

Chromosome Painting in Neotropical Long and Short-Tailed Parrots (Aves, Psittaciformes): Phylogeny and Proposal for a Putative Ancestral Karyotype for Tribe Arini

Ivanete de Oliveira Furo^{1,2,4}, Rafael Kretschmer³, Patrícia C.M. O'Brien⁴, Jorge Pereira⁴, Analía del Valle Garnero⁵, Ricardo J. Gunski⁵, Malcolm A. Ferguson-Smith⁴ and Edivaldo Herculano Corrêa de Oliveira^{2,6*}

1 Programa de Pós Graduação em Genética e Biologia Molecular, Universidade Federal do Pará, Belém, Pará, Brazil, **2** Laboratório de Cultura de Tecidos e Citogenética, SAMAM, Instituto Evandro Chagas, Ananindeua, Pará, Brazil, **3** Programa de Pós Graduação em Genética e Biologia Molecular, Universidade Federal do Rio Grande do Sul Porto Alegre **4** Cambridge Resource Centre for Comparative Genomics, Cambridge, United Kingdom, **5** Programa de Pós Graduação em Ciências Biológicas, Laboratório de Diversidade Genética Animal, Universidade Federal do Pampa, São Gabriel **6** Faculdade de Ciências Naturais, Instituto de Ciências Exatas e Naturais, Universidade Federal do Pará, Belém, Pará, Brazil.

* Email: ehco@ufpa.br

Abstract

Most Neotropical Psittacidae have a diploid number of $2n=70$, and a dichotomy in chromosome patterns. Long-tailed species have banded macrochromosomes, while short-tailed ones have telo/acrocentric macrochromosomes. However, the use of chromosome painting with chicken and white hawk probes has demonstrated that karyotype evolution in Psittacidae includes a high number of inter/intrachromosomal rearrangements. Hence, to determine the phylogeny of Long and Short-Tailed species, and to propose a putative ancestral karyotype for this group, we constructed homology maps of *Pyrrhura frontalis* (PFR) and *Amazona aestiva* (AAE) and compared them to other previously analyzed long-tailed species. Chromosomes were analyzed by conventional staining and fluorescent *in situ* hybridization (FISH) using whole chromosome paints of *G. gallus* (GGA) and *L. albicollis* (LAL). Conventional staining showed a karyotype with $2n=70$ in both species, with banded macrochromosomes in *Pyrrhura frontalis* and telo/acrocentric chromosomes in *Amazona aestiva*. Comparison of the results with the putative avian ancestral karyotype (PAK) showed fusions in *P. frontalis* of PAK1p/PAK4q (PFR1) and PAK6/PAK7 (PFR6) with a paracentric inversion in PFR6. However, in *A. aestiva* there was only the fusion between PAK6/7 (AAE7) with a paracentric inversion. Hybridizations with LAL probes confirmed these results. The results indicate that PFR retained a more basal karyotype than *Anodorhynchus hyacinthinus* (AHY), *Ara macao* (AMA) and *Ara chloropterus* (ACH), because these three species show the fusion PAK8/PAK9 that is not seen in PFR. Hence, we suggest that the ancestral karyotype of species with banded chromosomes have the fusions PAK1p/PAK4 and PAK6/PAK7 and, additionally, a pericentric inversion of PAK6/PAK7, while the fusion PAK8/PAK9 would have appeared in the common ancestor of *Anodorhynchus hyacinthinus*, *Ara macao* and *Ara chloropterus*. However, the species *A. aestiva* shows a characteristic plesiomorphic trait, since PAK1p/PAK4q and PAK8/9 fusions are absent. Our results base on chromosome rearrangements suggest the classification following the criterium of tail length may no reflect the real phylogenetic history of Neotropical Psittacidae.

Keywords: Parrots, Psittaciformes, Psittacidae, Chromosome painting, Phylogeny, Putative Ancestral Karyotype.

INTRODUCTION

The order Psittaciformes comprises cockatoos, parrots, macaws and parakeets, with approximately 350 species distributed between 84 genera. Currently, it is separated into two families – Cacatuidae, confined to Australia and proximity, and Psittacidae, which are found in tropical regions of the world and encompasses most species of the order [1-3].

For instance, in the Neotropics, the Psittaciformes are represented by the tribe Arini (Psittacidae, subfamily Psittacinae), with 140 species and 30 genera. Although Arini species are very diverse morphologically [1,2], they have been classified by the length of their tails, – long-tailed species and short-tailed species. This criterion was initially supported by mtDNA sequencing studies (12S rDNA, 16S rDNA and cytochrome b) in nine species of Arini [4].

The systematics of Neotropical Psittacidae are still controversial, although different types of data have been used in attempts to resolve them [5]. New phylogenetic models have been proposed, and some taxa have been relocated to other genera. For example, *Diopsittaca nobilis*, which was formerly included in the genus *Ara*, is now included in a new genus according to the evidence from morphology and mtDNA (6).

The most complete phylogenetic analysis of Arini has included 29 species of 25 different genera [5], grouped into three clades: Parrots of the genera *Bolborhynchus* and *Nannopsitta* (clade A), amazons and allies of the genera *Amazona*, *Pionus*, *Graydidascalus*, *Pionopsitta*, *Triclaria*, *Myiopsitta*, and *Brotogeris* (clade B), and macaws, conures, and relatives in the genera *Ara*, *Primolius*, *Orthopsittaca*, *Cyanopsitta*, *Nandayus*, *Aratinga*, *Guarouba*, *Diopsittaca*, *Anodorhynchus*, *Cyanoliseus*, *Rhynchopsitta*, *Enicognathus*, *Pyrrhura*, *Pionites*, *Deroptyus*, and *Forpus* (clade C). Additionally, it has been proposed that three of the genera were not monophyletic: *Aratinga*, *Pionopsitta* and *Amazona* [5,6]. The three major clades of Neotropical parrots originated about 50 Mya, coinciding with periods of higher sea level when both Antarctica and South America were fragmented with transcontinental seaways, and likely isolated the ancestors of modern Neotropical parrots in different regions in these continents. The diversification of amazons genera and allies occurred between 46 and 16 Mya suggests, however parrotlets and macaws, conures, and allies may have been isolated in Antarctica and/or the southern cone of South America, and only dispersed out of these southern regions when the climate cooled and Antarctica became ice-encrusted about 35 Mya [5].

Cytogenetic studies have shown that Neotropical Psittacidae have a constant diploid number, $2n=70$, with the exception of a few species, such as *Graydidascalus brachyurus*, *Forpus xanthopterygius* and *Brotogeris versicolurus* with $2n=64$, 82 and 84, respectively [7]. Differences in chromosomal morphology led Francisco and Galetti [8] to propose that reciprocal translocations and pericentric inversions were the main mechanisms of karyotype evolution in Arini. Recent studies based on chromosome painting in three species of two different genera - *Ara macao*, *Anodorhynchus*

hyacinthinus and *Ara chloropterus* – showed that fusions and fissions also have an important role in the karyotypical diversification of Neotropical Psittacidae [9,10]. These results confirm that despite apparent chromosomal similarity, macaws have very diverse karyotypes.

The chromosome painting with *Gallus gallus* (GGA) probes in some Neotropical Psittacidae species indicated that fissions and fusions played an important role in the karyotype evolution of Tribe Arini. Some of these associations have been found in all Psittacidae species analyzed so far - GGA1/4, GGA6/7 and GGA8/9 [10] and seem to play an important role in the karyotype evolution of Psittacidae species. However, with GGA probes it is not possible to detect intrachromosomal rearrangements, which can be useful for phylogenetic inferences [11]. The use of probes derived from species with highly derived karyotypes has been shown to be an important tool in the detection of intrachromosomal rearrangements and the correct assignment of chromosomal segments involved in rearrangements. For instance, whole chromosome painting probes from the white hawk (*Leucopternis albicollis*), with $2n = 66$ and multiple fissions involving ancestral avian syntenies, have highlighted inversions, undetected by GGA probes, as the most common rearrangements in Passeriformes and Columbiformes [12-15].

Hence, in view of the success of chromosome painting in answering some phylogenetic questions in birds [11,13,14,16], including Psittaciformes [9,10], we have analyzed the chromosome complement of two species of the tribe Arini, *Amazona aestiva* (AAE) and *Pyrrhura frontalis* (PFR), belonging to the short- and long-tailed groups, respectively, by chromosome painting using whole-chromosome paints of *G. gallus* and *L. albicollis*. The results are considered in relation to the classification based on tail length. They show that different rearrangements are present in species of the tribe Arini which help in understanding phylogenetic relationships and suggest a putative ancestral karyotype for Neotropical Psittacidae.

MATERIAL AND METHODS

Samples, Cell Culture and Chromosome Preparation

Experiments were approved by the ethics committee (CEUA- Universidade Federal do Pará) under no. 170/2013. The sample included two females of *Amazona aestiva* (AAE) and one male and female of *Pyrrhura frontalis* (PFR) (table 1). Tissue cell cultures from skin biopsies or feather pulp, performed according to Sasaki et al. [17], with modifications, were used to make chromosome preparations. For harvesting, we used a protocol including colcemid treatment (0.05% for 1h), followed by incubation with hypotonic solution (KCl 0.075 M) and fixation. For the determination of diploid number and chromosome morphology, slides were stained with Giemsa (5% in phosphate buffer pH 6.8), and analyzed with 100x objective. A minimum of 20 metaphases per individual were photographed and karyotyped using Genasis software.

Fluorescent *in situ* Hybridization

FISH experiments were performed according to de Oliveira et al. [12], using whole-chromosome probes from *Gallus gallus*, GGA (pairs 1-10) and *Leucopternis albicollis*, LAL (pairs homologous to GGA1 (LAL 3, 6, 7, 15 and 18), 2 (LAL 2, 4 and 20), 3 (LAL 9, 13, 17 and 26), 4 (LAL 1 and 16), 5 (LAL 5), 6 (LAL 3), obtained by

flow cytometry at the *Cambridge Resource Centre for Comparative Genomics* (Cambridge, UK). Probes were labeled with biotin or fluorescein by DOP-PCR. Chromosomes were counterstained with DAPI. Slides were analyzed and metaphases were photographed in a Zeiss-Axiophot microscope, using a 60x objective, and modulated by software Axiovision version 4.1. Chromosome mapping and comparisons were based on the putative ancestral avian karyotype (PAK), in which pairs PAK 1-11 correspond to GGA1-GGA3, GGA4q, GGA5-GGA9, GGA4p and GGA10 [18].

Phylogenetic analysis

In order to construct the phylogenetic tree and clarified the phylogenetic position of some species this group, as well as to propose a putative ancestral karyotype for Neotropical Psittacidae (PAK-NP) (Table 2 and 3), we considered the cytogenetic information for Psittaciformes species, taking into account the presence or absence of chromosomal characters as described by Dobigny et al., [19]. We gave special importance to chromosomal rearrangements which correspond to common features in Psittaciformes, such as associations PAK 1/4 (GGA1/4), PAK 6/7 (GGA6/7) and PAK8/9 (GGA8/9). In addition, we considered data from chromosome morphology description of 31 species belong to 14 genera of the Arini Tribe.

RESULTS

Karyotype Analysis

We found a $2n=70$ karyotype in *P. frontalis*. Pairs 1 and 2 were very similar in size; however, pair 1 is metacentric and pair 2 is acrocentric. Pair 3 was found to be heteromorphic in both individuals analyzed (Figure 1A), pairs 4 and 5 are acrocentric. The remaining autosomal pairs were telocentric. The Z was metacentric and the W submetacentric, corresponding in size to the 5th and 7th pairs, respectively.

Amazona aestiva had a $2n=70$ karyotype as described previously [20,21]. Pairs 5, 6, 7 and 8 were telocentric, pairs 1, 2 and 4 submetacentric, pair 9 metacentric, and pairs 3 and 10 acrocentric. Both Z and W chromosomes were metacentric, but the Z chromosome corresponded in size to the 4th pair, and W to the 9th pair (Figure 1B).

Comparative Chromosome Painting

Whole chromosome paints of pairs 1-10 of GGA produced 14 distinct signals in the karyotype of *P. frontalis* (PFR) (Figure 2). According to the nomenclature proposed for PAK, we have the following correspondence: PAK1 (GGA1)=PFR1q and PFR4; PAK2 (GGA2)=PFR2; PAK3 (GGA3)=PFR3; PAK4 (GGA4q)=PFR1p; PAK5 (GGA5)=PFR5q; PAK6 (GGA6)=PFR6q; PAK7 (GGA7)=PFR6q; PAK8 (GGA8)=PFR7; PAK9 (GGA9)=PFR8; PAK10 (GGA10)=PFR9 and PAK11 (GGA4p)=PFR10. Hence, we found two fusions in PFR: PFR1 (PAK1q/PAK4) and PFR6 (PAK6/PAK7). In the latter, there was also a paracentric inversion. The use of LAL probes confirmed these results (Figure 3). The resulting homology map of *P. frontalis* is shown in Figure 6A.

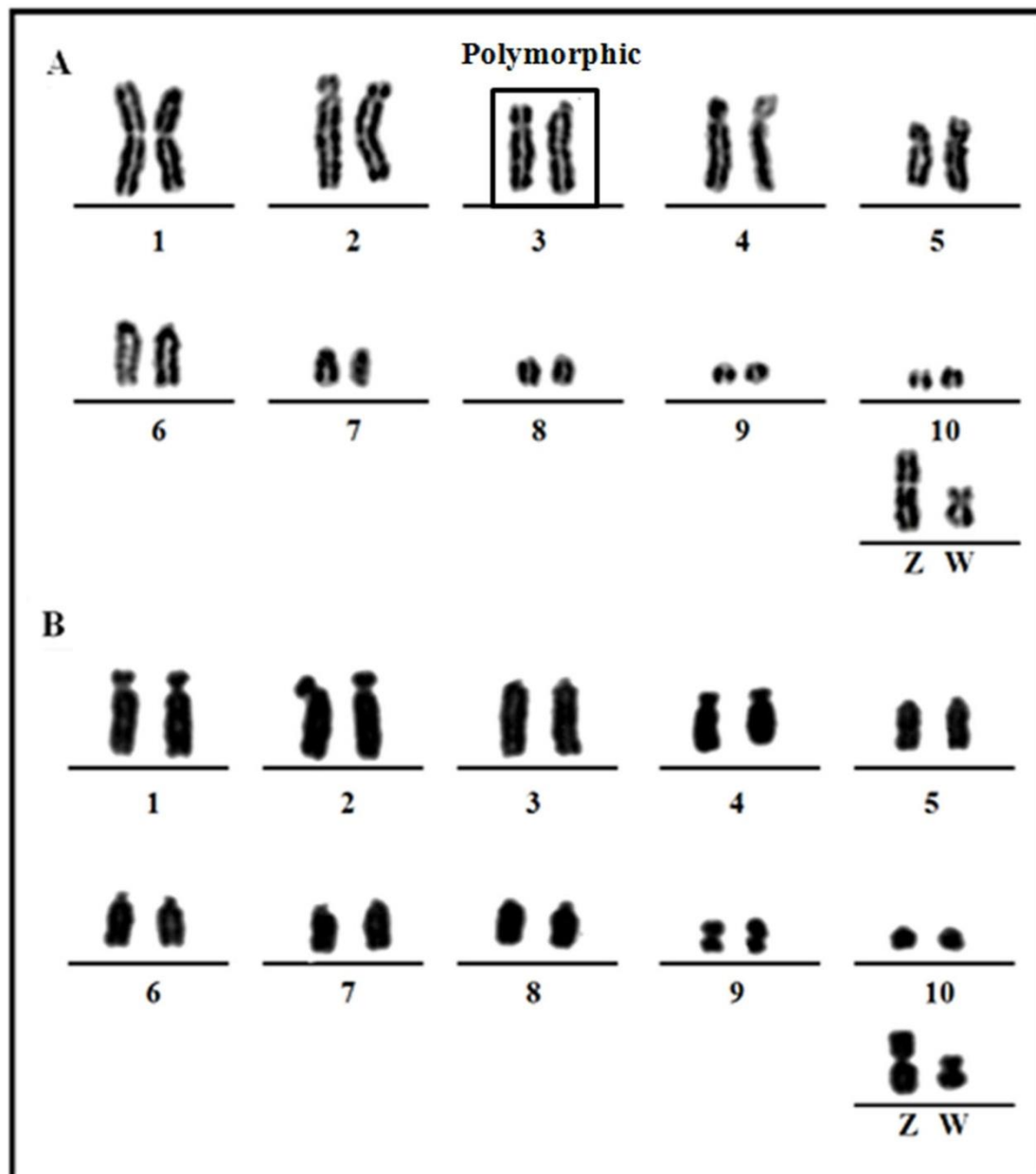


Figure 1 - Partial karyotypes conventionally stained of the two species: (A) *P. frontalis*, $2n=70$, (B) *A. aestiva* $2n=70$. Heteromorphic chromosome pair is boxed.

In *A. aestiva*, we found 16 segments homologous to GGA chromosome paints (Figure 4): PAK1 (GGA1)=AAE2 and AAE5; PAK2 (GGA2)=AAE1 and AAE12; PAK3 (GGA3)=AAE3q; PAK4 (GGA4)=AAE4q; PAK5 (GGA5)=AAE6; PAK6 (GGA6)=AAE7q; PAK7 (GGA7)=AAE7q; PAK8 (GGA8)=AAE11; PAK9 (GGA9)=AAE8; PAK10 (GGA10)=AAE9; PAK11 (GGA4p)=AAE10. In this species, only one fusion was detected: PAK6/PAK7, in AAE7, followed by a paracentric inversion. The experiments using LAL probes confirmed that the breakpoints occurred in syntenic groups according to GGA probes (Figure 5). The homology map of *A. aestiva* is shown in Figure 6B.

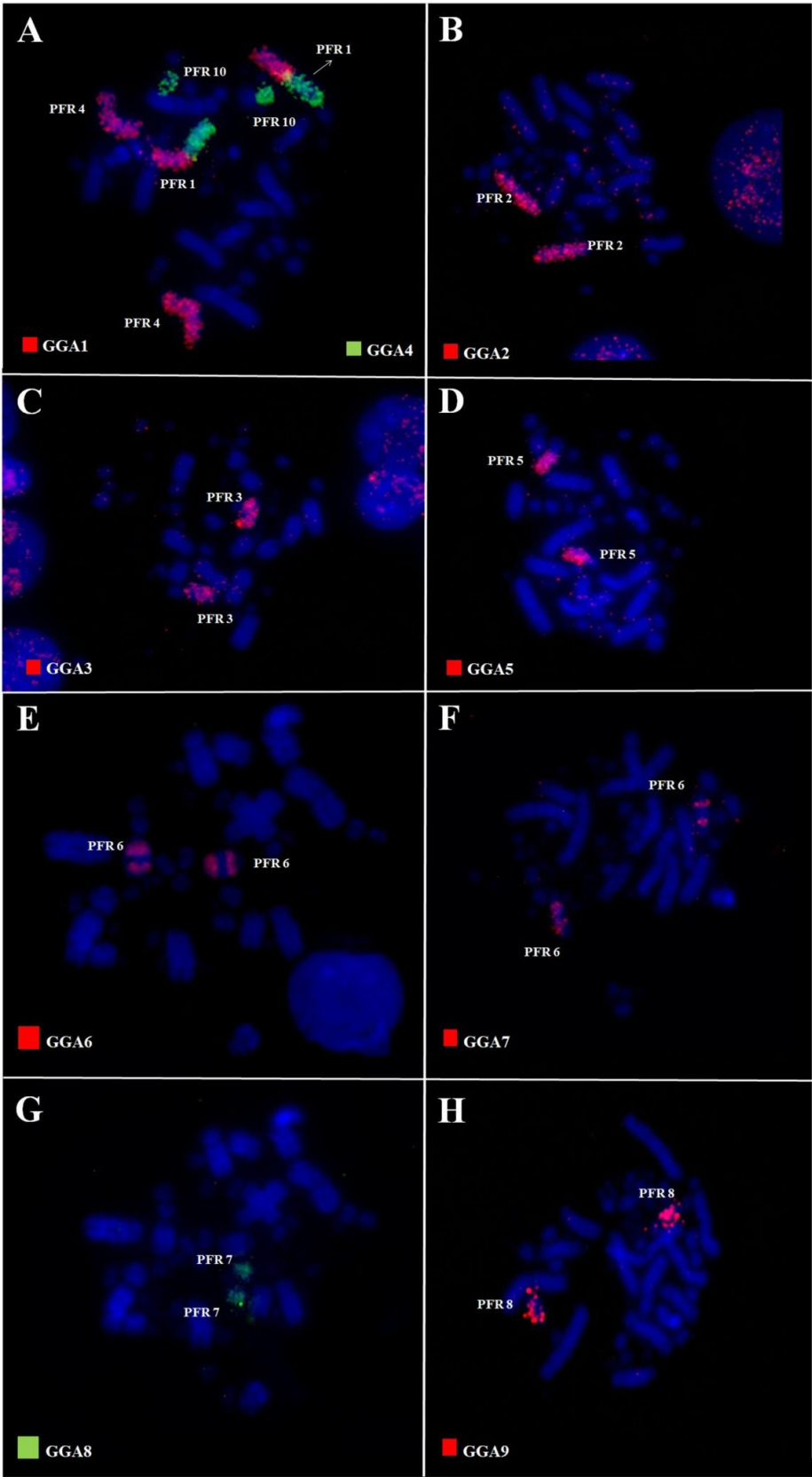


Figure 2 - FISH using whole-chromosome probes of *G. gallus* 1-9 in *P. frontalis*.

Furthermore, it was observed that the segment corresponding to GGA1q in both species (PFR4 and AAE2) also shows a gap in the terminal region, suggesting that

LAL7 does not cover all this area and there must be at least one more region of LAL which corresponds to GGA1 not identified by de Oliveira et al., [12], but suggested by Kretschmer et al., [15].

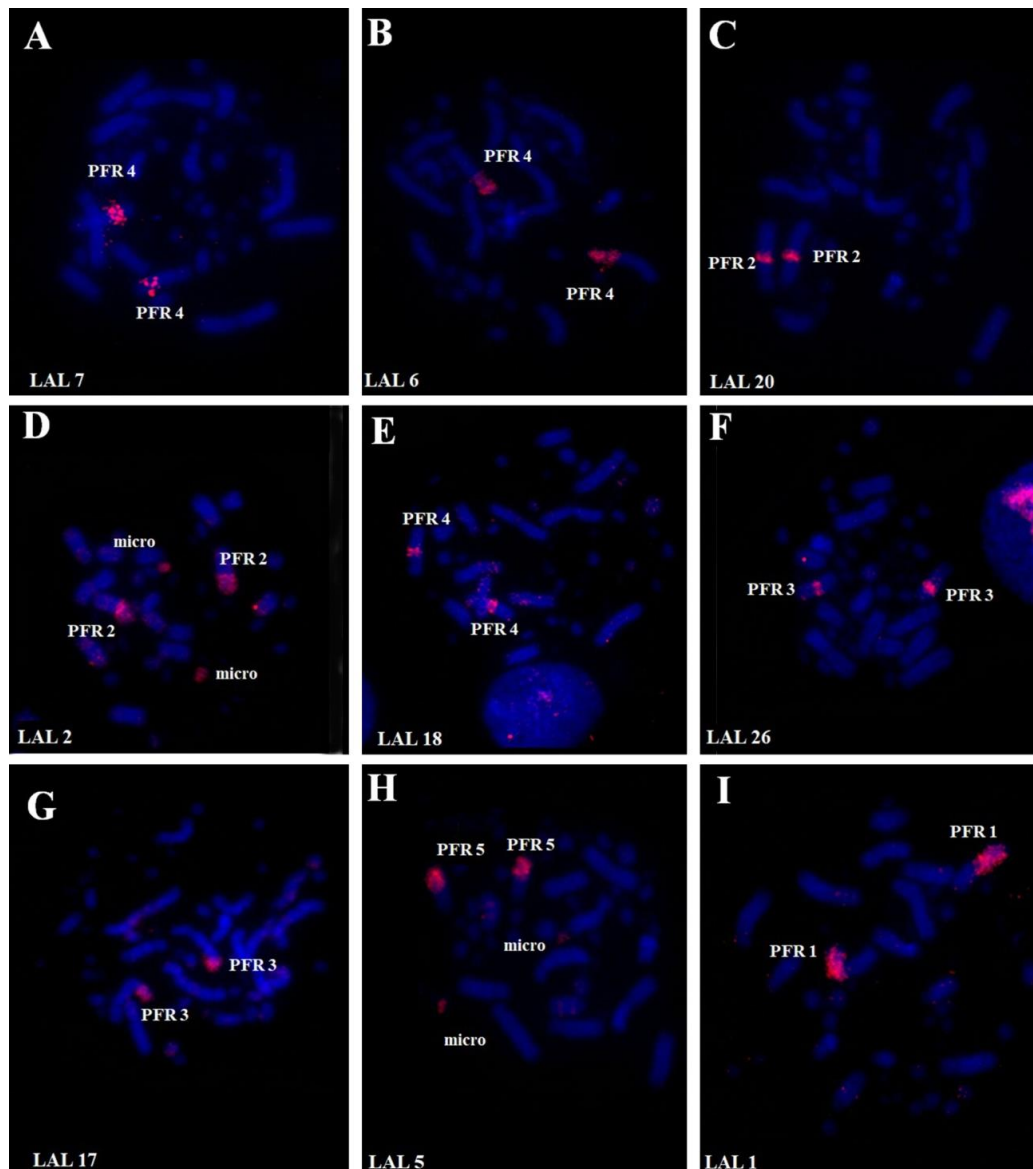


Figure 3 - FISH using whole-chromosome probes of *L. albicollis* macrochromosomes in metaphases of *P. frontalis*.

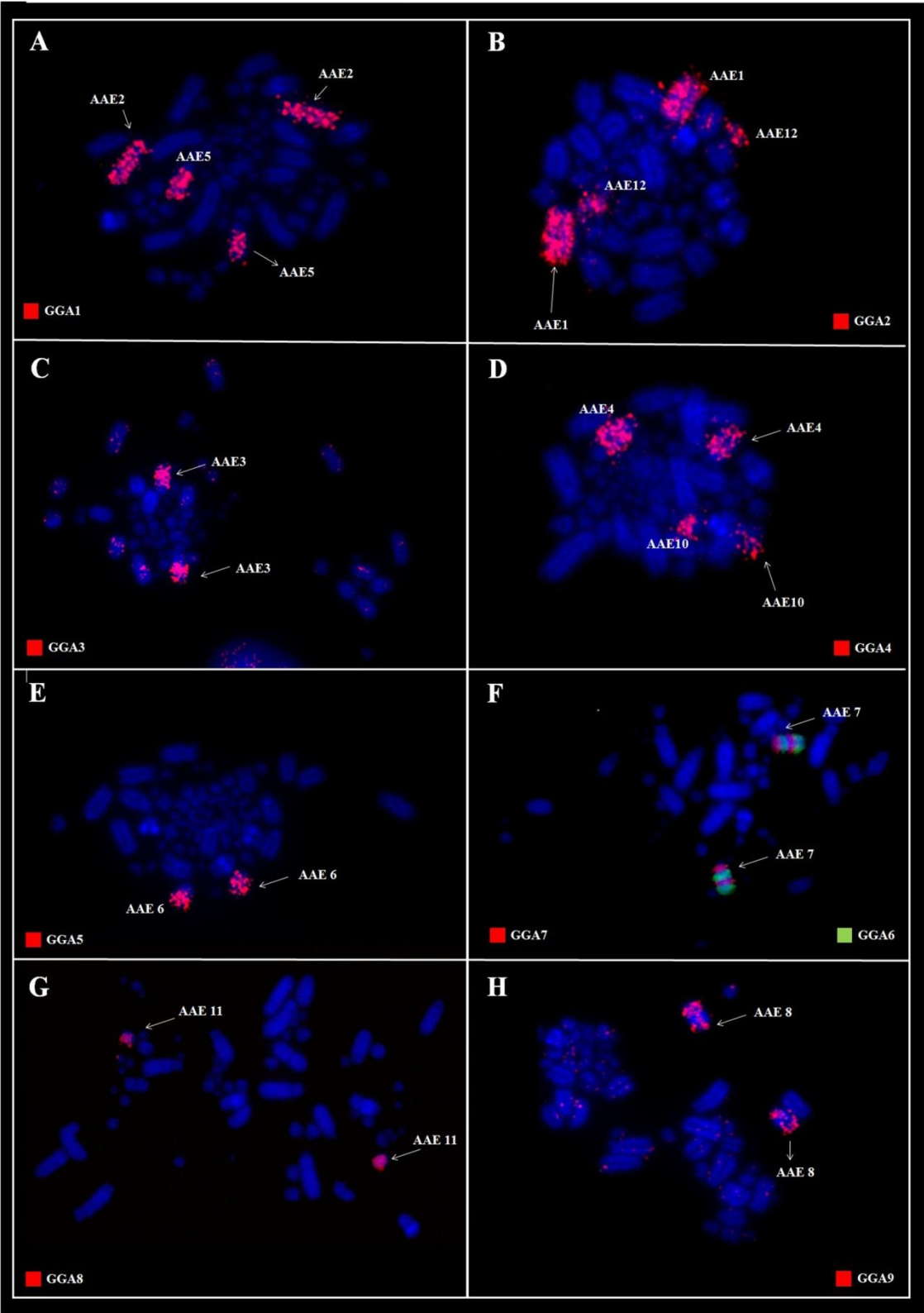


Figure 4 - FISH with whole-chromosome probes of *G. gallus* 1-9 in *A. aestiva*.

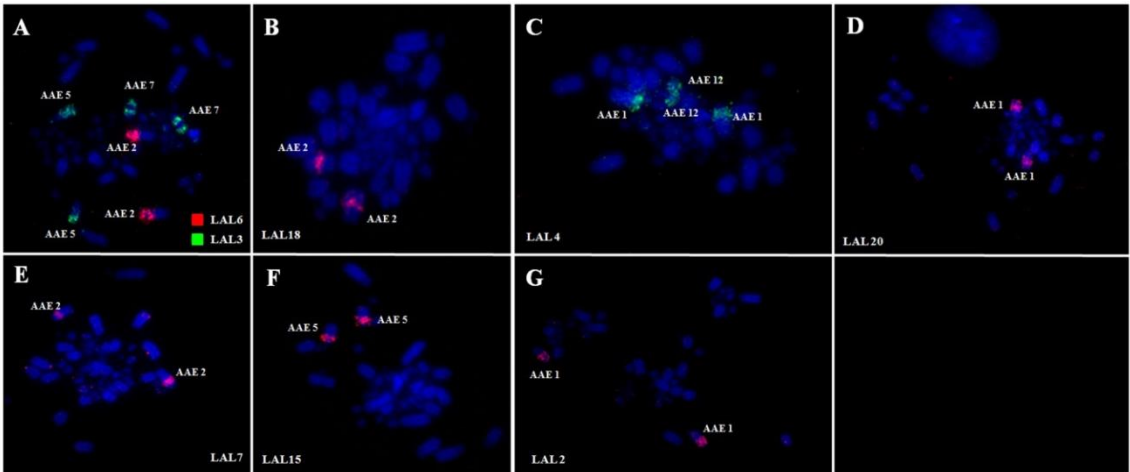


Figure 5 - FISH with whole-chromosome probes of *L. albicollis* in metaphases of *A. aestiva*.

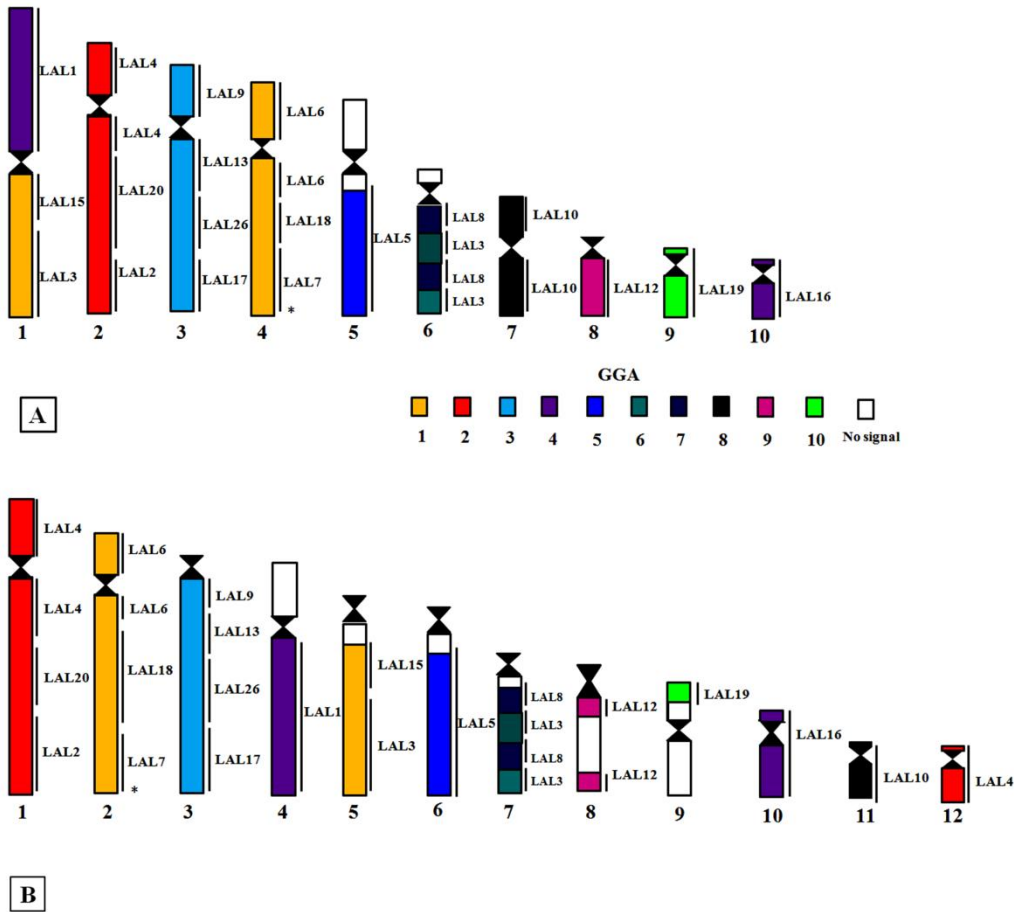


Figure 6 - Homology maps showing the correspondence between probes of GGA and LAL and chromosomes of (A) *P. frontalis* and (B) *A. aestiva*. Segments not hybridized with LAL probes are indicated with asterisks.

DISCUSSION

Cytogenetic studies based on classical cytogenetics have already revealed that parrots, macaws and related species show considerable chromosomal variability. With the advent of chromosomal painting, this variability has become even more apparent with examples of fusions, fissions, and paracentric inversions, which have raised issues concerning biogeographical aspects of this order [10].

This was the case of the two species analyzed herein, *P. frontalis* and *A. aestiva*: In comparison with the putative avian ancestral karyotype (PAK), our results using GGA and LAL paintings revealed the occurrence of rearrangements involving pairs PAK1, 6 and 7 in both species (Figure 2A, E and F; 4A and F), and PAK2 in *A. aestiva* (Figure 4B and 5C). Additionally, the segment resulting from the fusion of PAK6/PAK7 shows a paracentric inversion in both species. This has also been observed in other species of Neotropical Psittaciformes (*Ara macao*, *Ara chloropterus* and *Anodorhynchus hyacinthinus*) and in the African grey parrot (*Psittacus erithacus*) [9,10,22].

The presence of the centric fusion of PAK1 in *P. frontalis* and *A. aestiva* reinforces this rearrangement as a common feature for all the species of Psittaciformes analyzed up to now. Indeed, this centric fission is a recurrent rearrangement observed in species of different orders of birds, such as Passeriformes, Strigiformes and Accipitriformes [13,23-25]. Additionally, in *P. frontalis*, there is also a second rearrangement involving PAK1: the largest element, pair 1 (PFR1), arose by fusion of PAK1p/PAK4 (homologous to GGA1p/GGA4q) (Figure 2A). This rearrangement was observed in *Ara macao*, *Ara chloropterus*, *Anodorhynchus hyacinthinus* and *Psittacus erithacus* [9,10,22], indicating that it can be considered a cytogenetic signature reinforcing the monophyly of this group. For *Ara macao*, there is also a second fission, and PAK1 has three distinct segments [9].

However, not all the rearrangements are consistent with proposed classifications and biogeography. For instance, a fission of PAK2 (GGA2) is found in *A. aestiva* (AAE1 and AAE12). This fission occurred in PAK2p, in the median region of the segment corresponding to LAL4 (Figure 5C). A fission in PAK2 was also observed in *A. roseicollis*, a short tailed African Psittacidae [26], and in a Neotropical long tailed species, *Ara chloropterus* [10]. However, while in *A. chloropterus* and in *A. aestiva* the same breakpoint was confirmed by the use of LAL probes (a fission occurring in the region corresponding to LAL4), in *A. roseicollis* the available data include only the results obtained with GGA probes.

Other ancestral homologous syntenic blocks that show interesting reorganization in Neotropical Psittaciformes are PAK4 and PAK11 (GGA4q/GGA4p). In most species of birds with chromosome painting studies, these two blocks correspond to two distinct chromosome pairs (although they are fused in *G. gallus*) [18,23]. However, in Psittaciformes, although they correspond to two distinct segments, both are found fused to other chromosomes. For instance, species of genera *Ara*, *Anodorhynchus*, *Psittacus* and *Pyrhura* show PAK4 (GGA4q) fused to PAK1p (GGA1p) (Table 2). Interestingly, *A. aestiva* is the only Neotropical species in which PAK4 (GGA4q) is not fused, as observed in some African and Australian species (Table 2) [9,10,22,26].

Considering biogeographical issues, ancestral syntenic groups PAK6 and PAK7 (GGA6 and GGA7, respectively) are also interesting. These chromosomes are fused in all the Psittaciformes analyzed so far, and in most of them, the newly formed chromosome has undergone a paracentric inversion. This is the situation in Neotropical parrots and macaws and in the African *Psittacus erithacus* and *A. roseicollis* [9,10,22,26], which could be an indication that it represents a synapomorphy for this group. The fusion PAK6/PAK7 was also reported in Australian Psittaciformes, such as in *Melopsittacus undulatus*, but they do not show any inversion, or the inversion pattern is different to the one described for Neotropical and African species, as in *Agapornis roseicollis* [26] (Table 2). Hence, the fusion PAK6/PAK7 must represent a synapomorphy for Psittaciformes, but the different patterns of inversion and fusions with other segments may have occurred randomly. In addition, the fusion PAK8/PAK9, which was found in all the previously analyzed Psittaciformes, is not found in *P. frontalis* and *A. aestiva*.

Despite the intriguing distribution patterns of these rearrangements in species from different geographical locations, the information generated by chromosome painting allows us to propose a putative ancestral karyotype for Neotropical Psittacidae (PAK-NP) (Figure 7). In our proposal, PAK1 corresponds to two autosomal pairs, due to centric fission, and PAK6/PAK7 are fused and rearranged to form a paracentric inversion. Other macrochromosome pairs correspond to one pair each, as found in the putative avian ancestral karyotype [18,27]. As the karyotypes of Arini species diverged, a fusion of PAK1p/PAK4 appears in the common ancestor of species with a high number of banded chromosomes.

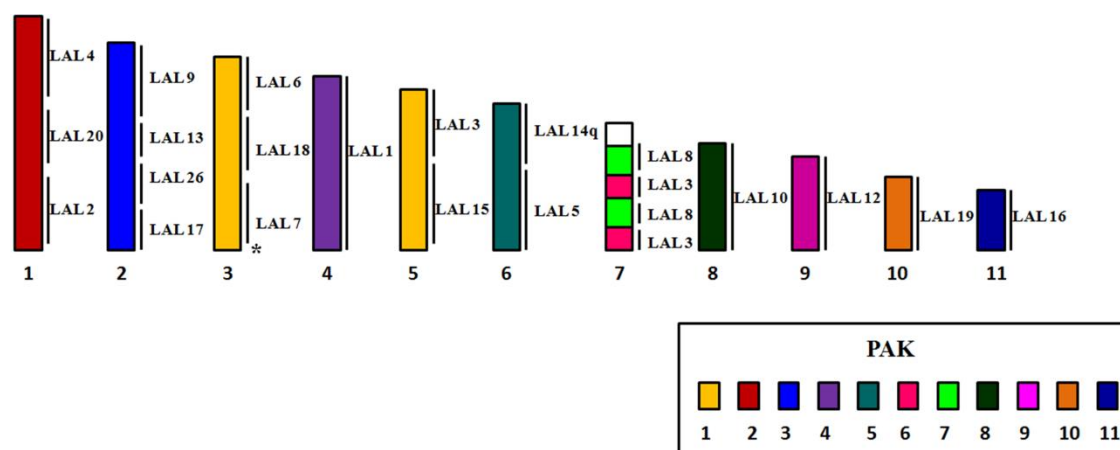


Figure 7 - Schematic representation of Putative Ancestral Karyotype of the Tribe Arini, showing fission in PAK 1 and association of PAK6/7, according to FISH results with probes of *L. albicollis* and *G. gallus* in species of Neotropical Psittacidae. Segments not hybridized with LAL probes are indicated with an asterisk.

Phylogenetic Analysis

Classification by tail length prior to molecular and cytogenetic studies was used to classify Neotropical Psittacidae into two groups – short- and long-tailed species [28]. This was supported by some minisatellite, behavioral, mtDNA and karyotypical studies [4,8,29,30]. However, results from more recent studies of mtDNA involving more genes and a higher number of species, and including genera which were not present in the first analyses are not in complete agreement with the division of tribe Arini based on tail length [5].

In a similar way, early cytotaxonomic studies, based on conventional staining, were initially concordant with this division according to tail length. However, as new analyses were performed in a larger number of species, it has become clear that chromosomal morphology does not support this classification, because there are short-tailed species which show a high number of banded macrochromosomes for example (*Forpus xanthopterygius*) (Table 4) [31,32].

In the case of Neotropical species, analyses by conventional staining were in agreement with mitochondrial DNA studies and morphological criteria, supporting their division into two groups: long-tailed species (genera *Ara*, *Cyanopsitta*, *Propyrrhura*, *Aratinga*, *Nandayus* and *Guaruba*), with most macrochromosomes banded, and only short-tailed species (genera *Amazona* and *Brotheris*), with most macrochromosome pairs classified as acro/telocentric [8,21]. However, some species of this tribe have shown karyotypes, such as genus *Pyrrhura* with long tails, transitional between the two formulae mentioned above, with pairs 1 metacentric, pairs 2, 4 and 5 acrocentric and remaining macrochromosomes telocentric in *P. frontalis* as well as in *P. molinae* (PMO) except that in PMO the 5th is submetacentric and 2th was classified with metacentric and 1th acrocentric (Table 2) [33]. Hence, although *P. frontalis* and *A. aestiva* (with the typical karyotype of long- and short tailed species) showed the same diploid number, $2n=70$, chromosomal morphology of some pairs is different. Additionally, chromosome painting experiments revealed that their karyotypes are highly divergent due to the occurrence not only of pericentric inversions, but also of paracentric inversions, fissions and fusions (Table 4). The analysis to chromosome morphology of species of Arini Tribe suggested that there is probably a group of Psittacidae with intermediate karyotypic characteristics that not corresponding to short or long tailed species, which includes genus *Pyrrhura* and *Forpus* (Table 4). However, despite this karyotype transition, and considering the current data, the genus *Pyrrhura* presents chromosomal synapomorphies involving PAK1/4 and PAK8/9, which allow its inclusion in one of the two groups of Neotropical Psittacidae as discussed below.

Up at this point, chromosome painting results support the existence of two groups within Arini, based on ancestral chromosomes PAK1p, PAK4, PAK8 and PAK9: one group is constituted of species with banded chromosomes, in which pair 1 is metacentric, and there are two fusions – PAK1p/PAK4 and PAK8/PAK9, with or without inversions and further fusions. The second group includes species with most macrochromosomes having single arms that show the fission in PAK1, but do not show fusion of PAK8/PAK9, as in genus *Amazona*. Hence, cytotaxonomically, this group has a more basal position, corroborating the mtDNA studies [4]. Consequently, species of the first group that share two more synapomorphies, i.e., the PAK1p/PAK4 and PAK8/PAK9 fusions, could be considered more derived. Further rearrangements in PAK8/PAK9 seem to clarify the phylogenetic relationships of some genera such as *Ara*, *Anodorhynchus*, *Psittacus* and *Pyrrhura* (Figure 8).

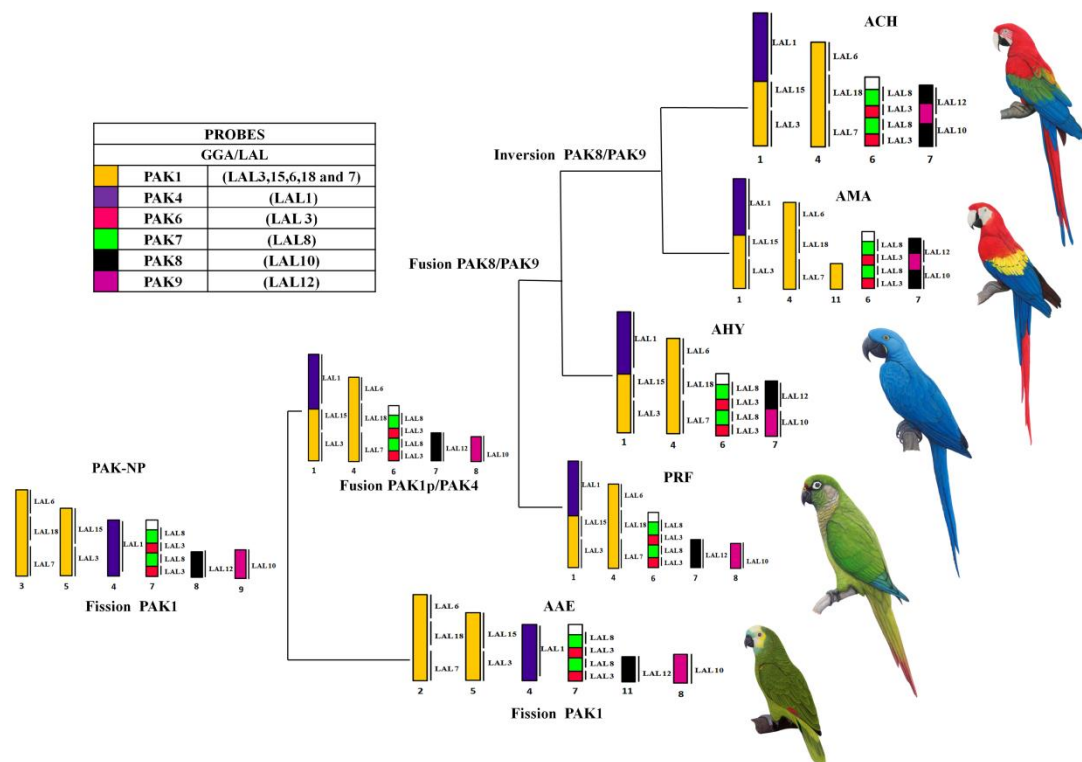


Figure 8 - Phylogenetic analysis based on rearrangements involving PAK1, PAK4, PAK6, PAK7, PAK8 and PAK9 in Neotropical Psittacidae, according to results obtained by FISH with probes of *G. gallus* and *L. albicollis* (Legend: PAK, putative ancestral avian karyotype; PAK-NP, putative ancestral karyotype of Neotropical Psittacidae; AMA, *Ara macao*; ACH, *Ara chloropterus*; AHY, *Anodorhynchus hyacinthinus*, PFR, *Pyrrhura frontalis*; AAE, *Amazona aestiva*; GGA, *Gallus gallus*; LAL, *Leucopternis albicollis*).

Together with *A. aestiva*, *P. frontalis* is the only species of Arini which does not have the fusion PAK8/PAK9, which could indicate that both species are more basal than the Neotropical *Ara* and *Anodorhynchus* and the African *Psittacus*. Additionally, *Anodorhynchus* is more basal than *Ara*, as it has the PAK/8/PAK9 fusion, while *Ara* and *Psittacus* have an additional inversion [10,22]. The similarity between these two genera, despite their divergent geographical distribution, lies in the fact that *Psittacus* is the sister-group of Neotropical Psittacidae. This suggests that African Psittacidae are not a monophyletic group, and may have colonized Africa twice – first from Southern Asia, and a second time from South America [34,35].

Although additional studies including other species of Psittacidae are necessary to clarify the phylogeny and biogeography of this group, chromosomal analyses so far suggest that the classification based on tail length do not reflect the real phylogenetic

history of Neotropical Psittacidae. Hence, we have found some chromosomal synapomorphies, such as associations PAK 1/4, 6/7 and absence of fusion 8/9, which strongly support their division in two different groups, not corresponding to the ones presented by these previous proposals.

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Conflict of interest: The authors declare that they have no conflict of interest.

Supplementary Materials

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