.018	0.0815	68.56
	GS:	0.11		0.79-0.90	0.10-0.11	0.10-0.11

[a] Mi = *M. incognita*; Ma = *M. arenaria*; Mj = *M. javanica*; Mh = *M. hapla*.
[b] Data taken from Esbenshade and Triantaphyllou (9). GD is calculated as number of isozyme band differences between each population pair, and GS as Jaccard's coefficient of similarity.
[c] Data taken from Xue et al. (28). GD is calculated by distance d and GS by the estimator *F* of proportion of shared fragments, both from Nei and Li (18).
[d] Data taken from Powers and Sandall (21). GD is calculated as nucleotide sequence divergence *p* and GS as proportion of shared fragments *F*, both from Nei and Li (18).
[e] Data obtained in this work. GD is the net divergence between species with the distances calculated by the model of Clark and Lanigan (5) and GS is the proportion of shared bands *F*, from Nei and Li (18).
[f] Data obtained in this work. GD is the net divergence between species with the distances calculated by analysis of molecular variance (10) and GS is the proportion of shared bands *F*, from Nei and Li (18).

ity value seems to increase slightly as more bands are scored when the pairs of species compared are less similar. The similarity variance of the more similar species clearly decreases when the number of scored bands increases and the pattern is consistent with the intuitive notion that the error estimation decreases when the number of bands increases. It is interesting to note that in the worst case the variance is almost constant from 200 bands on. Considering that one reaction with each primer can produce about eight to 10 bands, an experiment carried out with about 20 to 25 primers may be enough to obtain an adequate estimation of the genetic similarity.

The results of the reproducibility experiment suggest that the error induced by scoring some poorly reproducible bands is less than the error when F is estimated. This is so even when a band that appears once in four experiments is scored. In consequence, one replicate of the experiment seems to be enough to obtain an adequate set of data. This contradicts the intuitive notion that several replicates of each experiment are needed, but it is important to notice that, in the present work, the four replicates were made with the same DNA, thermocycler, and reagents. As a consequence, several important sources of variation were eliminated. However, in most of the studies of variability, the usual strategy is to try to minimize the sources of experimental variation. In consequence, one has to deal only with the variation inherent in the thermocycler functioning and the reaction setup, a problem addressed in the present experiment. Another result of the reproducibility experiment is to show that the visual scoring of the bands can introduce an important source of error. According to the degree of restrictiveness of the scoring, which is a subjective variable, different estimations of F can be obtained with the same set of data. On the other hand, the visual scoring produces results similar to those of automated scoring when a similar degree of restrictiveness (small in this case) is used with both methods.

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