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4840

Molecular Systematics of the Living Rhinoceros

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Classification of the living species of rhinoceros has been somewhat controversial. Traditionally, the two-horned rhinoceros, which includes the African (*Diceros* and *Ceratotherium*) and the Asian (*Dicerorhinus*) forms, has been included in one group separate from the one-horned rhinoceros (*Rhinoceros*). However, recently some authors have regarded the Asian species as a group separate from the African species, irrespective of the number of horns. Furthermore, others have split the living rhinoceros into three unrelated groups that include the African two-horned species in one group, the Asian two-horned rhinoceros in another group, and the Asian one-horned rhinoceros in a third group. We investigated the systematic relationships of the living rhinoceros using high-resolution restriction site mapping of the ribosomal genes of the mitochondrial DNA, and our results support the traditional subdivision of the living rhinoceros based on the number of horns. Few groups of mammals are more critically endangered than the rhinoceros, and the data obtained in this work should provide information relevant to their conservation. © 1994 Academic Press, Inc.

INTRODUCTION

The five extant species of rhinoceros are subdivided into four genera in one family, the Rhinocerotidae. These animals are the descendants of a once larger group of Tertiary rhinoceros that was one of the most successful radiations of mammals, spanning over 40 million years (Prothero *et al.*, 1986). Within this large mammalian radiation, most authors agree that the living forms should all be included in either a single subfamily (Groves, 1983) or a single tribe (Prothero *et al.*, 1986), suggesting a close relationship among the living rhinoceros relative to most of the Tertiary forms. Although numerous Old World forms from the Late Oligocene and Early Miocene have also been recognized as members of the group containing the living rhinoceros, today only four genera survive: *Rhinoceros* (Indian and Javan rhinoceros) and *Dicerorhinus* (Sumatran rhinoceros) in Asia, and *Diceros* (black rhinoceros) and *Ceratotherium* (white rhinoceros) in Africa. However, because of human population expansion, habitat fragmentation, and more recently, poaching for commercially valuable rhinoceros horn (Penny, 1988), even these remnant species are in serious danger of extinction over their entire natural distribution.

Systematic relationships among the surviving rhinoceros genera have been somewhat controversial. Although everyone agrees that the two African genera should be considered sister taxa, the relationships between the Asian genera are not as clear. Early systematists considered the two-horned African rhinoceros and the two-horned Sumatran rhinoceros to be closely related to one another (the subfamily Dicerorhininae of Simpson, 1945) and distinct from the one-horned rhinoceros (Rhinocerotinae). Groves (1983), on the other hand, in a cladistic analysis of morphological characters, regarded the Asian forms as sister taxa (regardless of the number of horns) in the tribe Rhinocerotini, placing the African genera in the tribe Dicerotini. Recently, Prothero and Schoch (1989b), in their classification of the Perissodactyla, grouped all extant rhinoceros into the tribe Rhinocerotini, but separated them into three different subtribes, without commenting on their interrelationships (Fig. 1).

The purpose of this study was to test the different hypotheses of living rhinoceros phylogeny, using high-resolution restriction site mapping of the mitochondrial DNA ribosomal region. Specifically, we examined whether molecular data support either the "number of horns" hypothesis, which places the African genera and the Sumatran rhinoceros as sister groups, or the "geographic split" hypothesis, which places both Asian genera in one group separate from the African rhinoceros.

MATERIALS AND METHODS

For this study, a total of 50 rhinoceros specimens were included as follows (sample size in parentheses). *Diceros bicornis*: Kenya (5); Zimbabwe (10); South Africa (13). *Ceratotherium simum*: South Africa (3); Zaire (1). *Dicerorhinus sumatrensis*: Malaysia-Borneo (Sabah) (2); Indonesia-Sumatra (2). *Rhinoceros unicornis*: Nepal (9); India-Assam (5). One individual of domestic horse (*Equus caballus*) was included as an outgroup.

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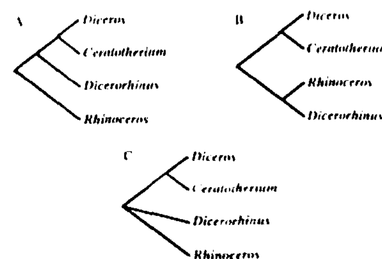


FIG. 1. Three major hypotheses on the systematic relationships of the genera of living rhinoceros. (A) Simpson (1945), (B) Groves (1983), (C) Prothero and Schoch (1989b).

It would have been preferable to include a tapir as an outgroup since these animals and the rhinoceros are considered to belong to one group (the Ceratomorpha), separated from the horse-like perissodactyls. Nevertheless, since the monophyly of the living rhinoceros with respect to the other odd-toed ungulates is not in question, using the next closely related group as an outgroup should not pose any significant problem. Moreover, a recent study of the amino acid sequence of pancreatic polypeptide in perissodactyls (Henry *et al.*, 1991) suggested a closer affinity of the tapir with the equids than with the rhinoceros.

Total genomic DNA was obtained mostly from blood samples following standard phenol/chloroform DNA extraction and ethanol precipitation (Maniatis *et al.*, 1982). A segment 1.6-kb long that includes most of the mitochondrial ribosomal region (12S, Valine tRNA, and 16S) was amplified for each specimen. The primers used were LGL284 (5'-TGGGATTAGATACCCCACTAT-3') and LGL384 (5'-TGATTATGCTACCTTTGCAC[A/G]GT-3') available from LGL Ecological Genetics, Inc. The resulting amplification products were subjected to restriction enzyme analysis with the following endonucleases: *Acc*I, *Aci*I, *Ase*I, *Bfa*I, *Bsm*AI, *Bsr*I, *Bst*NI, *Bst*UI, *Dde*I, *Dra*I, *Fok*I, HaeIII, *Hha*I, *Hin*fI, *Hpa*I, *Hph*I, *Mbo*I, *Mbo*II, *Nla*IV, RsaI, Sau96I, *Ssp*I, and *Taq*I. The resulting fragment patterns were resolved in 1.5 to 2.0% agarose gels stained with ethidium bromide, and each different haplotype was assigned an arbitrary letter score. For each haplotype, the corresponding sites were mapped onto the ribosomal segment using the partial endonuclease digestion mapping procedure (Morales *et al.*, 1993). With this method, the segment to be mapped is amplified again, but with one of the primers biotinylated (LGL284 in this paper). The product is subjected to partial digestion, run in an agarose gel, and then transferred to a nylon membrane. Those fragments with a biotin-labeled end are detected by chemiluminescence, and aside from the first band, which corresponds to the whole undigested fragment, the bands observed correspond to those fragments from the labeled primer position to the restriction site. Using this method, shared bands correspond to shared restriction sites. Fragment lengths were estimated with a known size standard (1-kb ladder from Gibco-BRL). All the haplotypes for each enzyme were run side by side to ensure that bands with the same mobility were in fact homologous and not an artifact of fragment length estimations. In this way, a binary matrix of the presence or absence of each site was constructed for all of the specimens.

Distance values (i.e., number of nucleotide substitutions per nucleotide site) between the different haplotypes were estimated according to Nei and Tajima (1981) using the computer program REAP (McElroy *et al.*, 1991). The resulting symmetric matrix was subjected to the Fitch–Margoliash least-squares tree building method (Fitch and Margoliash, 1967) with the programs FITCH and KITSCH from the computer package PHYLIP (Felsenstein, 1991).

Cladistic analyses were performed with the computer program PAUP, version 3.1.1 (Swofford, 1993), using the exhaustive search procedure. Because parallel site gains are considered to have a much lower probability than parallel site losses (Debry and Slade, 1985), the Dollo parsimony criterion was assumed for all characters. Finally, a bootstrap analysis was also performed with the branch-and-bound option through 1000 replications, and only those branch points that were represented in 50% or more of the replicates were retained for the bootstrap "consensus" tree.

RESULTS AND DISCUSSION

Among the 50 rhinoceros surveyed in this study, seven different haplotypes were identified in the 1.6-kb long segment of the mtDNA ribosomal region (Table 1). A total of 78 sites were mapped across all haplotypes (Table 2), which represents in total 22% of the sequence (about 352 nucleotides) and an average of 11.6% of the segment's nucleotides in each haplotype.

Phenetic Analysis

A matrix of estimates of nucleotide substitutions per nucleotide site (Nei and Tajima, 1981) is presented in Table 3. No intrapopulation variation was detected. Within species, a minimal amount of sequence divergence was observed between the northern and southern forms of the black rhinoceros (0.27%) and the white rhinoceros (0.3%) and between the two haplotypes found in the Sumatran rhinoceros (0.29%). No variation was found among the two populations sampled for the Indian rhinoceros. Interspecific sequence divergence varied from 2% between the black and white rhinoceros to 8% between the Sumatran and the Indian rhinoceros. Sequence divergence between the rhinoc-

TABLE 1

Haplotytpes Found in This Study

Haplotype number	Species	Procedence
1	*Diceros bicornis* (north)	Kenya (5)
2	*Diceros bicornis* (south)	South Africa (13), Zimbabwe (10)
3	*Ceratotherium simum* (south)	South Africa (3)
4	*Ceratotherium simum* (north)	Zaire (1)
5	*Dicerorhinus sumatrensis*	Malaysia: Borneo (2), Indonesia: Sumatra (1)
6	*Dicerorhinus sumatrensis*	Indonesia: Sumatra (1)
7	*Rhinoceros unicornis*	Nepal (9), India [Assam] (5)
8	*Equus caballus*	Domestic horse (outgroup)

Note. Sample size in parentheses.

eros and the domestic horse (outgroup) ranged from 15.1 to 17.1%, with an average of 15.9% sequence divergence between these two groups. The clustering methods used revealed similar topologies (e.g., Fig. 2), in which the African species (black and white rhinoceros) are most closely related to each other and the Sumatran rhinoceros is more similar to the African rhinoceros than to the Indian rhinoceros.

Parsimony Analysis

For the cladistic analysis, 60 variable characters were considered. The exhaustive search procedure of PAUP resulted in only one most parsimonious tree (Fig. 3) with a consistency index of 0.91 and a skewness index or g1 = −0.50, suggesting a strong phylogenetic signal in our data (Hillis, 1991). Species relationships in the cladistic analysis agree with the ones obtained in the phenetic analyses, in that the African rhinoceros are closest to one another, and the Sumatran rhinoceros is closer to the African forms than to the geographically more proximate Indian rhinoceros. Similarly, the bootstrap analysis supported the monophyly of two-horned rhinoceros in 57% of the replications.

TABLE 2

Binary Matrix of Site Data for the Eight Haplotypes Identified in This Study

Restriction enzyme	Map position	Haplotype 1	2	3	4	5	6	7	8
1. *Bfa*I	120	0	0	0	0	1	1	0	0
2. *Aci*I	150	1	0	0	1	1	1	1	1
3. *Nla*IV	150	1	1	1	1	1	1	1	1
4. *Taq*I	160	1	1	0	0	0	0	0	1
5. *Bfa*I	160	1	1	1	1	1	1	1	1
6. *Mbo*II	220	1	1	1	1	1	1	1	1
7. *Aci*I	240	1	1	1	1	1	1	1	1
8. *Nla*IV	250	0	0	0	0	0	0	1	1
9. *Rsa*I	250	0	0	0	0	0	0	0	1
10. *Aci*I	310	1	1	1	1	1	1	1	1
11. *Dde*I	330	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
17. *B*[illegible]I	[illegible]	[illegible]	[illegible]	[illegible]	0	1	[illegible]	1	1
18. *Hae*III	410	1	1	1	1	1	1	1	0
19. *Hinf*I	430	0	0	0	0	0	0	0	1
20. *Bst*NI	450	1	1	1	1	0	0	1	0
21. *Hha*I	450	0	0	0	0	0	0	0	1
22. *Hph*I	450	1	1	1	1	1	1	1	1
23. *Hae*III	470	1	1	1	1	1	1	1	1
24. *Bst*UI	470	0	0	0	0	0	0	0	1
25. *Rsa*I	470	1	1	1	1	1	1	0	0
26. *Rsa*I	500	0	0	0	0	0	0	1	0
27. *Dde*I	500	0	0	1	1	0	0	0	0
28. *Aci*I	500	0	0	0	0	0	0	0	1
29. *Aci*I	540	1	1	1	1	1	1	1	0
30. *Rsa*I	540	1	1	1	1	1	1	1	0
31. *Fok*I	550	0	0	0	0	0	0	0	1
32. *Hha*I	550	1	1	1	1	1	1	1	0
33. *Acc*I	550	0	0	0	0	0	0	0	1
34. *Bsm*AI	570	1	1	1	1	1	1	1	1
35. *Rsa*I	580	0	0	0	0	0	0	0	1
36. *Bsr*I	580	1	1	1	1	1	1	1	0
37. *Dde*I	630	0	0	0	0	1	1	0	0
38. *Bfa*I	640	1	1	1	1	1	1	1	1
39. *Bst*NI	670	1	1	1	1	1	1	1	0
40. *Dde*I	680	0	0	0	0	0	0	1	1
41. *Hph*I	680	0	0	0	0	0	0	0	1
42. *Bfa*I	700	1	1	0	0	1	1	0	0
43. *Hha*I	760	1	1	1	1	1	1	1	1
44. *Rsa*I	760	1	1	1	1	1	1	1	1
45. *Dra*I	770	0	0	0	0	0	0	1	0
46. *Mbo*II	770	0	0	0	0	0	0	0	1
47. *Bsr*I	790	1	1	1	1	0	0	0	0
48. *Hph*I	800	0	0	0	0	0	0	1	0
49. *Aci*I	800	1	1	1	1	1	1	1	0
50. *Bst*NI	870	1	1	1	1	1	1	1	1
51. *Dde*I	890	0	0	0	0	0	0	0	1
52. *Bfa*I	900	1	1	1	1	1	1	1	1
53. *Dra*I	960	1	1	1	1	1	1	1	1
54. *Hinf*I	970	1	1	1	1	1	1	0	1
55. *Hph*I	970	1	1	1	1	1	1	1	1
56. *Taq*I	990	1	1	0	0	0	0	0	0
57. *Mbo*II	1000	1	1	1	1	1	1	1	0
58. *Hpa*I	1020	1	1	1	1	0	0	1	0
59. *Bfa*I	1040	1	1	0	0	0	0	0	0
60. *Hae*III	1070	1	1	1	1	1	1	1	0
61. *Rsa*I	1080	1	1	1	1	1	1	1	1
62. *Hinf*I	1100	0	0	0	0	0	0	1	0
63. *Dde*I	1100	0	0	0	0	0	0	1	0
64. *Bst*NI	1110	0	0	0	0	1	1	0	1
65. *Mbo*I	1160	0	0	0	0	1	0	0	0
66. *Dra*I	1170	1	1	1	1	0	0	0	1
67. *Dde*I	1190	0	0	0	0	0	0	1	0
68. *Hpa*I	1230	1	1	1	1	1	1	1	1
69. *Mbo*I	1240	1	1	1	1	0	0	1	0
70. *Bsr*I	1240	1	1	1	1	0	0	0	0
71. *Hae*III	1290	1	1	1	1	1	1	1	0
72. *Sau*96I	1320	0	0	0	0	1	1	0	0
73. *Ssp*I	1340	[illegible]	1	1	1	1	1	0	0
74. *As*[illegible]I	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Note. Haplotype numbers [illegible] defined in Table 1. Map position corresponds to the approximate number of nucleotide pairs from the labeled primer (LGL284) to the restriction site.

TABLE 3

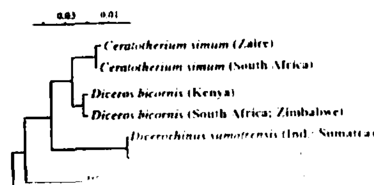
Percentage of Sequence Divergence of Haplotypes Used in This Study (See Text)

Haplotype	1	2	3	4	5	6	7	8
1	0.00							
2	0.27	0.00						
3	2.13	1.82	0.00					
4	1.78	2.13	0.30	0.00				
5	5.64	6.08	5.64	5.20	0.00			
6	5.35	5.78	5.34	4.90	0.29	0.00		
7	6.18	6.62	5.50	5.09	8.03	7.74	0.00	
8	15.29	16.02	17.11	16.25	15.85	15.55	15.14	0.00

Note. Haplotype numbers are identified in Table 1.

Intraspecific Variation

The levels of intraspecific variation observed in this study agree in general with previous molecular studies of rhinoceros. We found no variation within African rhinoceros populations, similar to the findings of Ashley *et al.* (1990) for the black rhinoceros of East and Southern Africa and of O'Ryan and Harley (1993) for black and white rhinoceros from South Africa. Our results are also consistent with the low level of intraspecific nuclear genetic allozyme variability observed in these species by Merenlender *et al.* (1989). However, we also found no variation among our samples of Indian rhinoceros, which is somewhat surprising considering that Dinerstein and McCracken (1990) found high levels of heterozygosity (9%) for the same individuals from the Royal Chitwan National Park in Nepal.



[illegible] surveyed in this study. The scale represent the estimated number of nucleotide substitutions per nucleotide site.

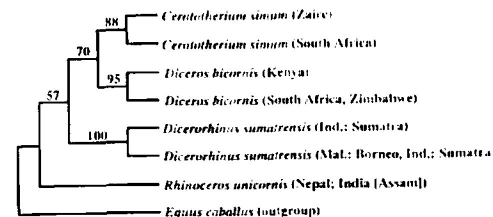


FIG. 3. Cladistic relationships of the seven haplotypes of representative forms of living rhinoceros surveyed in this study, with the Dollo parsimony criterion and the exhaustive search mode of the computer program PAUP. The numbers represent the bootstrap values after 1000 replications with the branch-and-bound option.

Considering the different modes of transmission of mitochondrial versus nuclear DNA, it is quite possible that the dramatic population bottleneck that these animals experienced at the turn of the century (Dinerstein and McCracken, 1990) had different effects on the level and distribution of variation in each genome. Furthermore, the low level of mitochondrial variation found in this study may also be a consequence of the conservative nature of the mitochondrial region surveyed (see below).

Among the major divisions within each of the African species surveyed, we found distributions of variation consistent with subspecific designations. However, within the Sumatran rhinoceros species, we found one of the haplotypes from Sumatra to be identical with the haplotype from Borneo, which is supposed to harbor a different subspecies. Our sample size, though, in this case is too small and the mtDNA region surveyed too conservative to allow for accurate inferences about intraspecific variation. This is confirmed by our recent detection of fixed differences in the mitochondrial D-loop region between haplotypes from the two different Sumatran rhinoceros subspecies (Morales, Salgaocar, and Melnick, unpublished).

Molecular Evolutionary Rate

Brown *et al.* (1979) suggest an evolutionary rate for the entire mtDNA molecule of 2% sequence divergence per million years of evolution. However, Allard and Honeycutt's (1992) review of recent studies suggests that the ribosomal 12S gene has a much lower evolutionary rate. The fossil record of the Perissodactyla is [illegible]

[illegible] ago (Prothero and Schoch, 1989a). The average percentage divergence observed between the horse and

the rhinoceros was 15.9%, which suggests an evolutionary rate of about 0.3% sequence divergence per million years. Fossil records of the African genera *Diceros* and *Ceratotherium* indicate that these genera have coexisted for about 7 million years (Merenlender *et al.*, 1989), and with a sequence divergence of 1.96%, it also suggests an evolutionary rate of 0.3% sequence divergence per million years. If we accept this two-point calibration as indicative of the "local" evolutionary rate of the mitochondrial ribosomal region within the rhinoceros, then the average divergence of 6.5% between the extant two-horned rhinoceros and the one-horned rhinoceros would indicate a cladogenic split between these two groups at about 21.7 million years ago, or early Miocene, which is consistent with the appearance of the first *Dicerorhinus* types in the fossil record from Europe (Heissig, 1989). An average divergence estimate between the Sumatran rhinoceros and the African species of 5.5% suggests a split between these two groups about 18.3 million years ago. This date is consistent with the appearance in the fossil record of the genus *Paradiceros* (Prothero and Schoch, 1989a), which is considered the ancestral form of the African branch of the two-horned rhinoceros (Heissig, 1989). Within the three variable species of rhinoceros, the divergence estimates suggest that the northern and southern populations of both the black and white rhinoceros have evolved independently for almost one million years, which gives some support to the subspecific designations. The same estimate of one million years could be given for the two haplotypes of the Sumatran rhinoceros found in this study. It is important to note here that these molecular clock estimates are approximate and may be subject to substantial error, particularly in cases of recent divergence (Melnick, 1990).

Phylogenetic Inferences

Simpson (1945) recognized two subfamilies for the living forms of the Rhinocerotidae. These included the subfamily Dicerorhininae, which allied the African two-horned rhinoceros (the black rhinoceros *Diceros* and the white rhinoceros *Ceratotherium*) with the Asian two-horned rhinoceros (the Sumatran rhinoceros *Dicerorhinus*), and the subfamily Rhinocerotinae, which included the Asian one-horned rhinoceros (the Indian and Javan species of the genus *Rhinoceros*). Pocock (1945) included the Asian forms (Sumatran, Indian, and Javan rhinoceros) in the subfamily Rhinocerinae, while including the African [illegible] white rhinoceros) in the subf[illegible] [illegible] 1983) grouped all living [illegible] [illegible], but he placed the African [illegible] Dicerotini and the Asian forms, regardless of horn number, into the tribe Rhinocerotini. He further subdivided this last group into the subtribe Rhinocerotina or the Indian and Javan rhinoceros (and some other extinct forms) and the tribe Dicerorhinina for the Sumatran rhinoceros. Recently Prothero and Schoch (1989b) considered all living forms of rhinoceros within a single tribe (Rhinocerotini) and placed the living forms in three different subtribes (Dicerorhinina, Rhinocerotina, and Dicerotina). In any case, the critical systematic question for the living forms is the placement of the Sumatran rhinoceros with the Indian rhinoceros (and its close relative, the Javan rhinoceros), with the African rhinoceros, or in a group by themselves.

Prothero and Schoch (1989b) included the genus *Dicerorhinus* with other fossil forms in the subtribe Dicerorhinina, separated from both the African and the one-horned Asian living forms. *Dicerorhinus* is also one of the oldest and most generalized forms within the rhinocerotids (Heissig, 1989), known from the early Miocene of Europe, Africa, and Asia. However, this study suggests that the present-day two-horned rhinoceros of Africa and Asia represent a different lineage from the present-day one-horned rhinoceros of Asia, and therefore the subtribe Dicerorhinina (which excludes the living African forms) should be considered a paraphyletic group. Obviously, we cannot test the phylogenetic relationships of the fossil forms in this subtribe, or any other rhinocerotid group, with current molecular methods; however, the results obtained in this study should motivate a reinterpretation of the morphological characters used to reconstruct the phylogeny of this once very successful mammalian radiation. In sum, the data obtained in this study for the living forms support the "number of horns" interpretation of Simpson (1945) in that the two-horned rhinoceros constitute a separate group (subfamily Dicerorhininae) from the one-horned rhinoceros (subfamily Rhinocerotinae) and not the "geographic split" interpretation of Pocock (1945) and Groves (1983).

Conservation Implications

Molecular genetics can contribute to the conservation of endangered wildlife in two interrelated ways: defining the distribution of genetic variation within and among conspecific populations and determining the evolutionary relationships among closely related taxa (see Avise, 1989). The data derived from both population genetics and molecular systematics can be crucial in determining both captive propagation and wildlife management strategies. Arguably, no group of mammals is more critically endangered than the rhinoceros, and the results of our analyses provide infor[illegible] [illegible] the Sumatran and Javan rhinoceros) are closely related, despite the fact that they differ in number of horns. If we assume that the Javan rhino is indeed a sister taxon of the Indian rhino (Groves, 1983), then

our results demonstrate clearly that the Southeast Asian species are not closely related and instead fall into two distinct, long-separated branches of the rhinoceros phylogenetic tree. Thus, any superficial morphological similarities between the two are likely to be the product of convergence. Although the world's population of the Javan rhinoceros is less than 100 (Sadjudin, 1992), the loss of this species would clearly mark the loss of a significant portion of what remains of the rhinocerotid radiation. Efforts to save this species, as well as the remaining populations of the Sumatran rhinoceros (IUCN/SSC Asian Rhino Specialist Group, 1989; Sabah Wildlife Department, 1993; Directorate General of Forest Protection and Nature Conservation and Indonesian Rhino Foundation, 1993), thus receive further empirical support from our results.

The mtDNA ribosomal region is an ideal segment of the molecule to examine when one wishes to reconstruct the "deep" branches of a phylogenetic tree. However, its highly conserved nature makes it difficult to use in intraspecific analyses. Nevertheless, ribosomal gene variation found among conspecific rhinoceros populations suggests that at least some of these populations have been separated for some time and thus, again, separate conservation for each major regional aggregate (e.g., the white rhinoceros of South Africa and Zaire) is warranted. Clearly, more research needs to be done to define the distribution of interpopulational, intraspecific variation in each extant rhinoceros species. Along these lines, we have begun to examine both a structural gene region (NAD3-NAD4) and the origin of replication (D-loop) in the mitochondrial genome, as well as increase our sample size for each population. Preliminary evidence from these faster evolving mitochondrial regions (Morales, Salgaocar, and Melnick, unpublished) supports the distinctiveness of different geographic populations within several of the rhinoceros species.

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Characterization of Random Amplified Polymorphic DNA (RAPD) Products from *Xanthomonas campestris* and Some Comments on the Use of RAPD Products in Phylogenetic Analysis

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As part of our research to determine phylogenetic relationships of organisms within the phytobacterial species *Xanthomonas campestris*, we have examined the use of the random amplified polymorphic DNA (RAPD) technique. The objective of this aspect of our research was to determine if a valid cladistic character analysis could be carried out by direct comparison of RAPD products separated on ethidium bromide-stained agarose gels. RAPD products were amplified from 47 *Xanthomonas campestris* DNA templates using a single oligonucleotide primer. These RAPD products were compared and variation was characterized by Southern analysis of both RAPD products and genomic DNA of the 47 bacterial strains using two cloned RAPD products as probes. Analysis of the data set revealed that the RAPD products were not necessarily homologous or independent, crucial prerequisites for characters to be analyzed in a cladistic phylogenetic analysis. It has been commonly assumed that RAPD variation occurs due to insertion/deletion events or alterations in the primer binding site. Within our data set, we demonstrate absence phenotypes arising from the apparent absence of corresponding loci and also due to the preferred synthesis of alternative RAPD products from unrelated loci. These different types of variation are a reflection of different types of genotypic variation, and direct examination of RAPD products did not allow us to distinguish by which mechanism a particular absence phenotype arose. Although this may not be important for phenetic analyses, for analyses of homologous characters using a cladistic approach it is critical. We also detected unrelated, co-migrating RAPD products and multiple related RAPD products within reaction mixtures. These could both contribute to errors in estimates of similarity, important in any phylogenetic analysis. All of these characteristics of RAPD products should be taken into consideration when RAPD products are used for phylogenetic comparisons. © 1994 Academic Press, Inc.

INTRODUCTION

The random amplified polymorphic DNA technique (RAPD; Williams *et al.*, 1990) and the closely related arbitrarily primed polymerase chain reaction (AP-PCR; Welsh and McClelland, 1990) have come into broad use (see Hadrys *et al.*, 1992; Williams *et al.*, 1993, for reviews). These techniques have been successfully applied to the production of genetic linkage maps (Williams *et al.*, 1990; Klein-Lankhorst *et al.*, 1991) and the identification of genetic markers linked to certain phenotypes (Martin *et al.*, 1991; Michelmore *et al.*, 1991; Paran *et al.*, 1991). In addition, several workers have adapted the techniques for organism identification (Caetano-Anolles *et al.*, 1991; Goodwin and Annis, 1991) and resolution of taxonomic groups (Crowhurst *et al.*, 1991; Welsh *et al.*, 1992). However, since the nature of the variation uncovered using RAPD is not well characterized or understood (Hedrick, 1992), how to best use RAPD data for some applications, such as paternity determinations (Riedy *et al.*, 1992) and phylogenetic analyses (Welsh *et al.*, 1992; Kambhampati *et al.*, 1992; Tibayrenc *et al.*, 1993), remains a subject of debate.

We are interested in phylogenetic relationships within *Xanthomonas campestris*, a phytopathogenic bacterial species that is classified below the species level into more than 125 pathovars (*pathovars*, [illegible] ants or pv.; Bradbury, 1984). We are studying [illegible] relationships in *X. campestris* pv. *poannua* (pv. nov.; Roberts *et al.*, in preparation), a bacterium that causes systemic wilt in annual bluegrass (*Poa annua* L.). Several strains of *X. campestris* pv. *poannua* are being developed in the United States for use as bioherbicides.

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