Genetic analysis of the Javan rhinoceros

in relation to its management

Final Report (Phase II)

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Introduction

This project was initiated for the development of molecular methods that would enable the collection of demographic and genetic data to guide the immediate and future demographic and genetic management, and conservation of the Javan rhinoceros (*Rhinoceros sondaicus*). The project was linked with a WWF Indonesia project titled 'Population Investigation of Javan Rhinos', also funded by the USFWS Rhinoceros and Tiger Conservation Fund.

The project commenced in September 1999. This report summarizes activities conducted in the second phase of the project (2001 – 2003). Although the funding received for the project was for one year (2001-2002), we were confronted with a number of obstacles in achieving the objectives (see below). Consequently, we have on our own initiative continued to work on the project, in order to attain the objectives.

Objectives Phase II

1. Genetic analysis of Javan rhino dung samples through extraction of DNA, PCR amplification and
    a) screening for variation at microsatellite loci to identify individuals.
    b) screening of sex chromosome markers to identify the sex of Individuals.
    c) sequencing of mitochondrial DNA to examine between-population differences.
2. Interpretation of genetic and field data to provide baseline demographic and genetic information for the design of appropriate management plans.

Accomplishment of objectives

1. a) Screening for variation at microsatellite loci to identify individuals.

As no fresh Javan rhino tissue was available, we used the Indian rhino as a surrogate species to develop the microsatellite primers (3 samples of horn and 1 sample of skin obtained from Vietnam provided mitochondrial DNA but amplification of nuclear DNA from them was poor). Using DNA extracted from blood of captive Indian rhinos, we developed 30 microsatellite primer sets. While most of these primers worked well for the Indian rhinos, their success at amplifying Javan rhino DNA was limited. The lack of Javan rhino tissue hampered optimization of primers. Initially, the primer reaction conditions were optimized for Indian rhino blood extracted DNA and then Indian rhino dung extracted DNA. Since no blood/tissue extracted DNA was available for Javan rhinos, we used 10 samples of Javan rhino dung extracted DNA with the total set of 30 primers, to identify primers that would amplify Javan rhino DNA by PCR amplification and ethidium bromide staining of agarose gels.

Through this process we identified 8 primer sets that gave reasonable amplification in Javan rhinos.

Microsatellite primers selected for Javan rhinos

IRMS10F	CTTGGCTGAGGCAATTCTCC
IRMS10R	CCCAAGGCTTTGCATACATTG
IRMS15F	CCTAGTAGTCAACGGCAAGG
IRMS15R	TGGACTCTTGCATAGGCTCC
IRMS16F	GCCAGGTCTTTGTTGGTCTC
IRMS16R	GTTCATCACGCGGTGTTAAC
IRMS 18F	ATGGTGGAAGAAGTGCAGCC
IRMS 18R	ACTTCTGTGTCTCTAGCGCC
IRMS 20F	GAATGCTGATCATTTAGTGAC
IRMS 20R	GGGTCCAGTTGAGATATCAC
IRMS 22F	GATGGCACATGAGAAAGCAC
IRMS 22R	ATCTCTGTAGGTAGAAGCCAG
IRMS 24F	GCCAGGTCTTTGTTGGTCTC
IRMS 24R	GTTCATCACGCGGTGTTAAC
IRMS 27F	AGCCTTCCAGCATCTACTTC
IRMS27R	CCTAGTAGTCAACGGCAAGG

These primers were then fluorescent labeled and further optimization done using an ABI 377 automated sequencer on acrylamide gels. Using the optimization conditions arrived at, we screened 46 samples for the 8 microsatellite loci. Although the percentage of samples that provided successful amplification for each locus was insufficient to provide individual identification, a surprisingly large number of alleles were identified at each locus. How ever, for many of the loci, amplification was poor and the alleles identified were very faint. Given the population history of the Javan rhino (a recently founded population and possibly a bottle neck of many generations), the finding of a high amount of diversity needed to be interpreted with caution. Therefore, we undertook an additional phase of optimization.

Extensive analysis and testing of samples suggested that the DNA extracted from the dung samples obtained was of low quality and quantity. Therefore, we re-optimized the extraction process and developed a method that processed larger amounts of dung.

New DNA extraction protocol

Approximately 2 g of dung samples stored in ethanol were placed in 5 ml collection tubes. Tubes were placed in an oven at 70˚ C till the sample was completely dry (approximately 2 hours) and 3 ml digestion buffer (100 mM NACl, 10 mM Tris, 25 mM EDTA and 2% SDS) and 20 µl of 20 mg ml^{-1} Proteinase K added. Samples were vortex mixed and incubated at 70˚ C for 12 hours in a mechanical shaker at 150 rpm. Samples were centrifuged for 30 minutes at 3,000 rpm in a clinical centrifuge and 2 ml of supernate transferred to clean 5 ml tubes. The DNA was

extracted by adding 2 ml of a 25:24:1 mixture of phenol/chlorofoam/iso amyl alcohol, incubating in a mechanical shaker for 30 minutes at 45˚ C and centrifugation at 3,000 rpm for 30 minutes. The aqueous supernate containing the extracted DNA was purified using a QIAGEN Gel Extraction kit and the manufacturer's protocol. As the capacity of the QIAGEN columns was only 800 µl, this involved loading each column approximately 10 times. Consequently, an ethanol precipitation step was conducted to concentrate the DNA from the PCI extraction step prior to the QIAGEN column purification. DNA was eluted in 100 µl of Tris pH 8.0 and stored at –20 ˚ C. This protocol yielded a slightly higher percentage of amplification with mitochondrial DNA and considerably higher amplification with microsatellites. However, compared to a large scale study we are conducting on the genetic analysis of Asian elephants where we have success of over 98% positive amplification with mitochondrial and microsatellite DNA the amplification from Javan rhino dung samples was consistently poor.

Further screening and testing of the microsatellite loci indicated that many of the loci previously selected were monomorphic or unreliable. Therefore by screening with a set of 10 samples, and optimization of conditions of amplification first on agarose and then on acrylamide using labelled primers, the following loci were selected for screening.

IRMS 14 F	GATGGCACATGAGAAAGCAC
IRMS 14 R	ATCTCTGTAGGTAGAAGCCAG
IRMS 12 F	GAATGCTGATCATTTAGTGAC
IRMS 12 R	GGGTCCAGTTGAGATATCAC

IRMS TET3 F	GCAGAAGCGGTTCAGACAAGC
IRMS TET3	CCTCTAGCAGAAGCCCTGTGG
IRMS 6 F	TCATTTCTTTGTTCCCCATAGCAC
IRMS 6 R	TTACAATAGCCAAGGCAT
IRMS TET4 F	TAGCCTCCAGAACTGTGAGAC
IRMS TET4	GGATCCTCTAGCAGAAGCAG
IRMS 13 F	TGGGTACACTGGGTGACTG
IRMS 13R	CAGGTAGAAGGAATTACAACCC

In order to increase the consistency of amplification, we screened over 300 samples from Ujung Kulon for mitochondrial amplification, then from the samples that amplified (approximately 50%), selected 96 samples for microsatellite screening. Since the sample from Cat Tien was small (total 30 samples) we used all samples for screening.

Results Microsatellite screening

Ujung Kulon

Sample	L	O	C	U	S
	IRMS 14	IRMS 6	IRMS 12	TET 3	TET 4
1	233	107/109	155	127	181
2	_	107/117	_	_	_
3	233	107/117	155	_	167
4	_	_	_	_	174
5	233	107/109	_	_	181
6	233	117	155	_	181
7	_	107	155	129	_
8	_	107/117	151/153	_	_
9	233	_	155	131	_
10	229	_	_	_	_
11	233	107	_	_	187
12	_	_	_	_	_
13	233	_	_	_	_
14	233	_	155	_	181
15	233	_	155	_	_
16	_	_	_	_	_
17	233	_	155	_	181
18	233	107/117	155	_	_
19	233	107/117	155	_	181
20	_	109	_	127	181
21	233	107	_	_	_
22	_	107/123	_	_	181
23	233	117	155	_	198
24	_	107	_	_	181
25	_	101/109	_	_	_
26	_	101/117	155	_	181
27	_	109/117	_	_	_
28	_	_	_	_	_
29	_	107/111	_	_	181
30	233	109	155	113	_
31	233	_	155	_	_
32	233	_	155	_	_
33	_	123	155	_	_
34	_	_	_	_	_
35	_	_	155	_	_
36	_	107	_	_	181
37	_	107	_	_	181
38	_	_	_	_	181
39	_	109	_	_	_
40	_	109	155	_	_

41	_	107	155	_	_
42	_	107/109	_	_	_
43	_	_	_	_	_
44	_	107	_	_	_
45	_	111	135	_	205
46	233	123	155	_	_
47	233	107	155	_	_
48	_	103	_	_	_
49	_	_	_	_	_
50	_	_	155	_	_
51	233	107	155	_	_
52	233	107	155	127	_
53	_	_	_	_	_
54	_	_	155	_	_
55	233	_	151	127	_
56	233	107	_	127	181
57	233	_	155	_	184/196
58	_	101	155	_	_
59	_	107	_	_	_
60	233	111	141	_	_
62	_	_	_	_	181
63	_	111/117	_	_	_
64	229	107	_	117	_
65	229/233	117/121	155	_	_
66	_	107	_	_	181
66	_	_	155	_	_
67	233	111	155	127	_
68	233	_	155	_	181
69	233	_	_	_	_
70	233	109	155	_	_
71	_	109	155	127	181/205
72	_	_	_	_	_
73	_	107	_	_	187
74	_	107/111	_	_	181
75	_	_	_	_	181
76	_	107	_	_	_
77	_	107/111	_	_	181
78	233	107	_	_	181
79	233	107	_	_	181
80	_	107	_	_	_
81	_	107/111	_	_	_
82	233	107	_	_	181
83	_	107	_	_	_
84	_	107	_	_	_
85	_	_	_	_	_
86	_	_	_	_	_

87	_	_	_	_	_
88	_	_	_	_	_
89	233	111	155	_	_
90	_	107	_	_	_
91	_	111/121	153	_	_
92	_	107	_	_	_
93	_	_	_	_	_
94	_	107	_	_	_
95	_	_	_	_	_
96	_	_	_	_	_

Summary of results for each locus

IRMS 14

No. amplified	Alleles	Genotypes
38	2	3

Homozygotes	Heterozygotes
229 - 2	229/233 - 1
233 - 33	

IRMS 6

No. amplified	Alleles	Genotypes
61	8	18

Homozygotes	Heterozygotes
101 - 1	101/109 - 1
103 - 1	101/117 - 1
107 - 26	107/109 - 3
109 - 6	107/111 - 4
111 - 4	107/117 - 5
117 - 2	107/123 - 1
121 - 0	109/117 - 1
123 - 2	111/117 - 1
	111/121 - 1
	117/121 - 1

IRMS 12

No. amplified	Alleles	Genotypes
39	5	6

Homozygotes	Heterozygotes
135 - 1	151/153 - 1
141 - 1	
151 - 1	
153 - 1	
155 - 34	

TET 3

No. amplified	Alleles	Genotypes
11	5	5

Homozygotes	Heterozygotes
113 - 1	
117 - 1	
127 - 7	
129 - 1	
131 - 1	

TET 4

No. amplified	Alleles	Genotypes
31	8	8

Homozygotes	Heterozygotes
167 - 1	181/205 - 1
174 - 1	184/196 - 1
181 - 24	
184 - 0	
196 - 0	
187 - 2	
198 - 1	
205 - 1	

Cat Tien

Sample	L	O	C	U	S
	TET 3	IRMS 6	IRMS 13	IRMS 12	TET 4
dung 2001.1	139	107	168	_	_
dung 2001.2	_	_	168	_	_
dung 2001.3	_	_	168	_	_
dung 2002.1	127	107	176	155	_
dung 2002.2	127	107	_	155	_
dung 2002.3	127	107	168/176	155	_
dung 2002.4	127	107	168/176	_	_
dung 2002.5	127	107	_	155	_
dung 2002.6	127	107	_	155	_
dung 2002.7	127	107	168	155	_
dung 2002.8	127	107	168	155	_
dung 2002.9	127/150	_	_	_	_
dung 2002.10	127/150	107	176	155	_
dung 2002.11	127	107	168	163	_
dung 2002.12	127	_	168	163	_
dung 2002.13	_	_	168/176	155	_
dung 2002.14	_	_	168	155	_
dung 2002.15	127	_	_	_	_
dung 2002.16	127	107	_	163	_
dung 2002.17	_	_	168	_	_
dung 2002.18	127	107	168	155	_
dung 2002.19	127	107	168	_	_
dung 2002.20	_	107	168	_	_
dung 2003.1	_	117	168	_	177
dung 2003.2	109/127	107	176	163	171
dung 2003.3	109/127	107	176	163	171
dung 2003.4	122/127	_	168	_	_
dung 2003.5	_	_	168	163	177
dung 2003.6	_	117	168	163	177
dung 2003.7	_	_	168	163	_

Summary of results for each locus

TET 3

No. amplified	Alleles	Genotypes
20	5	5

Homozygotes	Heterozygotes
109 - 0	109/127 - 2
122 - 0	122/127 - 1
127 - 14	127 - 150 2
139 - 0	
150 - 0	

IRMS 6

No. amplified	Alleles	Genotypes
19	2	2

Homozygotes	Heterozygotes
107 - 17	
117 - 2	

IRMS 13

No. amplified	Alleles	Genotypes
24	2	3

Homozygotes	Heterozygotes
168 - 17	168/176 - 3
176 - 4	

IRMS 12

No. amplified	Alleles	Genotypes
19	2	2

Homozygotes	Heterozygotes
155 - 11	
163 - 8	

TET 4

No. amplified	Alleles	Genotypes
5	2	2

Homozygotes	Heterozygotes
171 - 2	
177 - 2	

Microsatellite data

Overall, in both populations the analyses found low to moderate diversity in microsatellite DNA. This finding is consistent with the population history of the two populations and the mitochondrial data.

The data given above are from the most reliable loci that we have screened. However, even with these highly optimized loci, and samples that were pre-selected by screening for mitochondrial amplification, many samples still did not amplify for microsatellites. In the samples that amplified, while some of the 'alleles' – especially the ones that were scored from only a single sample - could be due to amplification artifacts, there appear to be variation at a number of loci. In addition, some of the genotypes scored as a homozygote could actually be heterozygotes that have been scored as homozygotes due to allelic dropout from the low DNA concentration (false homozygotes).

The analysis of samples from Ujung Kulon suggested that there was a fair amount of genetic diversity in the population with some allelic variation observed at each locus. Although the reliability and amplification percentage was too low to allow definitive identification of multilocus genotypes, the observed variation indicates that genotyping is possible.

Camera trapping, information from local residents, and the fact that no signs of young animals observed over the last few years in Cat Tien suggested that the surviving animals may be limited to just two females (Gert P. & VanStrien N. Pers. Com.). However, the number of genotypes observed at all loci, suggests that there are more than a couple of individuals. The number of multilocus genotypes that can be derived from the given data is 10. Given the problems of reliability from amplification artifacts, allelic dropout and non amplification, this could be somewhat of an overestimate. However, the true estimate is also unlikely to be as low as two, given that at least some of the genetic variation detected cannot be explained by amplification artifacts/allelic dropout. The sexing of samples demonstrated the existence of both males and females in the Cat Tien population (see below). In addition, we have only a limited sample from Cat Tien and given the difficulty of the terrain and habitat, it may not represent the entire population. Therefore, we strongly believe that there are likely to be at least 5-6 surviving rhinos in Cat Tien.

Constraints

The amplification of microsatellites from Javan rhino dung was in general poor. While many of the loci had low diversity and some were fixed, some of the loci appeared to have a number of alleles. However, repeatability of amplifications was low. There are two main constraints to amplification of DNA from dung

1. Low quantity and quality of DNA
2. Presence of inhibitors

In the case of the Javan rhino samples, the indications are that the main constraint is the quantity and quality of DNA. To test this, we ran experiments by mixing extracted DNA samples with a sample of good quality (tissue extracted DNA) and performing PCR (see attached publication Fernando et al. 2003), which confirmed the diagnosis that the main problem was the quality/quantity of DNA rather than inhibitors. Our studies on elephant dung samples suggest that it is possible to consistently amplify microsatellite DNA from dung samples, with a level of error that is less than 5%, and that the error is not related to the source of DNA, but rather to sporadic contamination/laboratory error (Fernando et al. 2003). Such a level of consistency can

be achieved with genotyping from DNA samples of nanogram-microgram (300 – 300,000 copies of a unique sequence) range (Navidi et al. 1992). However, when the DNA concentration is very low (picogram range), other causes such as allelic dropout and false alleles become important in addition (Navidi et al. 1992; Taberlett et al. 1999; Morin et al. 2001) and genotyping becomes unreliable. The new extraction protocol while improving the percentage of amplification, still failed to provide the level of consistency that we need to be able to screen the population for applications such as population estimation. The level of amplification and consistency from Javan rhino samples suggest that the amount of DNA extractable even with our improved protocol is closer to the pico gram range rather than the nanogram range. There is no reason to believe that this is due to some peculiarity in the Javan rhinos, as we have had high levels of success in applying this technique to a wide range of taxa including black rhinos, elephants, primates, canids etc. We have currently maximized the optimization of extraction protocol. Therefore, we are certain that the main problem lies in sample collection. To achieve further success in this endeavor, the critical step is to optimize the collection protocol. Therefore we suggest that sample collection optimization be done as the next step in this study as well as the first step in future genetic studies.

1. b) screening of sex chromosome markers to identify the sex of Individuals.

Based on the Zinc Finger Protein gene which has copies in both the X and Y chromosomes, we have developed a method of molecular sexing that is applicable to all placental mammals. (see attached publication 'Molecular Sexing Eutherian Mammals: Fernando and Melnick, 2001). This is a very sensitive test and we have had much success in applying this test to field collected samples of elephant dung.

While its broad applicability to mammals is a great advantage, it can also be a disadvantage in that it is not species specific and any contamination from a mammalian source would cause a false positive.

One of the constraints we had in sexing in the present study, was the possible human contamination of samples of Javan rhino dung.

We proposed to overcome this problem with two approaches.

1. Develop species specific primers
2. Improve collection protocols to prevent human contamination

The species specific primer development was of limited success because amplification success was too low to allow estimation of the sex ratio. Therefore, as in microsatellites, for sex identification also, improving the collection protocol is critical. Selection of the portion of dung to be collected, technique of collecting, storage and transport all can make a significant difference in the percentage of samples that amplify successfully. Therefore, this aspect has to be addressed before we can proceed with any more analysis.

Although we were not able to get amplification from a sufficient number of samples to obtain a reliable sex ratio for Ujung Kulon, using the species specific primers we were able to confirm that both males and females were present in both populations. While this is not surprising for the Ujung kulon population, as discussed above, it has management relevance for the Cat Tien population.

1. c) sequencing of mitochondrial DNA to examine between-population differences.

Samples were screened for variation in the hypervariable left domain of the mitochondrial D loop. All samples from Cat Tien Vietnam (n=20) have been of the same haplotype as was observed previously for the Vietnamese animals. The total number of samples screened for the Ujung Kulon animals now is 50 and still we have found only the two haplotypes observed previously (different from each other by one base), suggesting that these are the only two haplotypes present in that population. A phylogeny based on the left domain of the D-loop suggested that the Vietnam Javan rhinoceros were quite distinct from the Java population. This validates the respective putative subspecific status of the Javan and Vietnam populations (*Rhinoceros sondaicus sondaicus* and *Rhinoceros sondaicus anamaticus,* respectively) and underscores the need to conserve both populations. We are working on a publication detailing this work.

The Ujung Kulon population probably was founded by animals that migrated into the area subsequent to the explosion of Krakatoa in 1883, as most or all of the pre-existing population was likely to have been extirpated by the explosion. Information from the time that monitoring/study of the Ujung Kulong population was started suggests that the population has remained stagnant at around 50 animals. Founder events cause a genetic bottle neck and are especially severe on mitochondrial DNA diversity, due to the smaller effective population size of the mitochondrial genome. In addition, persistent small population size also leads to drastic reduction of diversity from rapid lineage sorting. Therefore, the finding of only 2 haplotypes different by one base in the Ujung Kulong population is consistent with the historical information on the population. Similarly, the Vietnamese population is now thought to be reduced to less than 10 animals. With such small numbers, again the finding of a single haplotype in the Cat-Loc samples is expected.

2. Interpretation of genetic and field data to provide baseline demographic and genetic information for the design of appropriate management plans.

We have not been able to achieve this objective completely, given the current state of the analysis. In order to go ahead from this point, a site visit is required, to meet with the scientists at the respective locations and work out a sampling protocol and strategy that would provide reliable data necessary for management planning.

In view of the ditinctiveness of the Ujung Kulon population and the Cat Tien population in terms of mitochondrial DNA they could be considered two sub species. The independent evolutionary trajectory of the two populations makes them two separate Evolutionarily Significant Units (ESUs). Therefore, as far as possible, they need to be managed as two separate populations. The genetic divergence observed suggests that interbreeding between animals from the two populations may have limited success. On the other hand, if the Vietnam population is found to have zero probability of survival (for example if all remaining individuals are one sex) then it would over ride subspecific considerations and the only chance of attempting to preserve at least some of the genetic diversity in Cat Tien rhinos would be to interbreed them with Ujung Kulon animals.