



## Research Article

# The Isolation, Cloning, Characterization and Conservation among the Psittacidae of a Sex-Specific, Tandemly Repeated DNA Component from the African Grey Parrot (*Psittacus erithacus*)

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### Abstract

Digestion of DNA of the female African grey parrot (*Psittacus erithacus*) with the restriction endonuclease *Msp*I revealed the presence of a tandemly repeated, female-specific component with a repeat monomer length of approx. 628 nts and a repeat copynumber of approx.  $1.3 \times 10^4$ . The nucleotide sequence of a cloned 628 bp repeat monomer, is characterized by short oligo dA2-6 segments which occur with an average periodicity of 10.5 bp. The sequence shows therefore some similarity with the W-specific repeat of the chicken, which is characterized by alternating dT3-5 and dA3-5 oligonucleotides occurring each

with a periodicity of 21 bp. Similar to the chicken sequence the psittacine monomers show a strong reduction in electrophoretic mobility in polyacrylamide gels. The component occurs widespread in females of many members of different subfamilies of the Psittacidae. Minor amounts of cross hybridizing material were found in males of most species tested. Only in a few species, including the Eclectus parrot (*Eclectus roratus*) and the Bourke's parakeet (*Neopsephotus bourkii*), DNA of males contained, in addition to an abundant female specific component, significant amounts of non-sex-specific *PAR*<sub>sex-1</sub>

like material, whereas DNA of male and female keas (*Nestor notabilis*) contains almost nothing.

**Keywords:** Curved; Repetitive; Sex-specific DNA; W chromosome; Psittaciformes: Sex determination

### Abbreviations

AMPPD=adamantine-methoxy-phosphoryloxy-phenyl-dioxetane; cpm=counts per minute; PVDF=polyvinylidene difluoride; SSC=0.15 M NaCl; 0.015 M sodium citrate pH 7.2; TE=0.01 M tris-HCl buffer pH 8.0; 0.001 M EDTA; TPE=0.08M Tris-phosphate; 0.008M EDTA pH 8

### 1. Introduction

Sex-specific, repeated DNA components have been identified in many higher animals including snakes, mammals and birds. Sex-specific repetitive DNA components can amount to between 0.3 and 3% of the total genomic DNA and can comprise a substantial part of the chicken female specific W chromosomal DNA [1]. The function, if any, of such sequences is unknown, as is their evolutionary origin.

In mammals, sex-specific, repeated DNA components are usually conserved as sex-specific elements only between relatively closely related species [2]. Related sequences may occur sex-nonspecifically elsewhere in the genome. The female-specific W-chromosome of virtually all colubrid species of snakes [3] contains long tandemly repeated GATA or GACA sequences but GATA or GACA repeats of shorter length occur again non-sex-specifically elsewhere in the snake genome. In birds, some repeated sex-specific sequence elements similar to a highly female-specific repeated DNA component of the chicken (*Gallus*

*gallus*) [1] are also found in DNA of the female Japanese pheasant (*Phasianus colchicus*) and turkeys (*Meleagris gallopavo*), but males of these latter species contain also a fairly large amount of material with a similar sequence [4]. Other repeated sequences resembling those found in gallinaceous birds have been found in more distantly related avian families such as owls (Strigiformes), cranes (Gruiformes) and Falconiformes [5]. In contrast again, a female specific repeated DNA component of the lesser black-backed gull occurs as a sex-specific component in the closely related herring gull (*Larus argentatus*) but in other birds outside the order Charadriiformes related sequences (identified by cross hybridization) do not show any sex-specificity [6]. A repeated sex-specific DNA sequence in the zebrafinch (*Taeniopygia guttata*) [7] is not found in the chicken and therefore supposedly specific for the former species.

Parrots and parakeets (Psittaciformes) form a well-defined, old avian family [8-14] of considerable interest to programs in avian conservation, since many species have become rare or endangered and are hence of considerable interest to captive propagation projects. Most psittacine species are sexually monomorphic, which means that there are no obvious external signals which differentiate males from females. The sex of these birds has therefore to be determined cytologically by detection of the female-specific W chromosome in blood cells or feathers, surgically by observing the internal reproductive organs by laparoscopy or by quantitative determination of steroid hormone levels in fecal matter, blood or serum. These methods can require considerable experimental skill and can also be of considerable risk to the survival of the bird or can

only be applied only to mature birds. More recently DNA-based methods have been developed for sex determination in birds. In an earlier paper [14] we have described a general method for the identification of sex-specific repeated DNA in parrots. In the present paper we describe this method, which relies of the identification, cloning and characterization of a highly sex-specific, tandemly repeated component in DNA of the African grey parrot (*Psittacus erithacus*) in more detail and report that it occurs widespread with only a few exceptions as a highly female specific element in the DNA of many psittacine species and is suitable for the practical application in gender determination of parrots and parakeets.

## 2. Materials and Methods

### 2.1 Blood samples were obtained from the following species (common English and scientific names) used in this investigation

African grey parrot (*Psittacus erithacus*), Alexander ringneck parakeet (*Psittacula eutrapi\**), Amboina king parrot (*Allisterus amboinensis\**), aymara parakeet (*Amoropsittaca aymara*), black-masked love bird (*Agapornis personata*), blue and gold macaw (*Ara ararauna*), blue-crowned conure (*Aratinga acuticauda*), blue-fronted amazon (*Amazona aestiva*), blue-headed pionus (*Pionus menstruus*), Bourke's parrot (*Neopsephotis bourkii*), brown-eared conure (*Aratinga pertinax*), budgerigar (*Melopsittacus undulatus*), chattering lory (*Lorius garrulus*), cherry-headed conure (*Aratinga erythrogenys*), cockatiel (*Nymphicus hollandicus\**), crimson-winged parrot (*Apromiscus erythropterus\**), eastern rosella (*Platycercus eximus\**), eclectic parrot (*Ecleetus roratus\**), gang gang cockatoo (*Callocephalon fimbriatum\**), Goffins cockatoo (*Cacatua goffini*),

greater sulphur-crested cockatoo (*C. galerita*), Indian ringneck parakeet (*Psittacula krameri\**), kea (*Nestor notabilis*), Mexican double yellowhead amazon (*Amazona ochrocephala*), moluccan cockatoo (*C. moluccensis*), palm cockatoo (*Probosciger aterrimus*), pesquets parrot (*Psittichas fulgidus*), Princess of Wales parakeet (*Polytelis alexandrae\**), red-tailed black cockatoo (*Calyptorhynchus magnificus\**), rose-breasted cockatoo (*Eolophus roseicapilla*), scarlet macaw (*Ara macao*), severe macaw (*Ara severa*), sun conure (*Aratinga solstitialis*) and white-tailed black cockatoo (*C. funereus baudinii\**).

### 2.2 Sex determination

The sex of the individual specimens was determined surgically, with the exception of the dimorphic species marked \*), of which the sex was determined by appearance.

### 2.3 Isolation of DNA

DNA was isolated from blood, obtained by brachial vein puncture [15], Tris-base was used instead of Tris-buffer during phenol deproteinization. Final precipitation with two volumes of ethanol from the aqueous phase was done after the addition of ammonium acetate (final concentration 2.5 M). Alternately, DNA was also isolated with cationic detergents [16] using kits provided by Washington Biotechnology Inc. (Bethesda, MD USA) or InVitrogen (San Diego Ca USA), and a modification of the protocol recommended by the manufacturers for the isolation of mammalian DNA. In this modification, 0.1 ml avian blood, diluted with 0.1 ml 0.15 M NaCl, 0.02 M EDTA pH. 8.0 was lysed in 2 ml of a solution containing 10% dodecyltrimethyl-

ammonium bromide, 1.5 M NaCl, 100 mM Tris-HCl buffer pH 8.8 and 60 mM EDTA, heated for 2 min at 68°C, cooled and shaken with 2 ml of chloroform. After centrifugation for 5 min at 3000xg, the aqueous layer was collected, and the DNA precipitated by the addition of 4 ml of 1.0% cetyl trimethyl- ammonium bromide and further purified by dissolving in 1.2 M NaCl and precipitation with ethanol as described in the protocols. DNA was dissolved in TE (0.01 M tris-HCl buffer pH 8.0, 0.001 M EDTA).

#### **2.4 Restriction enzyme digestion, gel electrophoresis, Southern and slot blot hybridization and radioactive labelling of DNA fragments**

Restriction endonucleases were obtained from Promega (Madison WI USA) or New England Biolabs (Ipswich MA USA) and used with the buffers and protocols provided by the manufacturers. Agarose (BRL-Life Technologies Gaithersburg MD USA) gel electrophoresis [1.0 or 1.2% gels, in TPE buffer [17] containing 0.5 µg/ml ethidium bromide] was carried out in a horizontal slab gel apparatus. DNA fragments were visualized with long wave UV light. For preparative isolation of sex-specific DNA fragments a 3% Nusieve agarose gel was used according to the recommendations of the vendor (FMC Bioproducts Rockland ME USA).

Polyacrylamide electrophoresis using 5% polyacrylamide gels for the detection of curved DNA was carried out as described by Kodama et al. [18] and Saitoh et al. [4]. Southern blotting, using nylon membranes (Nytran, Schleicher and Schuell Keene NH) was carried out as described by Maniatis et al. [17]. Slot blots were prepared, using the same nylon

membranes in an apparatus manufactured also by Schleicher and Schuell. For slot blotting, 10 µg of genomic DNA in 200 µl of TE was denatured at room temperature with 200 µl of 1 M NaOH for 10 min followed by neutralization with 400 µl 1 M NaH<sub>2</sub>PO<sub>4</sub> and addition of 200 µl of 10 x SSC (SSC = 0.15 M NaCl, 0.015 M sodium citrate pH 7.2), prior to application to the membrane. 1 µg DNA was applied per slot. Filters were baked for 60 min at 80° and UV irradiated for further immobilization of the DNA. Filter prehybridization and hybridization with <sup>32</sup>P-labelled radioactive probes was carried out overnight at 60°C in the presence of heparin as a blocking agent [19]. Filters were washed 6 times with 1 x SSC (0.15 M NaCl, 0.015 M sodium citrate pH 7.0) each time for 5 min at room temperature and for 2 min with 1 x SSC containing 0.1% sodium dodecyl sulphate at 55°C. In this study DNA was labelled with <sup>32</sup>P-α-dCTP by random primer extension [20], using a kit (BRL). Autoradiograms were made on Kodak-X-omat X ray film (Thermofisher, Atlanta, Ga, USA) with or without an intensifying screen (Cronex Dupont Boston MA). Blots were also analyzed with the aid of a Betascope 603 Blot Analyzer (Betagen Waltham MA).

#### **2.5 Detection of sex-specific DNA fragments in DNA of a female African grey parrot (*P. erithacus*)**

Southern blots of restriction digests of genomic DNA were screened for the presence of sex-specific DNA fragments with a genomic sex-specific probe prepared as described before [14], consisting of 50 ng heat denatured, <sup>32</sup>P-labelled female African grey parrot DNA, preannealed to 200 µg unlabelled male African grey parrot Cot 2 DNA [21] in 0.4 ml 5 x SSC for 20 min at 65°C [14].

### 2.6 Cloning of sex-specific DNA fragments

For cloning, sex-specific DNA fragments were visually identified in an *Msp*I digest fractionated by electrophoresis in 3% Nusieve (FMC) gels after staining with ethidium bromide. Gel slices containing the sex-specific monomers of approximately 600bp (identified as described above) were excised and the DNA fragment isolated by the powered glass procedure [22] using a gene clean kit (Bio 101 LaJolla CA). Fragments were ligated into *Acc*I cut, dephosphorylated vector pGEM 3Z+, the resulting recombinant plasmid was used to transform *E. coli* 31/17 by the  $\text{CaCl}_2$  procedure [17] and the bacterial suspension plated on LB agar containing ampicillin. Clones containing sex-specific inserts were identified by colony hybridization [17] using the same sex-specific probe as described above for the identification of sex-specific DNA fragments in Southern blots and the positive bacteria grown on LB agar containing ampicillin for plasmid isolation and sequencing of the insert.

### 2.7 Nucleotide sequence determination

Nucleotide sequences were determined with an automatic sequencer (ABI Foster City CA USA), using the Sanger procedure [23] and forward and reverse primers. The sequence of one of the sex-specific monomers (*PARsex-1*) has been entered into the Genbank data base under Accession number AF295781.

### 2.8 Quantification of the sex-specific DNA component

Repeat copy number of the sex-specific component was determined as previously described for the centromeric DNA component [24, 25]. Briefly,

genomic DNA was blotted onto a nylon membrane in graded amounts and probed with a  $^{32}\text{P}$ -labelled 37 bp fragment (268'-TTAAGCCGTTTTTGTCCATTTCTGACCCATCTT TGCC-305') of AF295781, 5'-labelled with  $\gamma^{32}\text{P}$ -ATP and polynucleotide kinase. Linearized plasmid DNA containing the 628 bp monomer insert served as a standard. The number of cpm bound to the membrane was determined with a Betascope (Betagen Waltham MA USA). The genomic content of the DNA repeat was computed by linear regression using a least square procedure from the radioactivity measured in the genomic DNA and the standard.

### 2.9 Cytological localization of the *PARsex-1* repeat

Chromosome spreads were prepared from lymphocytes of *C. moluccensis* as described by Belterman and de Boer [26]. This species was used because it produced better chromosome spreads than the female African grey parrots that were available. In situ hybridization of cloned, biotin labelled *PARsex-1* monomer to metaphase chromosomes and visualization of the hybrids with streptavidine and mouse fluorescein labelled antistreptavidine was carried out as described by van der Ploeg et al. [27]. The probe was labelled with biotin-16-dUTP (BRL) by random primer extension [20]. Chromosomes were counterstained with propidium iodide. Photographs were made with a Nikon fluorescence microscope. "Slow-fade" (Molecular Probes, Eugene OR) was used to retard fading of the fluorescence.

### 2.10 Non-radioactive identification of sex-specific fragments in slotblots

For the non-radioactive identification and quantification of sex-specific DNA similar to

*PARsex-1* the 37 bp fragment described above was conjugated at its 5'-terminus [28, 29] with alkaline phosphatase by Keystone Laboratories, Camarillo, CA. Slot blots of DNA were prepared as described above. Hybridization of this probe was carried out in 50% formamide containing buffer as described by Maniatis et al. [17]. Detection of the hybridized probe was done with the chemiluminescent substrate AMPPD (adamantine-methoxy-phosphoryloxy-phenyl-dioxetane) [30, 31]. Photographs of the blots were made using Kodak-X-omat X ray film (Thermofisher, Atlanta, Ga, USA).

### 3. Results

#### 3.1 Identification of sex-specific elements in DNA

#### of the female African grey parrot

In an electrophoretic analysis of an *Msp1* digest of male and female African grey parrot DNA staining with ethidium bromide reveals a very weak component of approximately 0.5 kB in female DNA which cannot be found in DNA of the male. In order to improve the detection a Southern blot of a *Msp1* digest of DNA of the African grey parrot was probed with a small amount (50 ng) of  $^{32}\text{P}$  labelled female DNA, preannealed to a 4000 fold excess of unlabeled male African grey parrot Cot-2 DNA. Figure 1 shows clearly the presence of a component of approximately 600 bp in female DNA which cannot be detected in digests of DNA of males of this species.



**Figure 1:** Identification of female specific DNA components in a restriction digest (*Msp1*) of DNA of the African grey parrot. A Southern blot of 2.5  $\mu\text{g}$ s DNA of male and female African grey parrot, digested with *Msp1* was probed with a genomic female specific DNA probe of the African grey parrot prepared as described under "Materials and Methods".



**Figure 2:** Cloning of female specific DNA components of the African grey parrot. Material containing the female-specific component in the *Msp*I digest of the African grey parrot (Figure 1) was ligated into *Acc*I cut plasmid pGEM, the recombinant plasmid used to transform *E. coli* 31/17 and colonies carrying the female-specific *PARsex-1* component identified by colony hybridization (dark colonies).

### 3.2 Cloning and nucleotide sequence of the sex-specific DNA component (*PARsex-1*) of the African grey parrot.

For further characterization, the sex-specific *Msp*I component of approximately 600 bp was isolated from the gel, ligated into the *Acc*I site of the vector pGEM 3Z+, cloned in *E. coli* 31/17, clones containing the sex-specific DNA component were identified by colony hybridization (Figure 2) using the radiolabeled probe described in the previous section [32] and the

cloned fragments (*PARsex-1*) used for the determination of the nucleotide sequence (Figure 3, Genbank nr AF295781), copy number, genomic organization and chromosomal location of the component. Nucleotide sequence analysis of one of the cloned fragments (*PARsex-1*, Figure 3) shows that the actual length of the cloned repeat monomer is 629 nt (A=124, C=160, G=81, T=264). The fragment is AT rich (67% A + T) and is asymmetric with a pyrimidine-rich strand



>AF295781.1 *Psittacus erithacus* chromosome W repeat

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PARsex-1 5' GTTTTCGTCC ATTCCTAACC ACATTAAAGC CTATTTTTCA CCCATTTCCA
PARsex-2 5' ----- C-----C-----A-----

PARsex-1 ACCAATTTTA AGCAATTTGT GGTCAATTTCA AACACAGTTT TCACCACTTT 100
PARsex-2 -----

PARsex-1 GAACAAGCTT TAAGTCCTTT TTGGTTGCTT GTAAATGATT TTTGGAGTTT
PARsex-2 -----

PARsex-1 TCTAACCCCT TTTGAGCCAT TTTTCTGTT TCTAACCCAT TTTTCAACA 200
PARsex-2 -----

PARsex-1 GTTCTAGCTCGGTTTAAGTA GTTTTGCTT TTTTCTAACC CATTGATCCC
PARsex-2 -----

PARsex-1 ATGACTAATC AGTTTAAAGC CGTTTTTGTTC CATTCTGAC CCATCTTTGC 300
PARsex-2 -----T-----

PARsex-1 CCAGCTCTAG CTTTGTTTAA GCCGTTTTTC TCCATTTCTA ACCGTTTTCT
PARsex-2 -----

PARsex-1 AGCCCATTC TGACCTGTTT TAAGCCTATT CCTAACCCAT TTCCAACCCA 400
PARsex-2 -----A-----T-----T-----G-A-

PARsex-1 TTTTGGTCT TGTCAAATGC ATTTTCACC TCTTCTGACT CGCTGAAGA
PARsex-2 -----C---A-----C---A-----T-----

PARsex-1 CCTTTTTGCCGGTTCCTAAA GCATTTTGG CGTTTTCTAA CCCGGTTTAA 500
PARsex-2 ----- --3' 460 NT

PARsex-1 5' TCCTTTTCTT GGCTCTTTCT CACCCATTC TCACATTTCT CGTCCATTTT
PARsex-1 5' AATCCATTTT TGGTCATTTT TAAACATTT CTGCCCATGT GCAACCTGTT 600
PARsex-1 5' TCAAGCAGGT TTTGGGGATT TCTAACCG-- 628 NTS

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**Figure 3:** The nucleotide sequences of a 628 NT female specific DNA fragment (*PARsex-1*) and an additional 460 NTS long subfragment of the African grey parrot (*PARsex-2*). CG dinucleotides are underlined. Non-conserved nucleotides in a second monomer sequence (*PARsex-2*) are indicated, (-) means the same base as in *PARsex-1*.

containing 67% (T + C). The corresponding sequence of a second monomer, generated by complete digestion with *Msp1* (*PARsex2*, see Figure 6) differs in the first 460 nts by 16 nts or approximately 4% from the sequence of *PARsex-1*. Sequence analysis reveals also the presence of an internal repeat structure (consensus repeat sequence = (C,G,T)A<sup>1.00</sup>A<sup>1.00</sup>A<sup>0.75</sup>A<sup>0.40</sup>A<sup>0.05</sup>NNNNN---) in which

60 oligo dA2-6 segments of *PARsex-1* are repeated at average intervals of 628/60 or 10.5 nts (Figure 4). Such a sequence is typical of a curved DNA structure [4, 18, 33-35]. Polyacrylamide gel electrophoresis using a 5% gel in which curved DNA has an anomalous, slow rate of migration (Figure 5) [e.g. 18, 34, 36, 37], showed that this is indeed the case

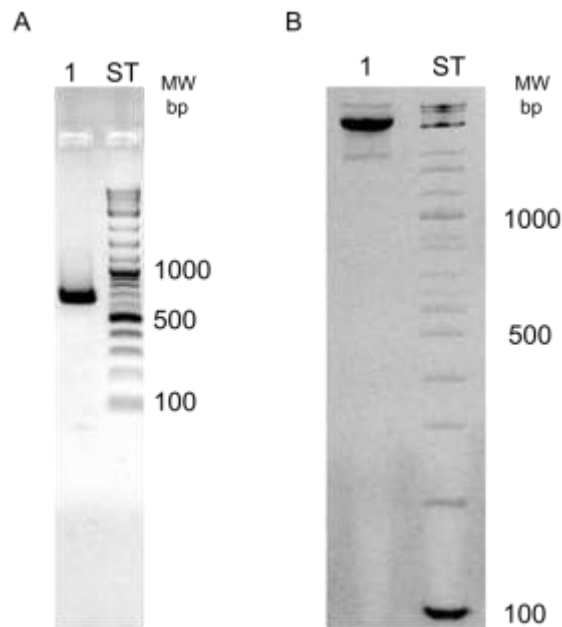


Fragment	Starting Position	Fragment Sequence	Fragment Length (nts)	Oligo dA length (nts)
1	5	GAAATCCC	8	3
2	13	CAAAAACCTGCTT	12	4
3	25	GAAACAGGTTGCACATGGCA	21	3
4	46	GAAATGGTTTA	11	3
5	57	GAAATGAC	8	3
6	65	CAAAAATGGAT	11	5
7	76	TAAAATGGACGA	12	4
8	88	GAAATGTGA	9	3
9	97	GAAATGGGTGA	11	3
10	108	GAAAGAGC	8	3
11	116	CAA	3	2
12	119	GAAAAGGAT	9	4
13	128	TAAACCGGTTA	12	3
14	140	GAAAACGC	8	4
15	148	CAAAAATGCTTTA	13	5
16	161	GAAACCGG	8	3
17	169	CAAAAAGGTCTT	12	5
18	181	CAAGCGAGTCA	11	2
19	192	GAAGAGGT	8	2
20	200	GAAAAATGCATTGA	14	5
21	215	CAAGAC	6	2
22	221	CAAAAATGGGTTG	13	5
23	234	GAAATGGGTTAG	12	3
24	246	GAATAGCT	9	2
25	255	TAAAACAGGTCA	13	4
26	268	GAATGGGCTA	10	2
27	278	GAAACCGGTTA	11	3
28	289	GAAATGGA	8	3
29	297	GAAAAACGGCT	11	5
30	308	TAAA	4	3
31	312	CAAAGCTAGAGCTGGG	16	3
32	328	CAAAGATGGGTCA	13	3
33	341	GAAATGGA	8	3
34	349	CAAAAACGGC	11	5
35	360	TAAAAC TGATTAGTCATGGGAT	22	4
36	382	CAATGGGTTA	10	2
37	392	GAAAAAAG	8	6
38	400	CAAAAAC TACT	11	5
39	411	TAAACCGAGCTA	12	3
40	423	GAAGTGT	8	2
41	431	GAAAAAATGGGTTA	14	6
42	445	GAAACA	6	3
43	451	GAAAAAATGGCT	12	6
44	463	CAAAAAGGGTTA	12	4
45	475	GAAAACTC	8	4
46	483	CAAAAATCATTTA	13	5
47	496	CAAG	4	2
48	500	CAAC	4	2
49	504	CAAAAAGGACT	11	5
50	515	TAAAGCTTGTT	11	3
51	526	CAAAGTGGT	9	3
52	535	GAAAAC TGTTTT	13	4
53	548	GAAATGACCA	10	3
54	558	CAAATTGCT	9	3
55	567	TAAAATTGGTTG	12	4
56	579	GAAATGGGT	9	3
57	588	GAAAAATAGGCTT	13	5
58	601	TAATGTGGTTAG	12	2
59	613	GAATGGAC	8	2
60	621	GAAAACCG	8	4

**Figure 4:** Internal repeat structure of the *PARsex-1* repeating unit. Position of the nucleotide preceding an oligo dA2-6 segment of a fragment, fragment sequence, length and oligo dA2-6 segment length in the fragments are indicated. A consensus sequence was generated from the individual fragment sequences = (C,G,T)A<sup>1.00</sup>A<sup>1.00</sup>A<sup>0.75</sup>A<sup>0.40</sup>A<sup>0.16</sup>A<sup>0.05</sup> N N N.

also for the sex-specific DNA component (*PARsex-1*) in the African grey parrot, in such gels, the cloned as well as the genomic sex-specific DNA fragments migrate, similar to the sex-specific DNA of chickens

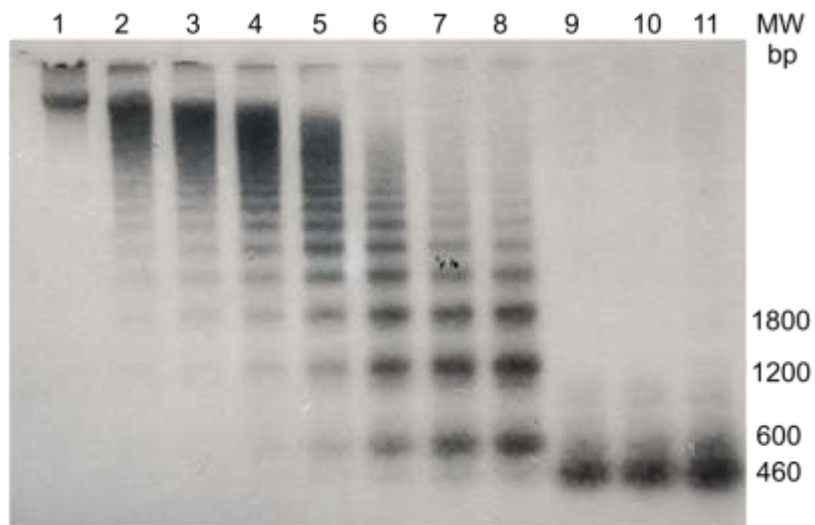
[18], as material of an apparent extremely high molecular weight. Figure 6 shows that in partial *Msp1* digests of female African grey parrot DNA,



**Figure 5:** Electrophoretic mobility of *PARsex-1* (= 1; 1 µg) in a 1,5% agarose (A) and 5 percent polyacrylamide gel (B).

the sex-specific component forms a transient ladder type pattern upon electrophoresis in an agarose gel, with an initial monomer length of approximately 630 bp, indicative of a tandemly repeated organization.

Exhaustive digestion results in the decrease of the amount this component, and the appearance of a component of approx. 460 nt, demonstrating that an



**Figure 6:** Genomic organization of *PARsex-1*-like material in DNA of the female African grey parrot. 15 µg of female African grey parrot DNA in 300 µl restriction buffer B was digested at 37°C with 4 units of the restriction endonuclease *Msp1*. 10 µl samples were removed every five minutes and added to 10ul of 2X loading buffer, heated for 10 minutes in a boiling water bath and applied to the gel (lanes 1 - 8). After 40 min an additional 10 units of the enzyme were added to insure complete digestion and additional samples were taken every ten minutes (lanes 9 - 11). The samples were fractionated by electrophoresis through a 1% agarose gel. A Southern blot was made and probed with P<sup>32</sup> labelled *PARsex1*.

additional *Msp1* site (GCCGG) at nt 460 is digested more slowly by the enzyme, most likely as a result of a difference in the sequence flanking the *Msp1* restriction site, as has been shown for other restriction enzymes [38]. Determination of the copy number shows that approximately 0.3% of the female African grey parrot DNA consists of elements with sequence similarity to the sex-specific repeat *PARsex-1*.

Assuming a diploid DNA content in parrots of 3.6 pg [39], this value corresponds to a copy number of approx.  $1.3 \times 10^4$  repeats per genome. Such experiments reveal also that the genome of the male African grey parrot contains at most 0.0045% or  $1.9 \times 10^2$  copies of material with some sequence similarity to the *PARsex-1* repeat.



**Figure 7:** In situ hybridization of biotin-labelled *PARsex-1* to metaphase chromosomes of a female *Cacatua moluccensis*. Site of hybridization is indicated by the arrow.

### 3.3 Chromosomal localization of the PARsex-1 repeat element

Figure 7 shows that PARsex-1 strongly hybridizes to one medium sized chromosome of a female *Cacatua moluccensis*. This species was chosen because it

yielded superior chromosome preparations as compared with the African grey parrot females in our collection. Some hybridization occurs with other chromosomes or with chromosomes of a male of this species (data not shown).

Subfamily and species	Female	Male
<b>Ariini:</b>		
<i>Amazona aestiva</i>	7.8 (3)	0.7 (3)
<i>Amazona ochracephala</i>	8.4 (2)	0.7 (2)
<i>Amoropsittaca aymara</i>	15.8 (3)	1.5 (2)
<i>Ara ararauna</i>	12.4 (4)	1.2 (2)
<i>Ara macao</i>	14.7 (2)	0.9 (2)
<i>Ara severa</i>	13.8 (1)	2.4 (2)
<i>Aratinga acuticauda</i>	12.4 (3)	1.6 (2)
<i>Aratinga erythrogenys</i>	15.8 (3)	2.3 (3)
<i>Aratinga pertinax</i>	14.2 (2)	2.1 (3)
<i>Aratinga solstitialis</i>	17.2 (3)	2.7 (3)
<i>Pionus menstruus</i>	12.4 (1)	0.9 (1)
<b>CACATUINI:</b>		
<i>Cacatua galerita</i>	33.7 (1)	1.1 (3)
<i>Cacatua goffini</i>	30.3 (1)	0.9 (3)
<i>Cacatua moluccensis</i>	35.0 (3)	1.3 (3)
<i>Callocephalon fimbriatum</i>	30.2 (2)	0.8 (1)
<i>Callyptorhynchus funereus</i>	38.3 (1)	0.7 (1)
<i>Callyptorhynchus magnificus</i>	35.3 (1)	0.9 (1)
<i>Eolophus roseicapella</i>	31.3 (3)	1.1 (2)
<i>Nymphicus hollandicus</i>	34.5 (4)	0.6 (2)
<i>Probosciger aterrimus</i>	12.7 (2)	0.7 (1)
<b>LORIINI:</b>		
<i>Lorius garrulus</i>	13.5 (2)	1.3 (1)
<b>NESTORINI:</b>		
<i>Nestor notabilis</i>	0.5 (1)	0.3 (1)
<b>PLATYCERCINI:</b>		
<i>Melopsittacus undulates</i>	23.5 (3)	0.7 (2)
<i>Platycercus eximus</i>	25.6 (2)	0.4 (1)
<b>PSITTACINI:</b>		
<i>Psittacus erithacus</i>	100 (3)	1.5 (2)

<b>PSITTACULINI:</b>		
<i>Agapornis personata</i>	8.3 (2)	0.3 (2)
<i>Alisterus amboinensis</i>	40.2 (2)	2.2 (1)
<i>Aprornis erythropterus</i>	16.4 (1)	1.3 (2)
<i>Eclectus roratus</i>	7.0 (2)	4.8 (4)
<i>Neospeophotis bourkii</i>	15.0 (3)	11.3 (4)
<i>Polytelis alexandrae</i>	24.3 (2)	0.5 (2)
<i>Psittacula estrapia</i>	20.6 (2)	1.3 (3)
<i>Psittacula krameri</i>	30.3 (2)	2.1 (2)
<b>PSITTRICHASINI:</b>		
<i>Psittrichas fulgidus</i>	8.5 (1)	0.3 (1)

**Table I:** Conservation of sex-specificity of *PARsex* in selected Psittaciformes. 1 ug of alkali denatured DNA of the species indicated in the Table was applied to Nytran filter paper in a slot blot apparatus and probed with P32-labelled *PARsex-1*. Values represent the average hybridization of *PARsex-1* with DNA of the species in the table, expressed as a percentage of the hybridization with DNA of the African grey parrot. Numbers in parenthesis indicate the number of individuals of each species used.

### 3.4 Conservation of *PARsex-1* among other psittacine species

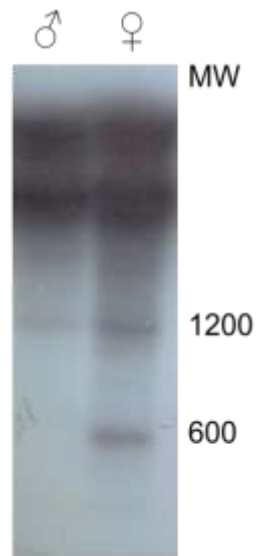
Table I shows that *PARsex-1* hybridizes in a sex-specific manner with DNA of many psittacine species, belonging to different subfamilies. Strong hybridization is observed with DNA of females of *Alisterus amboinensis*, *Psittacus erithacus*, *Polytelis alexandrae*, *Platyercus eximus* and females of the Cacatuini, with the exception of *Probosciger aterrimus*. A weaker but still highly sex-specific signal can be observed with the Ariini, Psittaculini and Loriini.

The data show also that *PARsex-1* hybridizes rather well to DNA of female as well as male *Eclectus roratus* and *Neospeophotis bourkii* but that only very little hybridization can be detected with DNA of the

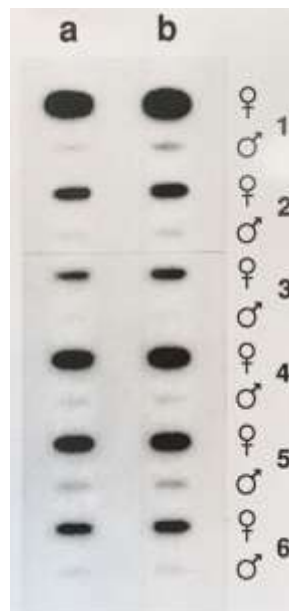
single male and female of *Nestor notabilis* which were available for analysis.

### 3.5 Sex-specific pattern of *PARsex-1* in restriction digests of *Eclectus roratus* and *Neospeophotis bourkii*

The data in Table I show that *PARsex-1* hybridizes at most only 1.6 times as strongly to DNA of females as compared to DNA of males of *Eclectus roratus* and *Neospeophotis bourkii*. The restriction pattern in Fig 8 shows that in the latter species (shown for *N. bourkii* only) distinct sex-specific as well as nonspecific *PARsex-1*-like components occur. Whereas a major component of approx. 600 bp is only observed in a *Msp1* digest of female DNA, components of a high molecular occur in the male as well as the female DNA



**Figure 8:** Pattern of *PARsex1*-like restriction fragments in DNA of male and female *Neopsephotis bourkii*. 1.5  $\mu$ g of DNA of male and female *Neopsephotis bourkii* was digested with the restriction endonuclease *MspI*, electrophoresed through an agarose gel and a Southern blot was made and probed with *PARsex-1*.



**Figure 9:** Non-radioactive detection of *PARsex-1*-like sex-specific DNA among the Psittacidae. 1  $\mu$ g (In a), 2  $\mu$ g (In b)  $\mu$ g of alkali denatured DNA of males and females of *Psittacus erithacus* (1), *Lorius garrulus* (2), *Agapornis personata* (3), *Cacatua galerita* (4), *Ara ararauna* (5) or *Amazona aestiva* (6) was applied to a Nytran filter with a slot blot apparatus and probed with alkaline phosphatase labeled *PARsex-1*.

### 3.6 Nonradioactive sex identification in different Psittacidae

The data in Figure 9 show that the use of an alkaline phosphatase labeled oligonucleotide probe derived from *PARsex-1* in combination with chemiluminescent detection allows the sensitive, non radioactive identification of the sex of several members of the Psittacidae. Again, where there is a strong signal obtained with DNA of females of these species, DNA of males is virtually devoid of this material.

## 4. Discussion

The results described in this paper demonstrate the presence of an abundant, tandemly repeated DNA component (*PARsex-1*) in the DNA of the female African grey parrot (*Psittacus erithacus*). The repeat monomer length of *PARsex-1*, cloned as an *Msp1* fragment obtained after exhaustive digestion, is approximately 460 nts. Apparently this fragment is derived from another *Msp1* fragment of approximately 628 bp as the result of a slow digestion of an additional *Msp1* site in this latter fragment. The copynumber of the repeat is approximately  $1.3 \times 10^4$ . Assuming that in parrots, as in chickens, the W chromosome represents approx. 1.0% of the diploid genome [40], this result indicates that in the African grey parrot the sex-specific repeat accounts for a substantial fraction, about 30% (or about 40% if one considers the 628 bp repeat from which the 460 bp repeat has been derived) of the DNA of the W chromosome. The size and copynumber of the repeat are similar to the values found for female specific repeated DNA components in the chicken (*Gallus gallus domesticus*) where a 0.7 kb *Xho1* fragment is repeated  $1.4 \times 10^4$  fold and the turkey (*Meleagris*

*gallopavo*) where a 0.4 kb *Pst1* fragment is repeated approx.  $10^4$  fold [1, 4]. They differ however somewhat from the values found for a sex-specific component in the DNA of the female lesser black-backed gull (*Larus fuscus*) where a much larger, 8.6 kb repeat is present in  $2.5 \times 10^4$  copies [6]. An analysis of the nucleotide sequence of *PARsex-1* revealed an internal repeat structure in which clusters of 2 to 5 adenine residues occur with an average periodicity of 10.5 bp. Such a repeat structure is considered to be typical for "curved DNA" [33, 41], and was revealed by polyacrylamide gel electrophoresis [34] where the *PARsex-1* monomer migrates as material of extremely high molecular weight (Figure 5). Why such curved DNA elements have a reduced electrophoretic mobility in polyacrylamide gels is not very well understood. Although it has long been thought that the curved nature itself and the associated altered dimensions of such DNA elements were directly responsible for the reduced electrophoretic mobility of such DNA segments upon polyacrylamide electrophoresis, currently direct interaction of the nucleotide sequence of these particular curved DNA segments with the polyacrylamide gel matrix is considered to be partly responsible [35, 42]. However, this explanation may be again only partly correct, since curved DNA elements migrate anomalously upon electrophoresis in free solution [43]. Further, additional complications arise, because the curved nature of DNA elements with phased dA tracts disappears again in solutions with high concentrations of monovalent cations [44]. The curved nature of *PARsex-1* however is not unique for a sex-specific DNA element, for the chicken, turkey and pheasant (*Phasianus colchicus*) sex-specific repeated DNA sequences have a similar bent



structure [4]. The difference between the psittacine and the chicken sequence is that whereas in the chicken the oligo dT3-5 and oligo dA3-5 sequence elements each alternate at a distance of approx. 21 bp on the same DNA strand, only oligo dA clusters (and consequently dT clusters on the opposite strand) are found in psittacine sex-specific W-DNA. Yet oligo dA and/or alternating oligo dA and oligo dT is also not a general property of all avian sex-specific DNA sequences, since the sequence of a repeated female specific 0.4 kb *Bam*HI fragment of the bobwhite quail (*Colinus virginianus*) or a sex-specific DNA sequence in the zebra finch [7] lack this feature. Another difference between the chicken XhoI repeat monomer and *PARsex-1* is the relative scarcity of CG dinucleotides in the latter [approx 11 (in *PARsex-1*) vs 34 per chicken repeat monomer]. Sequences related to the *PARsex-1* repeat element of the African grey parrot occur in the genome of females of many psittacines belonging to different subfamilies of the Psittacidae, like the Cacatuini, the Ariini, the Loriini etc. Again a limited amount of related sequence material can be detected in the genome of the males of these species under conditions of stringency applied in this investigation. Only in the Kea (*Nestor notabilis*) we could not detect a sex-specific hybridization signal with any certainty, because the hybridization values in males and females in this species were very low. Whether this observation reflects a distant phylogenetic relationship between this species and the other Psittacidae [8,-12] or whether it is the result of a loss of *PARsex-1*-like sequences from the W chromosome in this species, is unknown. More individuals of this species will have to be investigated to resolve this issue. Similar to what has been observed in some gallinaceous birds

[4] small, but variable amounts of material cross-hybridizing with *PARsex-1* occur also in the males of many psittacine species. In the African grey parrot this material amounts to at most 2.5% of that found in the female. Although minor amounts of *PARsex-1*-like material can be found as a non-sex-specific component in many psittacine species, only in *Eclectus roratus* and in the Bourke's parakeet (*Neopsephotus bourkii*) significant hybridization of *PARsex-1* occurs with male as well as female DNA. Southern blot experiments showed that *Msp*I digests of DNA of the latter species contain in addition to a female specific *PARsex-1* component of approx. 600 bp, a major amount of *PARsex-1*-like material occurs as material of very high molecular weight in DNA of males and females. A similar result was found in the case of the Eclectus parrot. Although it is probably coincidental, it may be of interest that the Eclectus parrot and the Bourke's parakeet are both among the dimorphic psittacine species. The widespread occurrence of a female specific component with sequence similarity in DNA of virtually all members of the Psittacidae suggests that this component arose early in the evolution of this avian family, whose oldest known fossils date from the lower Eocene [11]. The observation that the sex-specific repeat monomers of a member of the Psittacidae and some members of the Gallidae (and owls (AF231331.1, AF231326.1, AF291661.1, and de Kloet unpubl. data), share a similar sequence organization (A2-6 clusters at approx. 10.5 nt intervals vs. resp. alternating A3-5 or T3-5 clusters repeated at 21 nt intervals) suggests that this characteristic may be a rather general and conserved property of avian sex-specific DNA components. A strong conservation of a sex-specific, tandemly repeated DNA component in a

family of birds such as the Psittacidae) contrasts with male specific tandemly reiterated DNA components in mammals, where such sequences are often sex-specific only in a single species [45-48]. A conserved sex-specific repeated sequence has however been found among the Bovidae [49], but this sequence occurs interspersed. The unique nature of the Y chromosome, which limits recombinational exchange as a mechanism for the suppression of mutational change, has often been cited as the reason for the accumulation of species and chromosome specificity of Y linked, tandemly repeated sequences [49-51].

A similar reasoning may also explain the largely unique nature of the W-chromosome specific repeated sequences in parrots. Conservation of such sequences might only occur if such sequences are linked to positively selected genetic loci [52]. Whether the conservation of *PARsex-1* means a close linkage of these repeats to conserved elements involved in sex determination or fertility [53] in the Psittacidae, remains to be investigated.

## 5. Conclusion

The present investigation describes the isolation and partial characterization of a sex-specific repeated DNA component in parrots. The sex-specificity is widespread among many, often phylogenetically distantly related psittacine species, suggesting an ancient origin of the nucleotide sequence.

The sequence is further recognized by the occurrence of oligo dA sequence elements at regular intervals of about 10.5 bp, a feature which is associated with a bent structure.

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## Conflict of Interest

The authors declare no conflicts of interest.

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