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DNA Fingerprinting applied to parrot captive breeding programs

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RESUMO. **"DNA fingerprinting" (identificação individual pelo DNA) aplicada a programas de reprodução em cativeiro de psitacídeos**. A reprodução em cativeiro de papagaios do gênero *Amazona* é um evento raro no Brasil. Um dos fatores desta falta de sucesso poderia estar ligado ao endocruzamento. Nós estudamos este problema utilizando as sondas de minissatélite multilocos 33.6 e 33.15 e concluimos que a população cativa estudada ainda possui variabilidade genética e, portanto, outros fatores podem estar afetando a baixa taxa de reprodução. Nós também pudemos confirmar a filiação de dois filhotes de *A. brasiliensis*, três filhotes de *A. ocrocephala xantholaema* e seis filhotes de *A. aestiva* nascidos em cativeiro. Estes são os primeiros nascimentos ocorridos nestas espécies que foram documentados com o método de identificação individual pelo DNA.
PALAVRAS-CHAVE: *Amazona*, confirmação de filiação, papagaio, Psittacidae, reprodução em cativeiro, variabilidade genética.

ABSTRACT. Captive breeding of *Amazona* parrots is a rare event in Brazil. One of the possible reasons is that these birds may be inbred. We evaluated this problem using the human minisatellite multilocus probes 33.6 and 33.15 and concluded that this captive population still retains genetic variability. Other factors must be influencing their low rate of reproduction. We also confirmed the parentage of two chicks of the endangered *A. brasiliensis*, three chicks of *A. ocrocephala xantholaema* and six chicks of *A. aestiva*, born in captivity. This is the first time that DNA fingerprinting has been applied to document the birth of chicks of these species in Brazil.
KEY WORDS: *Amazona*, captive breeding, genetic variability, parentage assignment, parrot, Psittacidae.

Twenty nine species of the genus *Amazona* occur in the American continent from Mexico to Paraguay (Forshaw 1989). In Brazil, there are 11 species (Sick 1993), some of which have a broad distribution, as *Amazona aestiva*, while some inhabit restricted areas, as is the case of *Amazona brasiliensis*.

Recently, two taxonomic modifications have been proposed: a new species, *Amazona kawalli*, was characterized (Grantsau and Camargo 1989, Collar and Pittman 1996), and it has been concluded that *Amazona xanthops* should be excluded from the genus, based on karyotype, morphology and mitochondrial DNA differences (Valentine 1990, Birt *et al.* 1992, Duarte and Caparroz 1995) and its previous scientific name should be adopted (*Salvatoria xanthops*; Duarte and Caparroz 1995).

Twelve American amazons are threatened (Collar *et al.* 1992) and four Brazilian species (*A. brasiliensis*, *A. pretrei*, *A. rhodocorytha* and *A. vinacea*) are listed in Appendix I of CITES (Convention for the International Trade of Endangered Species). Habitat destruction, which affects nest-sites and food availability and which fragments populations, and illegal trading, are the most threatening factors causing the decrease in population sizes. Small populations can lose their genetic variability due to genetic drift. Also consanguineous matings frequently result in low fertility, resulting in extinction of local populations (Gilpin and Soulé 1986, Lacy 1987, Lacy *et al.* 1989).

Preservation programs *in situ* and captive reproduction are recommended by conservation specialists. Captive breeding in a safe environment should be established before the population is at risk. Although some parrots reproduce easily in captivity, amazons in general are difficult to breed. Even with a high input of effort and money, the rate of reproduction in captivity is usually low (Derrickson and Snyder 1992). One possible reason for the failure of these programs could be the low genetic variability and high consanguinity of the remaining populations.

With the exception of some Zoological Gardens, such as the Sorocaba Zoo, there is no official captive breeding program of parrots in Brazil. The best results are obtained by some private aviculturists, but they do not have the credibility of official institutions which have no means of discriminating between true breeders and those who use their activities to obscure pouching.

DNA techniques can be used to estimate genetic variability of wild and captive populations, as well as to determine parentage, confirm successful breeding and

prove illegal trading (Mathé *et al.* 1993, Ruth and Fain 1993). They are also important for monitoring genetic variability and to prevent consanguineous matings in captive populations. DNA fingerprinting, using the human minisatellite multilocus probes, was applied in captive breeding of amazons (Brock and White 1992). This technique is also useful to sex parrots of the genus *Aratinga* (Miyaki *et al.* 1992, 1995), *Ara* (Miyaki *et al.* 1993) and *Anodorhynchus* (Miyaki *et al.* 1997). The sex determination of parrots is essential for the success of captive breeding programs since there is no sexual dimorphism in many species.

In this work we present our results of DNA fingerprinting using human minisatellite multilocus probes 33.6 and 33.15 (Jeffreys *et al.* 1985a) in eight Brazilian species of the genus *Amazona*. We also present evidence for the success of captive breeding of *A. aestiva*, *A. brasiliensis* and *A. ochrocephala xantholaema* with the same method.

METHODS

The blood samples from presumably unrelated individuals were collected from birds belonging to aviculturists and official establishments in São Paulo, Brazil by venipuncture. Thirteen *Amazona aestiva aestiva*, four *A. aestiva xanthoperyx*, five *A. amazonica*, six *A. brasiliensis*, two *A. ochrocephala*, ten *A. pretrei*, eight *A. vinacea* and four *A. xanthops* were studied. Besides these birds, small families were available for *A. aestiva* (couple and six chicks), *A. brasiliensis* (couple and two chicks) and *A. ochrocephala* (couple and three chicks). The sex of some of the birds of each species was identified by karyotyping or laparoscopy.

The protocols applied to obtain multilocus fingerprints have been described in detail elsewhere (Bruford *et al.* 1992). Five µg of genomic DNA from each bird were digested with the restriction enzyme *Mbo*I. The fragments were separated by electrophoresis through a 30 cm long 1% horizontal agarose gel. Electrophoresis was stopped when the 2 kilobase (kb) marker had migrated to the bottom of the gel. The fractionated DNA fragments were transferred onto a nylon membrane (Hybond Nfp, Amersham) by capillary Southern blotting (Sambrook *et al.* 1989).

The human minisatellite multilocus probes 33.15 and 33.6 (Jeffreys *et al.* 1985a) were oligolabelled with [α-^{32}P]dCTP or [α-^{32}P]dATP. Pre-hybridization was undertaken by incubation in 0.263M NaPhosphate, 1 mM EDTA, 7% SDS, 1% BSA (Westneat 1990) at 65°C for four hours. One probe at a time was added to the solution and left overnight at the same temperature. The membrane was washed in 2X SSC, 0.1% SDS and in 1X SSC, 0.1% SDS at 65°C. The filter was then autoradiographed for one to ten days at -70°C using x-ray film and one or two intensifying screens.

Only the scorable bands were considered. The coefficient of band sharing (index of similarity) between individuals was calculated using the formula: $X = 2N_{AB}/(N_A + N_B)$, where N_A and N_B are the number of bands present in individuals A and B, respectively, and N_{AB} is the number of bands shared by A and B (Wetton *et al.* 1987, Bruford *et al.* 1992).

Assuming that the bands scored are independent markers, we can estimate the mean probability that all **n** bands in an individual's fingerprint are present in a second random individual conservatively as $< x^n$ (Bruford *et al.* 1992).

The probability (I) that all the chick's bands are shared with a couple by chance, is estimated as: $I = (1-(1-X)^2)^n$ (Jeffreys *et al.* 1985b), where **n** is the number of bands in common between the chick and putative parents.

Segregation analysis of the bands of six chicks of *A. aestiva* was performed as described by Miyaki *et al.* (1995).

RESULTS

The band patterns observed after the hybridization using probes 33.6 and 33.15 are characterized by a large number of bands (figure 1). Table 1 presents, for each species studied, the mean number of bands, the mean index of similarity, the mean band frequency and the expected probability of individual band pattern for unrelated birds using probes 33.6 and 33.15.

In the family of *A. brasiliensis*, all the bands present in both chicks' patterns are present in their parents; approximately 50% of the bands detected by probes 33.6 and 33.15 are shared with the mother and the other 50% are of paternal origin. The mean probabilities of this couple sharing all the bands with the chicks only by chance are 3.7×10^{-3} for probe 33.6 and 2.71×10^{-8} for probe 33.15. It was also possible to confirm the parentage in the families of *A. aestiva* and *A. ochrocephala*. All bands in the chicks detected with both minisatellite probes were also visualized in the parents; however, most of the bands detected with probe 33.6 were of paternal origin in *A. ochrocephala*.

The segregation analysis in the *A. aestiva* family showed that at least 9 maternal and 11 paternal loci were detected by probe 33.6 (figure 2).

DISCUSSION

DNA fingerprinting is being applied as a powerful tool for individual identification and paternity testing in different species of birds (Burke and Bruford 1987, Wetton *et al.* 1992). This technique can be applied to estimate consanguineous relationships between individuals and potentially can be used to monitor the genetic variability of captive birds (Brock and White 1992). Besides, it can be used to determine the sex of some parrots, including *Aratinga* spp (Miyaki *et al.* 1992, 1995), *Ara* spp (Miyaki *et al.* 1993), *Anodorhynchus* spp and *Cyanopsitta spixii* (Miyaki *et al.* 1997). It was also used to determine the sex

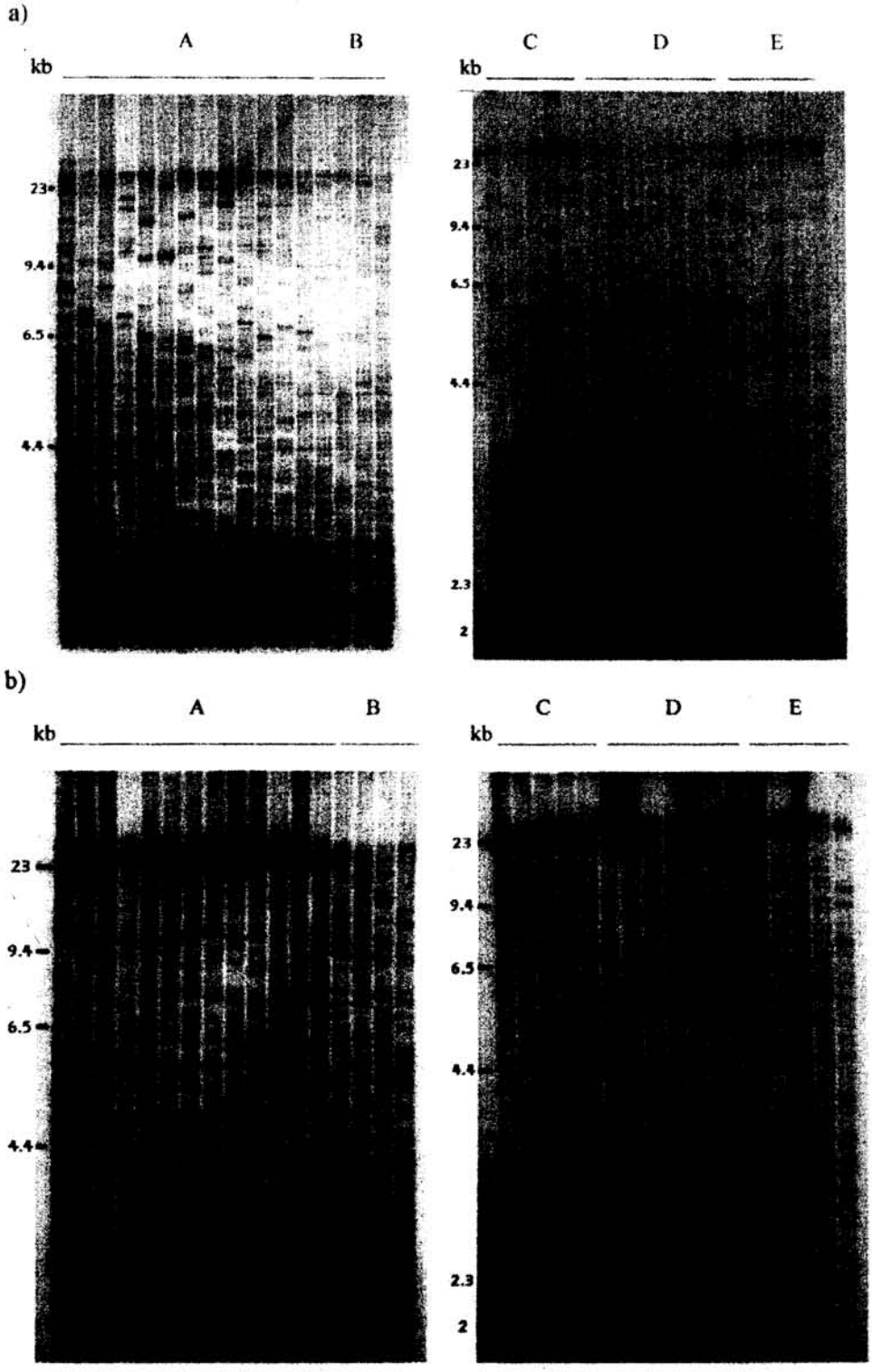

Figure 1. Multilocus DNA fingerprints of 34 individuals belonging to four *Amazona* species. a) Patterns obtained with human minisatellite multilocus probe 33.6. b) Patterns obtained with human minisatellite multilocus probe 33.15. A) *A. aestiva aestiva*, B) *A. aestiva xanthopteryx*, C) *A. amazonica*, D) *A. brasiliensis*, E) *A. ochrocephala xantholaema.*

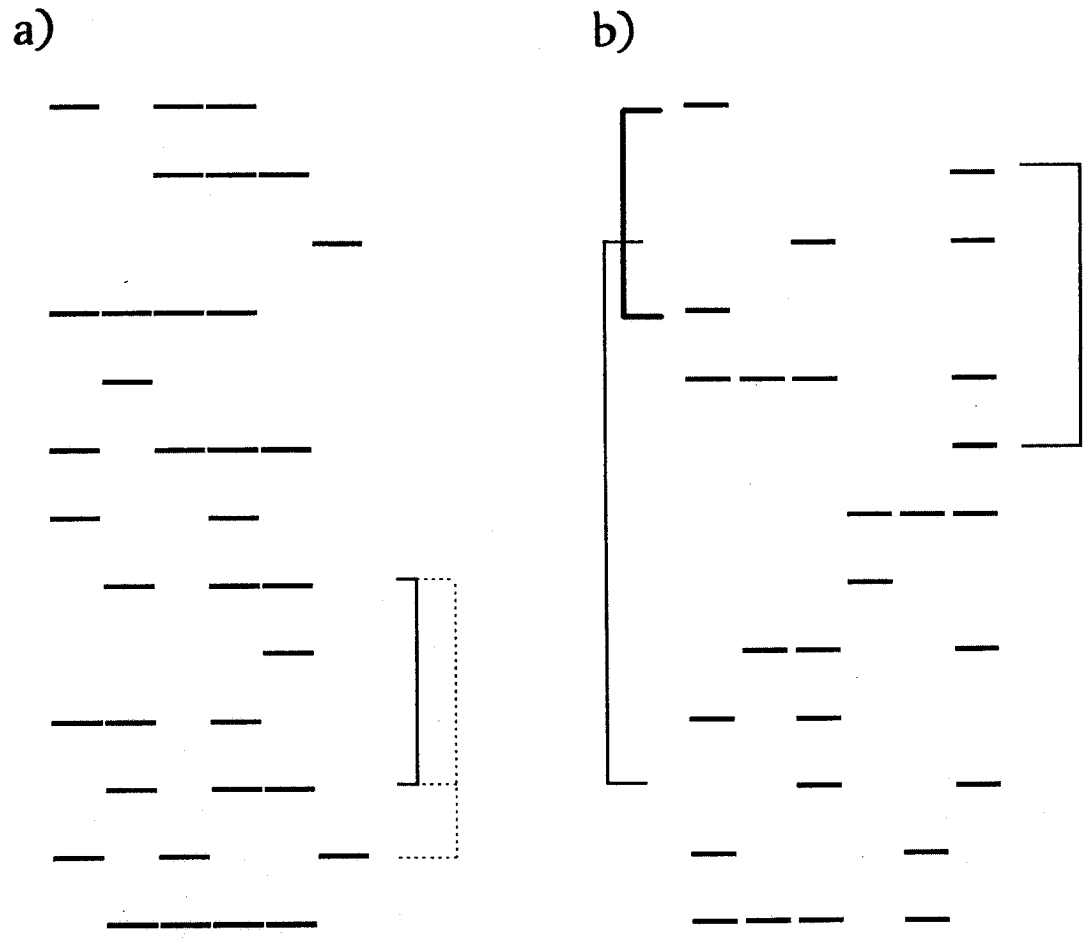

Figure 2. Segregation analysis of bands detected by minisatellite multilocus probe 33.6 in six full sibs of *Amazona aestiva*. Filled lines are co-segregating groups and dotted lines are allelic pairs. a) Paternal bands. b) Maternal bands.

ratio of chicks in a natural population of *Anodorhynchus hyacinthinus* (Miyaki *et al.* unpublished).

Interestingly, these W-chromosome linked bands could not be visualized in any of the *Amazona* species studied. This could be explained by the evolutionary history of this sex-linked minisatellite. The genera *Aratinga*, *Ara* and *Anodorhynchus* are phylogenetically closer to each other than to the genus *Amazona* and this may be related to the gain/loss of this minisatellite sequence.

The band sharing coefficients observed between unrelated individuals of the most endangered *Amazona* species (*A. brasiliensis*, *A. pretrei* and *A. vinacea*) are not higher than the ones found in other species of the same genus (table 1) and in various species of *Aratinga* parrots and other birds (Miyaki *et al.* 1995). This result indicates that the genetic variability among captive populations, measured by the DNA technique, is not depleted. These data indicate that the correct management of such populations should improve captive breeding efforts.

The similarity index (band sharing coefficient) between first degree relatives was higher, as expected, than those between unrelated birds (table 2). This coefficient can be used to advise breeders in their choice of less related pairs to mate.

In this work, we also report the successful captive breeding of *A. aestiva*, *A. brasiliensis* and *A. ochrocephala*, as demonstrated by DNA fingerprinting. All the bands present in the chicks were visualized in the parents, thus confirming the parentage of these families.

To our knowledge, successful captive breeding of *A. brasiliensis* has been claimed previously in only three occasions. The first one being of an Italian parrot breeder, G. A. della Riva, who owned an aviary in Miracatú, SP until his death in 1978 (Low 1986). N. Kawall (pers. obs.),

Table 1. Results of hybridization with human minisatellite multilocus probes 33.6 and 33.15 in unrelated individuals of *Amazona* species.

Probe	Species	N	n±sd	x±sd	x^n	q
33.6	*A. aestiva aestiva*	13	36.85±4.58	0.25±0.11	$6.5x10^{-23}$	0.134
	A. aestiva xanthopteryx	4	37.75±0.96	0.38±0.07	$1.4x10^{-19}$	0.212
	A. amazonica	5	27.8±7.3	0.22±0.09	$5.2x10^{-19}$	0.117
	A. brasiliensis	7	26.4±5.6	0.30±0.08	$1.6x10^{-14}$	0.163
	A. ochrocephala xantholaema	2	28.2±5.8	0.45	$1.7x10^{-10}$	0.258
	A. vinacea	8	20.9±4.6	0.14±0.07	$1.4x10^{-18}$	0.073
	A. xanthops	4	29.0±2.8	0.13±0.04	$2x10^{-26}$	0.067
33.15	*A. aestiva aestiva*	13	38.25±6.47	0.29±0.07	$2.7x10^{-21}$	0.157
	A. aestiva xanthopteryx	4	37.75±2.63	0.26±0.05	$8.2x10^{-23}$	0.140
	A. amazonica	5	45±7	0.28±0.09	$1.3x10^{-25}$	0.151
	A. brasiliensis	7	45.43±3.73	0.23±0.06	$1x10^{-29}$	0.122
	A. ochrocephala xantholaema	2	47.5±2.1	0.13	$2.3x10^{-39}$	0.067
	A. pretrei	10	26,00±3,46	0,12±0,07	$4,7x10^{-25}$	0,060
	A. vinacea	8	23.9±4.3	0.15±0.07	$2x10^{-20}$	0.078
	A. xanthops	4	27.5±3.5	0.15±0.06	$2.2x10^{-23}$	0.078

N - number of individuals; n - mean number of bands; sd - standard deviation; x - mean band sharing coefficient; x^n - probability for two unrelated individuals to show the same band pattern; q - mean band frequency (Jeffreys *et al.* 1985a).

Table 2. Mean number of bands of maternal and paternal origin, mean band sharing coefficient between parents and offspring and between full siblings of *Amazona* species.

Probe	Species	x_{p-o}±sd	n_f±sd	n_m±sd	x_s±sd	n_s±sd
33.6	*A. brasiliensis*	0.54±0.06	6 (54 %)	5 (46 %)	0.58	10
	A. ochrocephala	0.46±0.15	11.67±3.78 (70 %)	5±1 (30 %)	0.75±0.05	21±3
33.15	*A. brasiliensis*	0.49±0.02	20 (51 %)	19 (49 %)	-	-
	A. ochrocephala	0.46±0.10	17±3 (48 %)	18.67±6.35 (52 %)	0.53±0.06	42±5

x_{p-o} - mean band sharing coefficient between parents and offspring; sd - standard deviation; n_f - mean number of bands of paternal origin; n_m - mean number of bands of maternal origin; x_s - mean band sharing coefficient between full siblings; n_s - mean number of bands in common between full siblings

a reputed Brazilian parrot breeder, visited this aviary on many occasions and stated that he saw several hybrids between an *A. brasiliensis* male and various *A. aestiva* females, but he never saw evidence of or heard about a successful intraspecific mating of *A. brasiliensis* in della Riva's aviary. Diefenbach and Goldhammer (1986) stated that della Riva obtained 15 hybrid birds by mating *A. aestiva* males with *A. brasiliensis* females. N. Kawall himself successfully bred *A. brasiliensis* in his aviary in only two occasions; one chick was produced in 1980 (Diefenbach and Goldhammer 1986) and another chick from a different pair, in 1988. Because of the low reproductive success and possible behavioural peculiarities of the mating behaviour of this species in captivity, we describe here the special circumstances of successful breeding. The original couple of *A. brasiliensis* we studied belonged to N. Kawall and was previously sexed by karyotype analysis. A peculiarity in the behaviour of the male was observed constantly: whenever it was in visual contact with *A. aestiva* females, it started to attack his own female and the couple had to be separated. Because of the arrangement of the cages in this aviary, it was difficult to place the birds in such a way as to prevent eventual visualization of *A. aestiva* females. Therefore the pair was removed to the aviary of another breeder, L. Maluf, where it was better isolated from other birds. During the next breeding season, following this transference, the couple mated and two chicks were born in December 1992; one of them died within the first days, but was not removed from the nest to avoid disturbance of the pair.

We studied this pair, their surviving chick and another chick born in 1994. DNA samples from other four unrelated *A. brasiliensis* individuals belonging to other aviaries, were also hybridized with minisatellite multilocus probes

33.6 and 33.15. Band segregation data detected in fingerprints are not available for *A. brasiliensis*, since this is the first birth documented by DNA fingerprinting and big families are not available. These segregation data are important for estimating the number of loci scored for paternity assignment and other forensic use. Brock and White (1991) have reported that in *Amazona ventralis* there are only 6 independent loci detected by probe 33.15. Miyaki *et al.* (1995) detected 14 unlinked loci in *Aratinga aurea* using the same probe. In this report, at least 9 independent loci were detected in *A. aestiva*. Assuming that in *A. brasiliensis* the number of segregating loci lies between 6 and 9 independent loci, the probability that this couple of birds will share all the bands with the chick by chance, lies between 7.3×10^{-4} and 2×10^{-5}. Thus, even without precise estimation of the number of unlinked loci, it can be possible to establish the paternity of the chicks studied. Hopefully, future matings of this or another couple will allow a better estimation of the number of loci detected

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The applications of multilocus fingerprint analysis in parrots are multiple: it can be used to determine the sex of some species, determine the paternity of chicks born in captivity, for individual identification and to provide data for the reproductive management of captive populations.

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