Chromosomal Rearrangements of Psittacidae Evolution: Zoo-FISH in *Amazona vittata* and *in silico* analysis of chromosomal rearrangements validated by *de novo* PCR

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Abstract

The Puerto Rican parrot (*Amazona vittata,* AVI) is a critically endangered species and the only native parrot species remaining on all U.S. territories. After its genome was sequenced through a community-funded project, we studied the ALLPATH 2012 genome assembly largest 3,099 scaffolds using UCSC Genome Browser's BLAT tool against the chicken reference genome. The alignments of the first and last 25 kb of the scaffolds showed a total of 164 possible chromosomal rearrangements. A selection of the alignments with scores greater than 1,000 on both sides was made and primers were designed from the rearrangement joint regions. Out of the 19 putative rearrangements that did not involve sexual or unknown chromosomes, five were confirmed as true rearrangements in four Amazon parrots, including the Puerto Rican parrot. With our *de novo* PCR validation approach, we also confirmed that three of these rearrangements occurred before the evolutionary split of the African grey parrot, joining chicken chromosomes 2 with 15, 6 with 7, and 8 with 9. PCR further showed that the remainder two chromosomal rearrangements, joining chicken chromosomes 3 with 9 and 6 with 7, occurred after the split of the scarlet macaw. In order to revalidate these rearrangements, we used the well-established *Fluorescent In Situ Hybridization* (FISH) technique, hybridizing 10 chicken (GGA) whole-chromosome paints on Puerto Rican parrot metaphases. The FISH revealed three [6 with 7 (2) and 8 with 9] of the previously confirmed rearrangements through PCR and the rest of the chicken paints hybridized to unique segments or whole chromosomes in the parrot. Chicken chromosome probe 1 gave a signal on AVI3, GGA2 on AVI2 and a pair of microchromosomes, GGA3 on AVI1, GGA4 on AVI4 and to an arm of the metacentric AVI8, while GGA5 painted AVI7, GGA6 & 7 interestingly painted AVI6 in an alternated fashion, and GGA8 and 9 shared hybridization signals on a microchromosome pair. Furthermore, GGA9 also painted AVI9 and GGAZ painted its equivalent Z chromosome in the parrot. In addition, 100 of these metaphases were also used to describe A. vittata's karyotype, which averaged a chromosome diploid number of 76. All together, these results contribute as the first complete description of the karyotype evolution of an Amazon species.

Resumen

La cotorra puertorriqueña (*Amazona vittata*, AVI) es la única especie nativa en todos los territorios de los Estados Unidos y actualmente se encuentra en crítico riesgo de extinción. Luego de que su genoma fuese secuenciado, como parte de un proyecto subsidiado por la comunidad; nos dimos a la tarea de estudiar los 3,099 andamios más grande de su ensamblaje de ALLPATH del 2012, alineándolos contra el genoma de la gallina como referencia, mediante el uso de la herramienta de "BLAT" en el "UCSC Genome Browser". Los alineamientos de las primeras y últimas 25 kb de los andamios resultaron en un total de 164 posibles rearreglos cromosómicos. Se realizó una selección de los alineamientos con puntajes mayores a 1,000 en ambas puntas y se diseñaron cebadores a partir de la región donde se encontraba cada rearreglo aparente. De un total de 19 potenciales rearreglos cromosómicos que no incluían cromosomas sexuales ni tampoco cromosomas desconocidos, cinco rearreglos fueron confirmados en todas las cuatro especies de cotorras amazonas estudiadas, incluyendo la cotorra puertorriqueña. Mediante nuestro enfoque de PCR *de novo* para rearreglos cromosómicos, también se confirmó que tres de estos rearreglos ocurrieron antes de la divergencia del loro gris (*Psittacus erithacus*), uniendo así los cromosomas análogos en la gallina número 2 con el 15, el 6 con el 7 y el 8 con el 9. El PCR en adición probó que los dos rearreglos restantes, del cromosoma 3 con el 9 y otro del 6 con el 7, ocurrieron después de la divergencia evolutiva del guacamayo macaco (*Ara macaco*). Con el fin de revalidar estos rearreglos, utilizamos la muy conocida técnica de Hibridación Fluorescente In Situ o FISH por sus siglas en inglés, hibridando unas 10 sondas de cromosomas completos de la gallina (GGA) con los cromosomas de la cotorra puertorriqueña. La técnica reveló tres de los rearreglos previamente confirmados por PCR [6 con el 7 (2) y el 8 con el 9] y el resto de las sondas hibridó un único segmento o a un cromosoma en su totalidad en la cotorra. La sonda perteneciente al cromosoma 1 en la gallina mostró señal en el AVI3, el GGA2 en el AVI2 y en un par de cromosomas indistinguibles o microcromosomas, el GGA3 en el AVI1, el GGA4 en el AVI4 y en uno de los brazos del cromosoma metacéntrico AVI8. A su vez, el GGA5 hibridó en el AVI7, y el GGA6 y 7 hibridaron ambos en el mismo cromosoma AVI6 de una forma alternada, mientras que el GGA8 y 9 compartieron señales en un par de microcromosomas, aunque el GGA9 también dio señal en el cromosoma AVI9 completo. Finalmente, GGAZ pintó a su equivalente Z en la cotorra. Mas aún, 100 de las metafases de la cotorra se utilizaron a su vez para describir el cariotipo de *A. vittata* por primera vez, resultando en un promedio de número de cromosomas diploides de 76. Todo el trabajo en conjunto representa el primer estudio descriptivo completado del la evolución del cariotipo de una especie de cotorra amazona.

To my mom, dad and sister, you mean the world to me.

To my beautiful island of Puerto Rico, hope I have made my contribution.

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There were several other people that made this project possible. Without them it would have been impossible to produce results. Among these people, I begin thanking Ricardo Valentín of Bosque Río Abajo Aviary for providing blood samples and showing me for the first time a beautiful Puerto Rican parrot. Special thanks to Jafet Veléz-Valentín of Luquillo Aviary for being always happy to collaborate with the project sending me numerous samples from *Amazona vittata* and wishing me luck. Also expressing gratitude to the following undergraduate students at the University of Puerto Rico at Mayagüez, which performed the parrot to chicken DNA alignments, and helped validate chromosomal rearrangements through PCR: Adriana Gordon, Gustavo Rivera, Ghildres Tejedor, Roberto Calzadilla, Michelle García, Arnaldo López, Carlos Estévez, Jaileen Rivera and Carlos Zamora. Many thanks! Thanks to Dr. Mi Ok Lee of Texas A&M University for collecting chicken blood and going trough the lymphocyte collection protocol by my side. It was a pleasure working with you. At Texas A&M University, I would also like to give very special thanks to Dr. Terje Raudsepp for collaborating directly with the project, teaching me many cytogenetic techniques and guiding me along the way. I owe a lot of my growth as a scientist to what I learned at your lab. I have no words to describe how much I appreciate your contribution. I additionally wish to thank Jason Howard and Dr. Erich Jarvis of Duke University, you believed in me and I feel so grateful for all the attentions received during my work period at North Carolina. Thank you Jason for teaching me so much!

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Introduction

"In an age when we experience so much of our world through glass-screens, windows, windshields; birds are a vital connection to the wild. They reach across any barrier, flitting, surprising, and dazzling, always there to refresh my sense of wonder."

-Thor Hanson
Author of *Feathers: The Evolution of a Natural Miracle*

All throughout human history, birds have been accompanying humans while occupying the most remote places in the world. Their origins are still uncertain; nevertheless evolutionary scientists, based on fossil and biological evidence, attribute the rise of birds to the subgroup of Jurassic dinosaurs known as the coelurosaurian theropods. Approximately two hundred million years later, birds are primarily characterized as egg-laying, warm-blooded animals with feathers, wings (even for species that do not fly), and toothless beaks.

Taxonomically, Aves are one of seven vertebrate classes in the Animalia kingdom; with over 10,000 species alive today. Disappointingly, over the years many bird species have been brought to extinction by many factors; human activity being the predominant of them. The Amazon Parrots are but one example of number depletion over the years, with 2 of its species gone extinct, 13 being vulnerable, 5 endangered, 1 critically endangered and only 11 near threatened or least concern (IUCN Red List 2013). Over the years, more efforts are being directed onto the conservation of species employing statistical breeding analyses and genetic management.

The Puerto Rican parrot, *Amazona vittata*, is the first parrot among the 30 species of the large Neotropical genus *Amazona*, to have its genome sequenced (Oleksyk et al., 2012). It is the only endemic parrot of the island of Puerto Rico and the only native parrot remaining in the United States territory (Snyder et al., 1987). Being a critically endangered species, more efforts are being performed with the vision of contributing to the recovery program with genomic information of this parrot. With no previous comparative chromosome analysis, our goal was to evaluate inter-chromosomal synteny between the chicken and the Puerto Rican parrot for the first time. Since it is well known that birds have a high degree of conservation; then, we expected to have few chromosomal rearrangements when analyzing macro-chromosomal segments of the two birds. Finally, we also aimed to explore the possible presence of rearrangements found in other Amazon parrots and other Neotropical parrots to elucidate the evolution arrangement of the Psittacidae family.

Literature Review

Avian origins and evolutionary events

Avian origins are a topic of great debate as paleontological and mitochondrial DNA studies do not always agree on the time of divergence. While two fossils of *Protoavis texensis* specimens suggest birds to have diverged about 210 million years (Rodionov 1997), molecular data pushes back bird origin to 310 million years ago (mya), based on a common ancestor for birds (synapsids) and mammals (diapsids) (Kumar and Hedge, 1998; Burt et al., 1999). Even so that solely relaying on mitochondrial DNA of present crocodilians and bird species gives an estimation of 210 to 250 mya; much more closer to what fossil evidence suggests (Timetree.org).

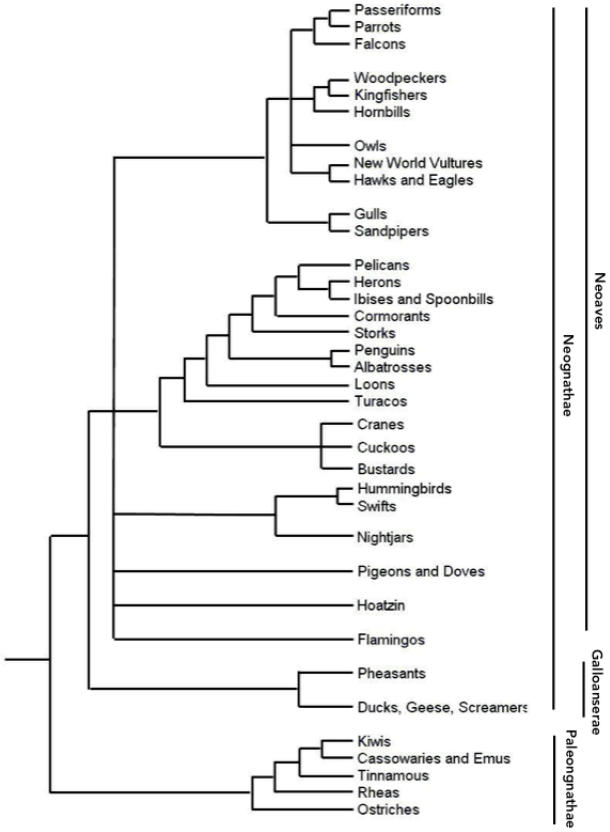

Figure 1. Phylogeny of birds. The tree was retrieved from the Fort Worth Zoo website which showed a redrawn version from the report by Hackett et al. (2008). To show evolutionary events, we further modified it, by adding the orders on the right side of the tree.

Another subject that is also full of discrepancy is that of avian lineage phylogeny. However, van Tuninen and Hedges (2001) used mtDNA, DNA-DNA hybridization and transferrin immunological distances to "calibrate" avian molecular clock and consequently attributed the avian lineage diversity to three major events. The first event led to the present order of ratites Struthioniformes that includes species such as the emu, the ostrich and the rhea and Neognathae (the rest) about 100-120 mya (Tuninen and Hedges, 2001, cited in Griffin et al., 2007) (Figure 1). Following this event, Neognathae diverged into Galloanserae, including modern chickens, goose, turkey, and duck about 100 mya; and finally the remainder of the Neognathae became what are known as Neoaves or modern birds some 70-80 mya (Tuninen and Hedges, 2001, cited in Griffin et al., 2007) (Figure 1).

Avian karyotype

It has been long established that birds possess small and evolutionarily conserved genomes. The Avian genome is the smallest among the vertebrate group (www.genomesize.com) and it is

37.5% smaller than the human genome. Yet, the majority of the bird genomes are distributed along a high number of chromosomes (2n=80), with about 9 pairs of macrochromosomes (medium-sized, easily recognizable through microscopic evaluation) and around 30 pairs of dot-like chromosomes (microchromosomes) and a ZZ(males)/ZW(females) pair of sex chromosomes (Christidis 1990).

Perhaps the most complete compilation of avian karyotypes was done by Christidis (1990), with over 700 species for which he saved diploid chromosome number and complete karyotypes. In the book, Christidis stated that the avian karyotypes range from 2n = 66 to 2n=86; which is surprising when compared to other class like mammals which are known to vary extensively between species. One of the best examples for the pronounced chromosome number change among mammals is the comparison between the Indian Muntjac (*Muntiacus muntjak vaginalis*) which has an incredibly low diploid chromosome number of only 6 in females and 7 in males; and on the other extreme, the Viscacha rat (*Tympanoctomys barrerae*) with 102, making it the species with the highest chromosome number in the class (Graphodatsky et al., 2011). This is rather not the case for birds as they usually retain the classic 2n = 66-86 karyotype, although there are some species that do not follow the norm such as the stone curlew *Burhinus oedicemus* (2n=40) and the gray or Southern "Go-away-bird" *Corythaixoides concolor* (2n=136-142) among others (Christidis 1990).

Moreover, the avian order Falconiformes appears to be the order with most evident change of genome organization between chromosomes, since its Falconidae (falcons and caracas) family shows great variation among some of its 60 species (e.g. Laggar falcon *Falco jugger* 2n=50 and Crested caracara *Polyborus plancus* 2n=84-86) (Christidis 1990). Nevertheless, Falconidae is known to have followed a more uniform karyotype with fewer microchromosomes than the norm, and thus larger chromosomes involved in their peculiar karyotypes (Griffin et al., 2007).

The first avian karyotype ever to be completed was that of the chicken (*Gallus gallus*) by Masabanda et al. (2004) describing the chicken as having a diploid chromosome number of 78. Being studied extensively, not only to explore genome evolutionary dynamics, but also for its agricultural importance, the chicken became the avian model of excellence and thus its karyotype became the most used for avian comparative cytogenetics. Its karyotype has also been divided into groups by several authors, however Griffin et al. (2007) created a system to follow in order to avoid confusion. The authors in the paper classified the chicken chromosome composition into four groups: A, B&C and D (Griffin et al., 2007). Group A included the biggest ten chromosomes (macrochromosomes) that are the ones that can be separated using flow sorting. Group B&C are comprised of chromosomes 11-32, all microchromosomes, and they are separated by chromosome

16, which is known as the Nucleolar Organizing Region (NOR) chromosome (Griffin et al., 2007). The final group, D, was designed as the one containing chromosomes that were not anchored to the chicken assembly at the time the paper was written (33-38) and seven years later, there are still unanchored (Griffin et al., 2007).

The reasons for which avian microchromosomes are not easily anchored to genomes are still unknown and this represents a bigger problem due to the evidence that microchromosomes have a high amount of functional genes. The GC content, CpG islands, hypermethylated cytosines and histone H4 hyperacetylation, all suggest higher gene density than macrochromosomes (McQueen et al., 1996, 1998; Smith et al., 2000; Grützner et al., 2001).

Interchromosomal rearrangements

Several authors have demonstrated that the avian evolutionary process gave rise to a low number of chromosomal rearrangements; therefore, a high degree of conservation between avian chromosomes is well known (Burt et al., 1999, Guttenbach et al., 2003, Hansmann et al., 2009 and Völker et al., 2010). Many of them have come to this conclusion after applying techniques, such as *Fluorescent In Situ Hybridization* (FISH), which initiates with chromosome preparations fixed on slides to essentially hybridize a DNA probe to its complementary sequence. In order to study the chromosomal rearrangements that took place in avian evolutionary history by identifying regions of synteny in the chromosomes of closely or distant related species, a type of FISH, called the Zoo-FISH have been successfully implemented. Also known as cross-species chromosome painting, this technique has been widely described as "*hybridizing libraries of DNA sequences, also known as chromosome paints, from one species to the chromosomes of another species, to identify regions of synteny*" (Volpi and Bridger, 2008).

In 1999, only five years after Scherthan et al. performed the first Zoo-FISH in history (painting several mammal species with human chromosome paints), the technique was applied to the emu (*Dromaius novaehollandiae*) chromosomes (Scherthan et al., 1994; Shetty et al., 1999). By utilizing flow-sorted chicken whole-chromosome paints, Shetty et al. found extraordinary homology between the emu and the chicken chromosomes despite the many million years that separate the ratites from the pheasants (Shetty et al., 1999). Chicken paints from 1 to 9 and Z & W were found to be homologous to the same chromosome pair in the emu, with the exception of chicken chromosome 4 that hybridized to two chromosomes (4 and a pair of microchromosomes) in the emu (Table 1) (Shetty et al., 1999).

Moreover, chicken chromosome 4 has proven to be a very peculiar chromosome since it was found to be the most ancient chromosome for the birds after showing striking evolutionary conservation with human chromosome 4 (Chowdhary and Raudsepp, 2000). Likewise, chicken chromosomes 1-5 and Z through comparison with soft-shelled turtle cDNA libraries showed no rearrangements for any of these two species (Matsuda et al., 2005) Thus, taken together the above studies, chromosomes 1-5 & Z would have appeared approximately 230 million years ago and chromosome 4 about 320 million years ago; and therefore it would be expected that they remained conserved throughout the evolutionary splits that followed avian speciation.

The members of the family Phasianidae are indeed the most studied avian species by Zoo-FISH with 9 of circa 35 published species to this day (Table 1). Only two predominant rearrangements involving chicken chromosome paints (GGA) 2 and 4 have been described for the majority of the Phasianidae species studied, GGA2 hybridizing to species' 3 + 6 or 7 corresponding chromosome and, as the emu, GGA4 splitting into chromosome 4 and a pair of microchromosomes. On the other hand, even though they are classified as belonging to a different family (Turnicidae) than the chicken, quails show perfect conservation for all 1-9 macrochromosomes and sexual chromosome Z (Schmid et al., 2000; Guttembach et al., 2003; Shibusawa et al., 2004a).

Besides the chicken, the other bird that has been widely studied for the song learning ability of its males and thus its huge importance for neurological studies is the zebra finch (*Taeniopygia guttata*). Only two interchromosomal rearrangements have been reported between the chicken and the zebra finch macrochromosomes using cytogenetic comparisons (Itoh and Arnold 2005, cited in Völker et al., 2010). According to Itoh and Arnold (2005), chicken chromosomes 1 and 4 suffered fissions that produced zebra finch chromosomes 1 and 1A and 4 and 4A, respectively. Even though the zebra finch and the chicken diverged at least 100 million years ago (timetree.org), their chromosome conservation is outstanding. Meanwhile, on the side of the spectrum, the vulture family Accipitridae appeared to have undergone massive chromosomal rearrangements as shown in Table 1. All together, with the exception of the Accipitridae family, interchromosomal rearrangements have not appeared to be as extensive for birds as for others classes such as mammals, where the evolutionary dynamics involved numerous chromosome additions (fusions) and chromosomal breaks (fissions).

Table 1. Avian Interchromosomal Rearrangements Detected by Zoo-FISH Using Whole-chromosome Chicken Paints

Species	Family	GGA1	GGA2	GGA3	GGA4	GGA5	GGA6	GGA7	GGA8	GGA9	GGA10	GGAZ	GGAW	2N	Reference:
Rüppell's Vulture *(Gyps ruepelli)*	Accipitridae	7 + 12 + 15 + 19 + 20 + 22	2 + 3 + 23	8 + 16q + 21 + 24	1 + 13	14q + 17	4q	6q	10	9q	18q	n/a	n/a	66	Nanda et al., 2006
Griffon Vulture *(Gyps fulvus)*	Accipitridae	7 + 12 + 15 + 19 + 20 + 22	2 + 3 + 23	8 + 16q + 21 + 24	1 + 13	14q + 17	4q	6q	10	9q	18q	n/a	n/a	66	Nanda et al., 2006
Bearded vulture *(Gypaetus barbatus)*	Accipitridae	7 + 8p + 11 + 12q	1q + 2 + 14q +23q	8q + 13 + 21q + 22q	3+ 16	15q + 20	4q	6q	10	5q	9q^	n/a	n/a	60	Nanda et al., 2006
Duck (*Cairina moschata*)	Anatidae	?	?	?	?	?	6	?	?	?	n/a	?	n/a	78	Schmid et al., 2000
Greylag goose (*Anser anser*)	Anatidae	?	?	?	?	?	6	?	8	?	n/a	?	n/a	80	Schmid et al., 2000
Cockatiel (*Nymphicus hollandicus*)	Cacatuidae	3 + 6	1	2	4q + micro (11)	7p	5q^	5q^	4p^	4p^ + micro (10)	9	n/a	n/a	72	Nanda et al., 2007
California condor (*Gymnopyps californianus*)	Cathartidae	1	2	3 + 2 (weak)	4	5 + Z (weak)	6	7	8	4	9	Z	Z (p)	80	Radusepp et al., 2002
African collared dove (*Streptopelia roseogrisea*)	Columbidae	1	2	3	P and q arms of one of the chrs from 4-7				?p	?p	n/a	?	n/a	78	Schmid et al., 2000 / Guttembach et al., 2003
Domestic pigeon (*Columba livia*)	Columbidae	1	2	3	4 + 10	5	6	7	8	9	11	Z	n/a	80	Derjusheva et al., 2004
Plain chachalaca (*Ortalis vetula*)	Cracidae	1	2	3	4 + micro	5	6	7	8	9	n/a	Z	n/a	?	Shibusawa et al., 2004b
Emu (*Dromaius novaehollandiae*)	Dromaiidae	1	2	3	4 + micro	5	6	7	8	9	n/a	Z	W	80	Shetty et al., 1999
Zebra finch (*Taeniopygia guttata*)	Estrildidae	1 + 1A	2	3	4 + micro (4A)	5	6	7	8	9	n/a	Z	n/a	82	Itoh and Arnold, 2005
Chaffinch (*Fringilla coelebs*)	Fringillidae	3q + 4	1	2	5 + micro	6	7	8	9	10	micro	Z + Wq	n/a	80	Derjusheva et al., 2004
Guinea fowl (*Numida meleagris*)	Numididae	1	2	3	4q + micro	5	5p	5q	7	4p	n/a	Z	n/a	78	Shibusawa et al., 2002
Pheasant (*Phasianus colchicus*)	Phasianidae	1	3 + 6	2	4 + micro	5	6 / 7?	7	8	9	n/a	Z	n/a	82	Schmid et al., 2000 / Guttembach et al., 2003
Golden pheasant (*Chrysolophus pictus*)	Phasianidae	1	3 + 6	2	4 + micro	5	6 7?	7	8	9	n/a	?	n/a	82	Schmid et al., 2000 / Guttembach et al., 2003
Silver pheasant (*Lophura nycthemera*)	Phasianidae	1	3 + 6	3-Feb	4 + micro	5	6	7	8	9	n/a	Z	n/a	80	Schmid et al., 2000 / Shibusawa et al., 2004b
Red-legged partridge (*Alectorious rufa*)	Phasianidae	1	2	3	4	5	6	7	8	9	n/a	Z	n/a	78	Kasai et al., 2003
Lady Amherst's pheasant (*Chrysolophus amherstiae*)	Phasianidae	1	3 + 6	3	4 + micro	5	6	7	8	9	n/a	Z	n/a	?	Shibusawa et al., 2004b
Turkey (*Meleagris gallopavo*)	Phasianidae	1	3 + 6	3	4 + micro	5	6	7	8	9	n/a	Z	n/a	?	Shibusawa et al., 2004b
Western capercaillie (*Tetrao urogallus*)	Phasianidae	1	3 + 7	3	4 + micro	5	5q	7	5p	9	n/a	Z	n/a	?	Shibusawa et al., 2004b
Chinese bamboo-partridge (*Bambusicola throracica*)	Phasianidae	1	2	3	4	5	6	7	8	9	n/a	Z	n/a	?	Shibusawa et al., 2004b
Common peafowl (*Pavo*	Phasianidae	1	2	3	4	5	6	6q	7q	7p	n/a	Z	n/a	?	Shibusawa et al., 2004b

cristatus)															
Scarlet Macaw (*Ara macao*)	Psittacidae	1q + 4 + 9q	2	3	8q	5p	6q ^	6q ^	7^	7q^	n/a	Z	no signal	62-64	Seabury et al., 2013
Peach-faced lovebird (*Agapornis roseicollis*)	Psittacidae	3 + 4p	2p + 9p^	1	4p^ + 7	8p^	6p^	6p^	5p^	5p^ + 9p^	10p ^	n/a	n/a	48	Nanda et al., 2007
Budgerigar (*Melopsittacus undulatus*)	Psittacidae	3 + 6	1	2	5p^ + 7q	4q	4p^ + 8p	4p^	5^	5q^	9q	n/a	n/a	62	Nanda et al., 2007
Rhea (*Rhea americana*)	Rheidae	?	?	?	?	?	6	7	8	9	n/a	?	n/a	80	Schmid et al., 2000
Great grey owl (*Strix nebulosa*)	Strigidae	1	?	3	4 + micro ?	5	6	7	8	?	n/a	?	n/a	82	Schmid et al., 2000
Eagle owl (*Bubo bubo*)	Strigidae	?	?	?	1p?	?	?	?	8	?	n/a	?	n/a	80	Schmid et al., 2000
Blackbird (*Turdus merula*)	Turdidae	2 + 5	1	3	4 + micro	6?	6	7	8	9	n/a	n/a	n/a	80	Guttembach et al., 2003
Redwing (*Turdus iliacus*)	Turdidae	2 + 5	1	3	4 + micro	6	7	8	9	10	micr o	Z + Wq	n/a	80	Derjusheva et al., 2004
Japanese quail (*Coturnix coturnix japonica*)	Turnicidae	1	2	3	4	5	6	7	8	9	n/a	Z	n/a	78	Schmid et al., 2000 / Guttembach et al., 2003
Blue-breasted quial (*Coturnix chinensis*)	Turnicidae	1	2	3	4	5	6	7	8	9	n/a	Z	n/a	78-80	Shibusawa et al., 2004a
California quail (*Callipepla californica*)	Turnicidae	1	2	3	4	5	6	7	8	9	n/a	Z	n/a	78-80	Shibusawa et al., 2004a

^ Interstitial

? Not clear or not shown in the refer

N/A not applicable (probe not used i

Objectives

1. To reveal and describe the karyotype of the Puerto Rican Parrot
2. To unravel the inter-chromosomal rearrangements using Fluorescent In Situ Hybridization with chicken flow-sorted chromosomes.
3. To confirm such rearrangements using *in silico* methods and a PCR-based *de novo* approach.

Materials and Methods

Avian Genomes database

The assemblies used as reference for the chicken genome and zebra finch were May 2006-WUGSC 2.1/galGal3 and Jul. 2008-WUGSC 3.2.4/taeGut1 respectively.

Scaffold Alignments

The first and last 25 kb of the Puerto Rican parrot biggest 3,099 ALLPATH 2012 assembly scaffolds were used to generate alignments against the chicken reference genome using USCS Genome Browser's BLAT (http://genome.ucsc.edu/cgi-bin/hgBlat?command=start). The chicken chromosome number, the genome coordinates, the direction of DNA strand (+/-) and the alignment score were documented. The text editor software used to open the scaffolds FASTA files was TextPad® version 5.

Scaffold chromosomal rearrangement filtering

Out of the 164 putative chromosomal rearrangements (Supplemental Table 1), a selection was made of the ones with a score equal or higher than 1000 at both ends and that not involved a sex chromosome. As a result, only 23 passed (Supplemental Table 2) the filter and a screen for their chromosome-joint position was followed. The screening was performed in the UCSC BLAT database by aligning 25 kb at a time. Once the position where the chromosome number changed was identified, smaller fragments were continued to be aligned against the chicken genome in order to increase the resolution of the rearrangement site (preferably down to less than 20 kb nucleotides long).

Primer design

Integrative DNA Technologies' Primer Quest tool (http://www.idtdna.com/Primerquest/Home/Index) was used to generate primer sets with the following custom parameters: Primer size (min 21, opt 24, max 27) and Max 2˚ TM difference. The chromosomal joint segment was entered as query and the most centralized primer set, which amplified the smallest segment possible from sites at each ancestral chromosome, was selected (Supplemental Table 3). Further alignments of the selected primers were performed against the zebra finch genome and double-checked with the chicken as well to confirm their evolutionary conservation and their single copy presence in these genomes.

Bird DNA samples

Blood samples of one specimen of the Puerto Rican parrot (*Amazona vittata*, AVI), blue-fronted amazon (*Amazona aestiva*, AAE), double-yellow headed amazon (*Amazona oratrix*, AOR), yellow-naped amazon (*Amazona auropalliata*, AAU), African grey parrot (*Psittacus erithacus*, PER), scarlet macaw (*Ara macao*, AMA) and blue-and-gold macaw (*Ara ararauna*, AAR) each were obtained from Bosque Río Abajo Aviary in Arecibo and Sr. Rosado's Pet Shop in Aguada, Puerto Rico. DNA was isolated using Qiagen's DNeasy Blood & Tissue Kit.

PCR validation

Because of large amounts of repetitive sequences in the "rearrangements zones", the necessity to amplify segments longer than appropriate for the standard Taq DNA Polymerase was evident. Thus, New England Biolabs' LongAmp® Taq DNA Polymerase was chosen for the PCR reactions. Supplemental table 4 displays the primer sets designed, the amplicons sizes and the respective annealing temperatures.

AVI Lymphocyte cell culture and harvest

-Lymphocyte separation

Two methods were tested for lymphocyte separation. The first one was the slow-spin centrifugation technique described in Lavoie and Grasman, 2005, (adapted from Hovi et al., 1976 and Weber, 1990). PR parrot blood was centrifuged at 120 g in the heparin collection tube for 20 min. With a sterile plastic Pasteur pipet the buffy coat was swirled up and off the red blood cells bottom layer and the supernatant (containing the lymphocytes) was transferred carefully to a sterile tube. The second method used Histopaque 1077 solution adding 1:1 of Hystopaque to blood in a 15 mL Falcon tube and centrifuging for 30 min at 400 g. Following centrifugation, four layers are formed (plasma, lymphocytes, Histopaque and red blood cells), and using also a sterile plastic Pasteur pipet the opaque interphase containing the lymphocytes was transferred to a clean falcon tube. The latter yield more lymphocyte cells per volume of blood.

Lymphocyte cell culturing and harvesting were performed following the unpublished Chicken Lymphocyte Protocol of Dr Darren Griffin's Laboratory in Kent University, UK. Lymphocytes collected were then washed with 10 mL of PBS and centrifuged at 250 g for 10 min at room temperature. The supernatant was discarded and the wash was repeated with 5 mL with further centrifugation. Again, the supernatant was discarded leaving about 0.5 mL and resuspending the pellet in it. The cells were then transferred to a 25 cm^2 culture flask previously filled with 10 mL of media (RPMI 1640 Glutamax, 10% Chicken serum, 1% antibiotic-antimycotic (100X) and 100

μg/mL Concanavilin A type IV) and incubated at 39.5˚ C (5% CO_2) for 72 hours in a cell culture incubator.

After the incubation period, 50 μL of demecolcine solution in HBSS (10 μg/mL) was added to the flask and was left in the incubator for another hour. Next, the medium with cells was transferred to a 15 mL Falcon tube and centrifuged for 5 min at 400 g at room temperature. The supernatant was discarded. With a timer set for 15 min and a plastic Pasteur pipet on hand, 6 mL of 0.075 M KCl hypotonic solution (pre-warmed at 39˚ C) were added drop-wise while re-suspending the cells by gently flicking the tube with the fingers. For the rest of the 15 min, the tube was incubated in a water bath at 39˚ C.

In order to fix the cells, 8 mL of ice-cold 3:1 Methanol:Acetic acid solution were poured along the side of the tube and gentle mixing was performed by tube inversion. The tube was centrifuged at 400 g for 5 min at room temperature. The supernatant was discarded, leaving only about 1 mL and the cells were re-suspended by gently pipetting up and down with a plastic Pasteur pipet. In a drop-wise manner, 5 mL of fixative were added and the tube again was centrifuged at 400 g for 5 min at room temperature, discarded the supernatant (leaving about 1 mL) and resuspended with Pasteur pipet. These steps were repeated 3 times or more, until the cell suspension would appear clear and cells would turn white.

After the final centrifugation, the supernatant was discarded leaving about 500 μL and this was resuspended and transferred to a 1.5 mL tube. The tube was then centrifuged at 6,000 rpm for 5 min at room temperature and the supernatant was carefully removed leaving about 100 μL of cell suspension. Slides were prepared as followed: a pre-warmed at 37˚C clean slide in ddH_2O was hold in a 45˚ angle, while 6 μL of cell suspension (previously mixed by flicking the tube) was added to the top part of the slide and the drop was mouth-blown to spread along the slide. The slide was left to air dry still in the 45˚ angle. Finally, metaphases were screened using the phase-contrast filter of a Zeiss Axioplan2 microscope with objective 20X.

Fibroblast cell culture and harvest

-Feather pulp

Upon the arrival of 5 AVI feathers, they were wrapped in 70% ethanol towels and left overnight at 4^{o}C. In a sterile environment, the contents of the feathers were then removed and washed with HBSS + 1% antibiotic-antimycotic (100X) solution 6-8X. Using a scalpel, the pulps were cut into small pieces and incubated in 500 μL of 5% Collagenase at 37˚C for approximately 1hr or as long as it took for the pulp to disintegrate; taping the tube to mix every 15 min. These were transferred to a

T25 flask, to which 5 mL of α-DMEM media with nucleosides, 15% FBS and 1% of 100X antibiotic-antimycotic were added. They were incubated at 39.5˚C and 5% CO_2 changing the media every three days and checking for contamination under a phase-contrast microscope.

-Skin biopsy

Dr. Bernardo L. Soler-Mena kindly performed a skin biopsy on a Blue-fronted Amazon. The tissue was sent overnight to the laboratory and the next day it was washed with HBSS + antis solution 6-8 times and cut into small pieces. Twelve pieces per T25 flask were arranged, each flask containing a 1 mL of α-DMEM media with nucleosides, 15% FBS and 1% of 100X antibiotic-antimycotic. They were incubated at 39.5˚C and 5% CO2 changing the media (3 mL) every three days and checking for contamination under a phase-contrast microscope.

-Embryo

After 2 recently dead embryos were shipped to the lab, their skin was carefully removed and processed in the same manner as for the skin biopsy tissues.

Puerto Rican Parrot karyotyping

The metaphases were stained with 5% Giemsa stain and captured with the CCD camera of a Zeiss Axioplan2 microscope and chromosomes were arranged in a size decreasing order using the software Isis V5.2 (MetaSystems GmbH).

Zebra finch BAC DNA acquisition, isolation and labeling

The PCR-confirmed chromosomal rearrangements regions were aligned against the zebra finch genome and the orthologous chromosomal segment of both sides of the rearrangements were searched for. The genomic coordinates were then used to search for the BAC clones containing the DNA segments in the TGMCBa zebra finch BAC library of the Clemson University Genomic Institute (CUGI). The BAC ID clones and the zebra finch genomic coordinates for each chromosomal rearrangement are found in Supplemental Table 4.

Upon arrival of the zebra finch (zf) BAC clones, cultures were set up in agar plates with 25 µg/mL chloramphenicol overnight. To isolate the DNA, the Mini Scale Preparation of the PhasePrep™ BAC DNA Kit (Sigma-Aldrich®) was followed. Zebra finch DNA recovery was confirmed through agarose electrophoresis and to verify precise cloning, the cloning site was sequenced.

Biotin-16-dUTP and Digoxigenin-11-dUTP Nick Translation kit (Roche®) was utilized to label the zf BAC DNA. A mixture of 10 µL of zf BAC DNA, 4 µL of ddH_2O and 4 µL of the kit master mix was prepared and for the reaction to occur the tube was incubated in a water bath at 15° C for 1 hr and 30 min. Using Sephadex columns the contents of the labeling reaction were purified by centrifuging for 3 min at 3,600 rpm at room temperature. A 1% agarose gel confirmed the labeling. Furthermore, to reduce non-specific binding, 20 µL of 1ng/µL zebra finch genomic DNA were added to the tubes and dried the contents completely using a Vacufuge machine at 60° C. Finally, ddH_2O and hybridization master mix (70% deionized formamide, 14% dextran sulfate sodium salt, and 3X SSC) were added in the ratio of 3:7 respectively.

Chicken whole-chromosome paints acquisition

The following flow-sorted whole-chicken chromosome paints were kindly provided by the lab of Dr. Darren K. Griffin (Cambridge University, UK) and Dr. H. Sherthan (who is was the first person to ever perform a Zoo-FISH experiment): GGA1, GGA2, GGA3, GGA4, GGA5, GGA6, GGA7, GGA8, GGA9 and GGAZ. N=10.

1st Round DOP-PCR:

	Volume per sample	Initial concentration	Final concentration
• ddH_2O	30.0 µL	-	-
• PCR buffer	5.0 µL	10X	1X
• $MgCl_2$	4.0 µL	25 mM	2.0 mM
• dNTPs	5.0 µL	2 mM	0.2 mM
• Universal Primer I	1.5 µL	50 µM	1.5 µM
• Taq Gold DNA Pol	0.5 µL	5 U/µL	2.5 U (0.05 U/µL)
• GGA chr DNA	4.0 µL		
	50.0 µL total		

The PCR program was set as following: 95˚ C 10 min, 25 cycles (94˚ C 1min, 56 ˚ C 1 min, 72˚ C 1min), 72 ˚ C 5 min, and rest at 12 ˚ C until removed.
Results were confirmed through 1% agarose gel-electrophoresis.

2nd Round DOP-PCR

Two reactions per GGA chromosome paint were prepared (two 50 µL reactions per GGA chromosome).

	Volume per sample	Initial concentration	Final concentration
• ddH_2O	34.0 µL	-	-
• PCR buffer	5.0 µL	10X	1X
• $MgCl_2$	4.0 µL	25 mM	2.0 mM

• dNTPs	0.5 µL	2 mM	0.2 mM
• Universal Primer I	1.5 µL	50 µM	1.5 µM
• Taq Gold DNA Pol	1.0 µL	5 U/µL	5 U (0.1 U/µL)
• GGA chr DNA	4.0 µL		
	50.0 µL total		

The PCR program was set as following: 95° C 10 min, 25 cycles (94° C 1min, 56 ° C 1 min, 72° C 1min), 72 ° C 5 min, and rest at 12 ° C until removed.

The two reactions for each chromosome were pooled together and purified the DNA through Sephadex columns. Subsequently, the contents were dried to a final volume of 50 µL using a Vacufuge at 60° C. Results were confirmed through 1% agarose gel-electrophoresis. Following the vacuum centrifugation the PCR products were purified a second time using Qiagen's QIAquick PCR Purification kit.

Labeling DOP-PCR

Chicken whole chromosome paints label arrangement

Chicken chromosome paint:	Label:
GGA1	Biotin-16-dUTP
GGA2	Biotin-16-dUTP
GGA3	Biotin-16-dUTP
GGA4	Biotin-16-dUTP
GGA5	Biotin-16-dUTP
GGA6	Digoxigenin-11-dUTP
GGA7	Biotin-16-dUTP
GGA8	Digoxigenin-11-dUTP
GGA9	Biotin-16-dUTP
GGAZ	Biotin-16-dUTP

The labeling PCR reaction with Biotin-16-dUTP (green) and Digoxigenin-11-dUTP (red) was performed as follows:

	Volume per sample	Initial concentration	Final concentration
• ddH_2O	30.5 µL	-	-
• PCR buffer	5.0 µL	10X	1X
• $MgCl_2$	4.0 µL	25 mM	2.0 mM
• dNTPs label*	0.5 µL	2 mM	0.02 mM
• Universal Primer I	1.5 µL	50 µM	1.5 µM
• Taq Gold DNA Pol	1.0 µL	5 U/µL	5 U (0.1 U/µL)
• Label (Bio or Dig)	3.5 µL	1 mM	0.07 mM
• GGA chr DNA	4.0 µL		
	50.0 µL total		

*2mM for dCTP, dATP and dGTP. 1.3mM for dTTP.

The PCR program was set as following: 95° C 10 min, 25 cycles (94° C 1min, 56 ° C 1 min, 72° C 1min), 72 ° C 5 min, and rest at 12 ° C until removed.

Furthermore, the labeled DNA was purified with Sephadex columns and labeling was confirmed through 2% agarose gel electrophoresis. To prepare the hybridization probes, 20 µL of chicken *Cot-1* DNA were added to the products of the labeling PCR and they were dried completely by using a Vacufuge at 60˚ C. Finally, 3 µL of ddH2O and 7 µL hybridization buffer (70% deionized formamide, 14% dextran sulfate sodium salt, and 3X SSC) were added to each tube and stored at -20˚ C for later Zoo-FISH use.

Fluorescent In Situ Hybridization

For the technique, Raudsepp & Chowdhary protocol in Methods in Molecular Biology: Phylogenomics book (Murphy WJ, 2008) was followed.

Hybridization zones with good metaphases were identified under a phase-contrast objective and marked with a square by scrapping the glass with a diamond tip pen. Next, 500 µL of RNAse 1X solution, prepared from 10X stock solution (Fisher Scientific 1 mg/mL in 2X SSC), was added to the slide and the slide was coverslipped, put in a moist chamber and incubated at 37˚ C for one hour. After the incubation, the coverslip was slid off and discarded and the slide was rinsed for 2 min with 2X SSC. Subsequently, the chromosomes in the slide were denatured for 2 min in a 70% formamide solution pre-warmed and kept at 70˚ C in a water bath. Immediately after, the slide was dipped for 2 min in ice-cold 70% ethanol and dehydrated in 80%, 90% and 100% ethanol series by immersing the slides in each solution for two min each. Meanwhile, the probes were denatured by incubating them at 80˚ C for 12 min and pre-annealed at 37˚ C for 40 min. On the slide, 1.2 µL of each probe was added to a respective hybridization square and covered with "minicoverslips" that were prepared by cutting large ones into tiny squares with a diamond tip pen. The coverslipped hybridization zones were sealed with plenty amount of rubber cement and the slide was placed in a moist chamber and incubated overnight for same species hybridization (control) and 72 hr for Zoo-FISH.

For dual-color FISH, three antibody-for-detection layers were prepared per slide as follows:
1st layer- 100 µL of 1X blocking solution, 300 µL of 0.1M Na_2HPO_4-0.1M KH_2PO_4-0.1% IGEPAL CA-630 (Sigma Aldrich) buffer (pH 8), 1.2 µL of avidin FITC (fluorescein-avidin D) stock solution and 1.6 µL of antidig stock solution. 2nd layer- 100 µL of 1X blocking solution, 300 µL of buffer, 4 µL of antiavidin D stock solution and 0.8 µL of antimouse Ig-dig stock solution. 3rd layer- 100 µL of 1X blocking solution, 300 µL of buffer, 1.2 µL of avidin FITC (fluorescein-avidin D) stock solution and 2 µL of antidig-rhodamine stock solution. The 1X Blocking solution was prepared by diluting 5X in-

situ hybridization blocking solution (Vector Laboratories) with 0.1M Na_2HPO_4-0.1M KH_2PO_4-0.1% IGE-PAL CA-630 (Sigma Aldrich) buffer (pH 8).

After incubation time finished, the slide was taken out from the moist chamber and the rubber cement was removed using tweezers. The slide was rinsed in 2X SSC for 2 min in order to remove the coverslips and washed three times with 50% formamide in a water bath at 40˚ C for 5 min each. Then, the slide was immersed in 3 jars of 4X SSC + 0.05% Tween-20 and a jar of 4X SCC for 2 min each with gentle shaking. The 1st antibody layer was added and the slide was coverslipped and incubated inside a moist chamber at 37˚ C for 30 min. Again the slide was immersed in 3 jars of 4X SSC + 0.05% Tween-20 and a jar of 4X SCC for 2 min each with gentle shaking. The latter was repeated with 2nd and 3rd layer with the washes after each incubation period. Finally each hybridization zone was mounted in 10 µL DAPI-antifade, coverslipped (24x50mm) and visualized and/or stored in dark at -20˚ C. The pictures were taken with the CCD camera of a Zeiss Axioplan2 microscope with DAPI, FITC and Dig filters using the software Isis V5.2 (MetaSystems GmbH).

**In silico* analysis and PCR validations were performed at Dr. Juan Carlos Martínez-Cruzado's & Dr. Taras Oleksyk's laboratory in the University of Puerto Rico at Mayagüez; while Zebra finch BAC isolation was executed in Dr. Erich Jarvis' laboratory at Duke University; and the cell culturing, karyotyping and Zoo-FISH parts took place at Dr. Terje Raudsepp's laboratory in Texas A&M University.

Results

Karyotype

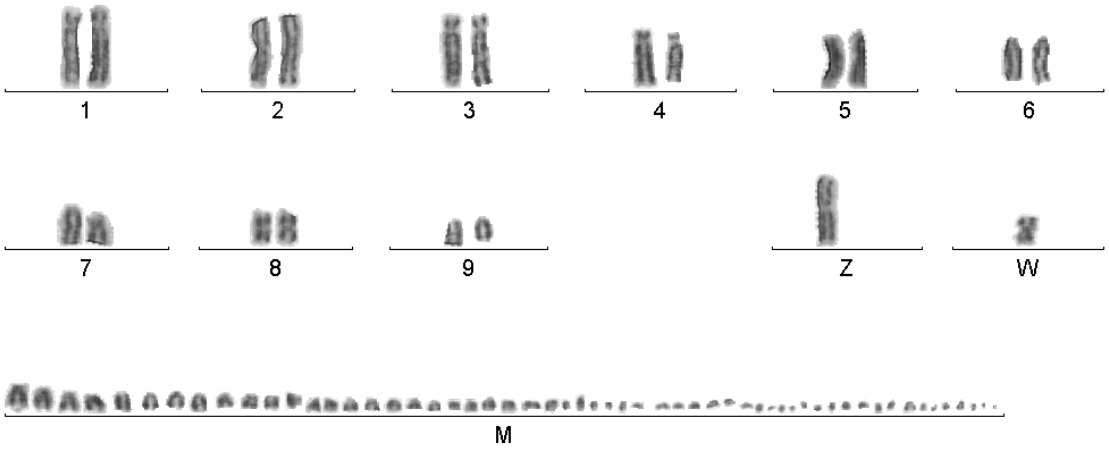

Figure 2. Karyotype of *Amazona vittata.* Giemsa stained**.**

Out of the methods applied for the acquisition of metaphases, only the lymphocyte separation method yield sufficient metaphases for appropriate karyotyping and comparative

cytogenetic studies. Giemsa-stained Puerto Rican parrot chromosomes of a hundred lymphocyte cells studied revealed a consistent diploid number of 76 (Figure 2). Table 2 shows the mean, mode and median calculated giving a total of 76 for the three statistics.

Only 10 pairs of chromosomes were notably distinguishable under light-microscope evaluation. Thus, 9 pairs of macrochromosomes were arranged in size descending order and sexual chromosomes Z and W were identified. However, it was impossible to distinguish more pairs of chromosomes in all metaphases, due to their short size. Nevertheless, microchromosomes were positioned all together and all 56 microchromosomes were accounted for.

Table 2. *Amazona vittata* chromosome counting

Cell #	Animal ID	Gender	Slide ID	Macros	Micros	Total (2N)	Picture #	Location on slide
1	3♀	Female	7-30-14(1)	20	56	**76**	8	M24.1
2	3♀	Female	7-30-14(1)	18	56	**74**	9	L25.2
3	3♀	Female	7-30-14(1)	20	55	**75**	10	M22.1
4	3♀	Female	7-30-14(1)	20	53	**73**	11	N22.3
5	3♀	Female	7-30-14(1)	20	59	**79**	14	K25.3
6	3♀	Female	7-30-14(1)	18	59	**77**	16	L28.1
7	3♀	Female	7-30-14(1)	18	57	**75**	18	L30.3
8	3♀	Female	7-30-14(1)	20	56	**76**	19	K30.1
9	3♀	Female	7-30-14(1)	20	54	**74**	20	Q31.1
10	3♀	Female	7-30-14(1)	20	54	**74**	21	P31.3
11	3♀	Female	7-30-14(1)	18	59	**77**	22	L32.4
12	3♀	Female	7-30-14(1)	20	55	**75**	23	N33.4
13	3♀	Female	7-30-14(1)	20	57	**77**	24	M33.2
14	3♀	Female	7-30-14(1)	18	58	**76**	25	O35.1
15	3♀	Female	7-30-14(1)	18	58	**76**	26	N35.3
16	3♀	Female	7-30-14(1)	18	58	**76**	27	N35.1
17	3♀	Female	7-30-14(1)	20	52	**72**	28	L35.4
18	3♀	Female	7-30-14(1)	20	56	**76**	29	N35.2
19	106314♂	Male	7-30-14(2)	20	52	**72**	30	L33.3
20	106314♂	Male	7-30-14(2)	18	56	**74**	31	O28.1
21	106314♂	Male	7-30-14(2)	18	53	**71**	32	L28.3
22	3♀	Female	7-30-14(6)	20	54	**74**	33	O54.4A
23	3♀	Female	7-30-14(6)	20	53	**73**	34	O54.4B
24	3♀	Female	7-30-14(6)	20	56	**76**	35	O54.4C
25	3♀	Female	7-30-14(6)	20	59	**79**	37	N54.4
26	3♀	Female	7-30-14(6)	20	56	**76**	38	M55.1
27	3♀	Female	7-30-14(1)	20	56	**76**	39	Q37.2
28	3♀	Female	7-30-14(1)	20	57	**77**	40	Q38.2
29	3♀	Female	7-30-14(1)	20	59	**79**	41	N38.4
30	3♀	Female	7-30-14(1)	22	54	**76**	42	O39.3
31	3♀	Female	7-30-14(1)	20	57	**77**	43	N39.2
32	3♀	Female	7-30-14(1)	20	58	**78**	44	O39.4
33	3♀	Female	7-30-14(1)	20	54	**74**	45	L40.1

34	3♀	Female	7-30-14(1)	20	56	**76**	46	M39.2
35	3♀	Female	7-30-14(1)	20	56	**76**	47	N40.3
36	3♀	Female	7-30-14(1)	20	56	**76**	48	L41
37	3♀	Female	7-30-14(1)	20	55	**75**	49	N41
38	3♀	Female	7-30-14(1)	22	51	**73**	50	O41.1
39	3♀	Female	7-30-14(1)	20	56	**76**	51	P41
40	3♀	Female	7-30-14(1)	20	56	**76**	52	M41.4
41	3♀	Female	7-30-14(1)	20	58	**78**	53	L41.4
42	3♀	Female	7-30-14(1)	20	56	**76**	55	L43.3
43	3♀	Female	7-30-14(1)	20	58	**78**	56	M43.1
44	3♀	Female	7-30-14(1)	22	54	**76**	57	N42
45	3♀	Female	7-30-14(1)	20	56	**76**	58	P42.2
46	3♀	Female	7-30-14(1)	20	55	**75**	60	P43.4
47	3♀	Female	7-30-14(1)	20	56	**76**	61	M43.4
48	3♀	Female	7-30-14(1)	20	58	**78**	62	M43.2
49	3♀	Female	7-30-14(1)	22	56	**78**	63	P44.4
50	3♀	Female	7-30-14(1)	20	56	**76**	64	P44.2
51	3♀	Female	7-30-14(1)	22	53	**75**	65	N44.2
52	3♀	Female	7-30-14(1)	20	55	**75**	66	M44
53	3♀	Female	7-30-14(1)	22	50	**72**	67	M44.2
54	3♀	Female	7-30-14(1)	20	56	**76**	69	M45.3
55	3♀	Female	7-30-14(1)	18	60	**78**	70	N45.3
56	3♀	Female	7-30-14(1)	18	57	**75**	71	Q45.2
57	3♀	Female	7-30-14(1)	20	56	**76**	72	N45.4
58	3♀	Female	7-30-14(1)	20	56	**76**	74	N46
59	3♀	Female	7-30-14(1)	20	55	**75**	75	N47.3
60	3♀	Female	7-30-14(1)	20	56	**76**	78	N48
61	3♀	Female	7-30-14(1)	20	56	**76**	79	M47.2-4
62	3♀	Female	7-30-14(1)	20	56	**76**	81	P49.1
63	3♀	Female	7-30-14(1)	20	56	**76**	84	P51.3
64	3♀	Female	7-30-14(1)	20	56	**76**	85	N50.4
65	3♀	Female	7-30-14(6)	20	55	**75**	88	K54.3
66	3♀	Female	7-30-14(6)	20	54	**74**	89	K52
67	3♀	Female	7-30-14(6)	20	56	**76**	90	K53.1
68	3♀	Female	7-30-14(6)	20	56	**76**	91	M53.1
69	3♀	Female	7-30-14(6)	20	56	**76**	93	K55.2B
70	3♀	Female	7-30-14(6)	20	57	**77**	94	K48.4-K49.3
71	3♀	Female	7-30-14(6)	20	54	**74**	96	K50
72	3♀	Female	7-30-14(6)	20	56	**76**	97	K53.4-K54.3
73	3♀	Female	7-30-14(6)	22	57	**79**	98	K55
74	3♀	Female	7-30-14(6)	20	56	**76**	99	L48
75	3♀	Female	7-30-14(6)	20	55	**75**	100	L50.4
76	3♀	Female	7-30-14(6)	20	58	**78**	101	M47.2
77	3♀	Female	7-30-14(6)	20	58	**78**	105	M48.1
78	3♀	Female	7-30-14(6)	20	59	**79**	107	O48.2
79	3♀	Female	7-30-14(6)	20	56	**76**	109	P47
80	3♀	Female	7-30-14(6)	20	56	**76**	110	P53.4
81	3♀	Female	7-30-14(6)	20	58	**78**	111	Q54
82	3♀	Female	7-30-14(6)	20	56	**76**	112	Q54.1

83	3♀	Female	7-30-14(6)	19	55	**74**	113	Q52-Q53
84	3♀	Female	7-30-14(6)	22	54	**76**	114	R52A
85	3♀	Female	7-30-14(6)	22	54	**76**	114	R52B
86	3♀	Female	7-30-14(6)	20	56	**76**	115	R51.2
87	3♀	Female	7-30-14(6)	20	56	**76**	116	R51-R52
88	3♀	Female	7-30-14(6)	18	58	**76**	117	P47.4
89	3♀	Female	7-30-14(6)	18	57	**75**	118	P53.2
90	3♀	Female	7-30-14(6)	20	56	**76**	119	M54-M55
91	3♀	Female	7-30-14(6)	20	56	**76**	120	R49.4
92	3♀	Female	7-30-14(6)	20	56	**76**	121	R49.2
93	3♀	Female	7-30-14(6)	20	53	**73**	124	R50.4
94	3♀	Female	7-30-14(6)	18	56	**74**	128	O54.3
95	3♀	Female	7-30-14(6)	20	56	**76**	129	O54.1
96	3♀	Female	7-30-14(6)	20	57	**77**	131	P54.1
97	3♀	Female	7-30-14(6)	20	56	**76**	133	O48.4
98	3♀	Female	7-30-14(6)	20	56	**76**	134	N47
99	3♀	Female	7-30-14(6)	20	56	**76**	135	M47
100	3♀	Female	7-30-14(6)	20	56	**76**	136	M47-M48

Average:	**75.79**
Median:	**76**
Mode:	**76**
STD	**1.563937339**

Furthermore, the first three pairs of macrochromosomes are very similar in size and although pair 2 is telocentric, pair 1 and 3, are both submetacentric and are hard to distinguish solely on Giemsa-stained metaphases. Still, pairs 1 and 3 can be differentiated when using DAPI-inverted filter (Barros e Silva AE and Guerra M, 2010), since an AT-poor heterochromatic region at the end of the q arm of chromosome 1 serves as a feature to distinguish it among the three pairs (Figure 3).

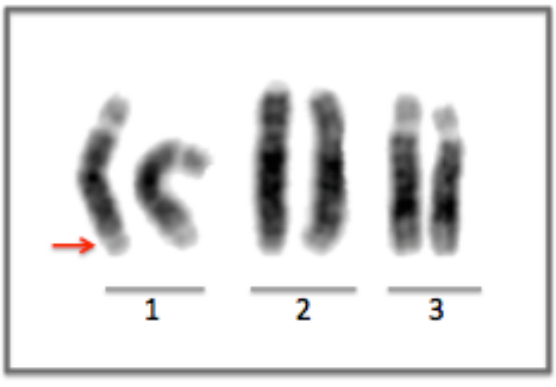

Figure 3. AVI 1-3 DAPI-inverted chromosomes. Red arrow shows DAPI (-) banding in AVI1.

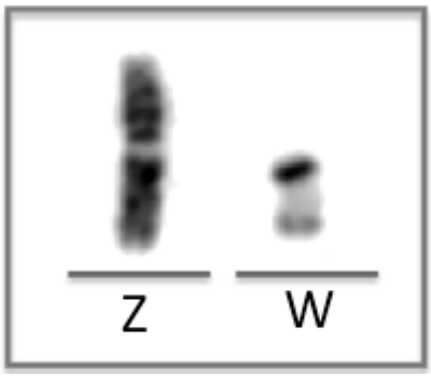

Figure 4. DAPI-inverted AVI Z and W chromosomes.

Pairs 4 and 5 are submetacentric and are also very similar in size. In some cells their size difference was enough to arrange them, however that was not the general case. Thus, these two pairs of chromosomes could only be truly distinguished in the future through the use of chromosome-specific molecular probes. On the other hand, pairs 6 and 7 are both telocentric but their size difference is notable. Pair 8 is metacentric and pair 9 telocentric.

Perhaps, pair 8 and sex chromosome W can also cause identification problems due to their similar size and centromere position (metacentric). Yet, DAPI-inverted filtering reveals differences in heterochromatin that facilitates rapid identification. Finally chromosome Z is about the size of the first three chromosomes. It was designated as a large, metacentric chromosome (Figure 4).

PCR validation

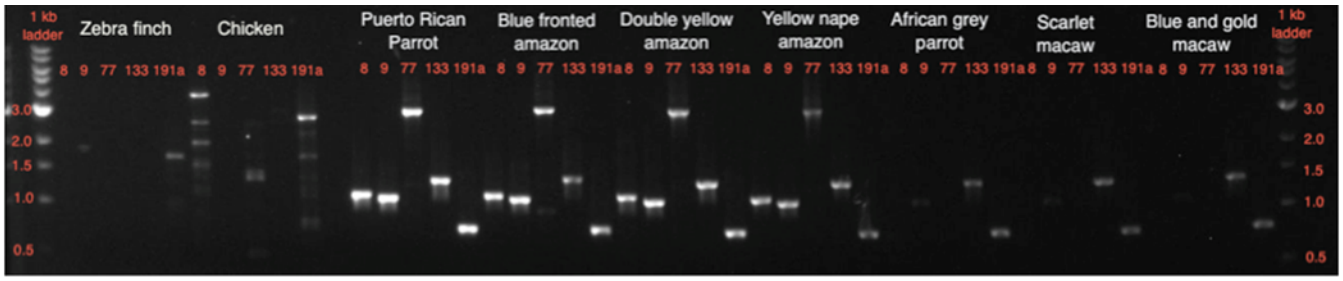

Figure 5. PCR validation of chromosomal rearrangements analyzed through a 2% agarose gel electrophoresis. Primers are labeled according to their scaffold and are shown horizontally in red. Vertically in red are the bands of 1kb NEB® ladder. The predicted sizes of the amplicons were 1151 bp (Sc. 8, joining chromosomes 2 and 15), 1120 bp (Sc. 9, joining chromosomes 3 and 9), 2948 bp (Sc. 77, joining chromosomes 6 and 7), 1351 bp (Sc. 133, joining chromosomes 6 and 7) and 793 bp (Sc 191a, joining chromosomes 8 and 9).

Five Puerto Rican parrot chromosomal rearrangements predicted by BLAT alignments were confirmed in ALLPATH 2012 scaffolds 8, 9, 77, 133 and 191a (first of two rearrangements predicted in the scaffold) (Figure 5). As expected, zebra finch and chicken controls gave unspecific or no amplification. In addition, the five predicted chromosomal rearrangements were also confirmed for the three other species of Amazon parrots. The African grey parrot and the two species of macaws studied also gave positive amplification for the three rearrangements in scaffolds 9, 133 and 191a. The sizes of the amplicons were as predicted from the distances between the primer sequences in the scaffolds (supplemental table 4), confirming that these were not unspecific amplicons.

Consequently, chromosome 3-9, 2-15, 6-7(2) and 8-9 rearrangement types were confirmed among the Amazon parrots using the PCR approach. The rearrangements uncovered for the African grey parrot and the macaws were 2-15, 6-7 (1) and 8-9 correspondingly.

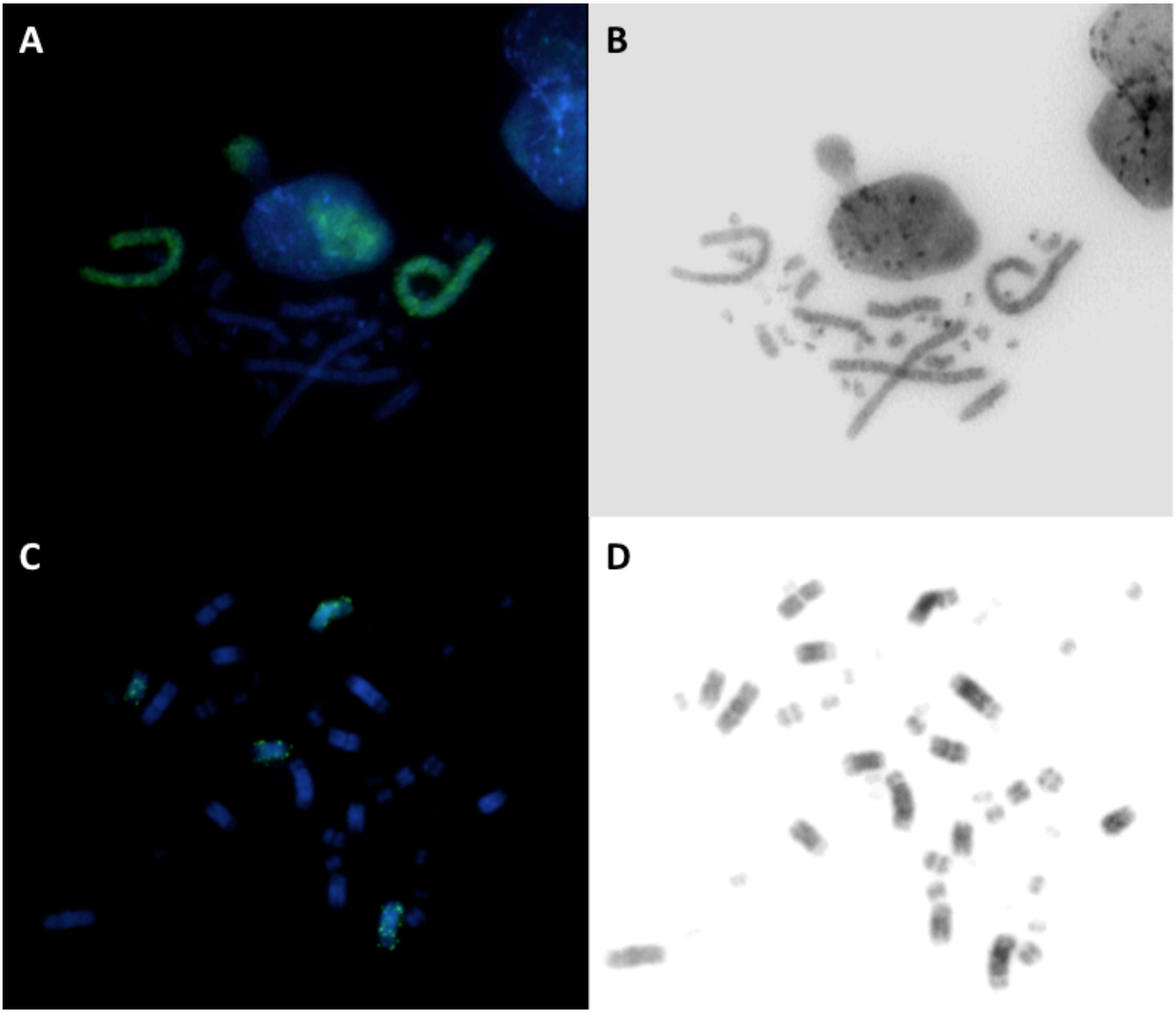

Figure 6. Chicken (GGA) whole chromosome paint 1 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

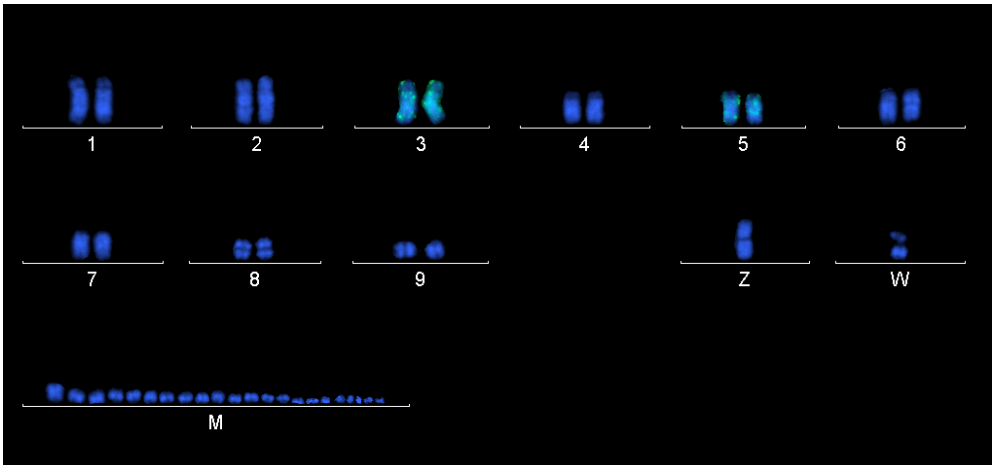

Figure 7. *A. vittata*'s karyotype result of GGA whole chromosome 1 Zoo-FISH.

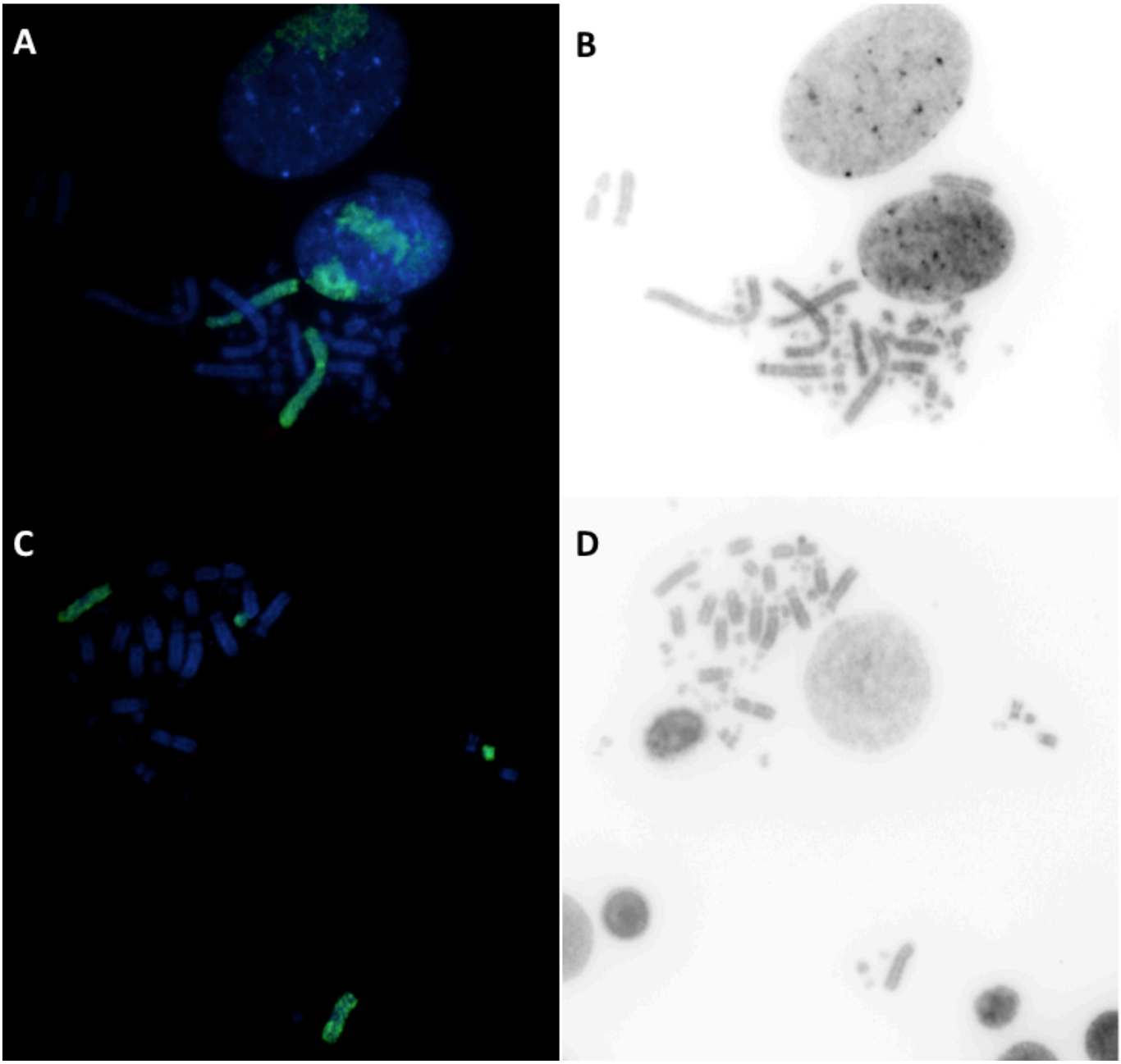

Figure 8. Chicken (GGA) whole chromosome paint 2 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

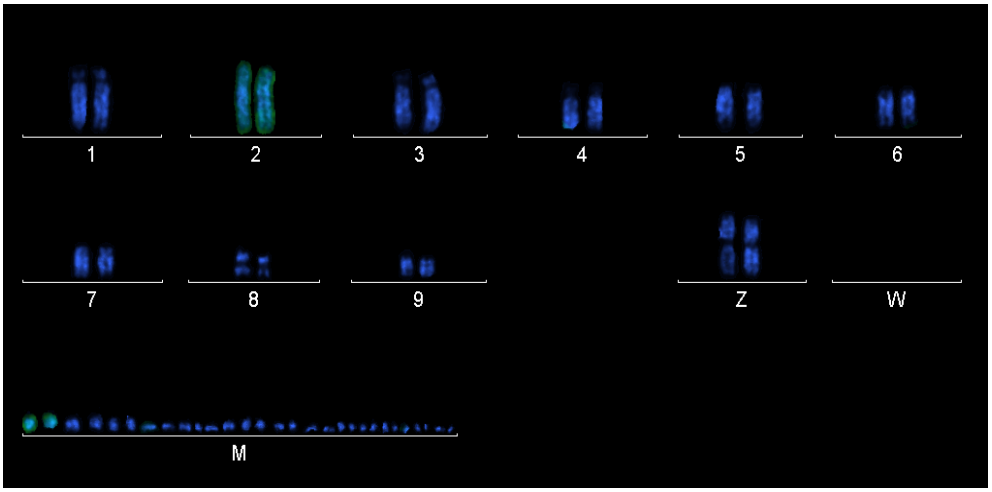

Figure 9. *A. vittata*'s karyotype result of GGA whole chromosome 2 Zoo-FISH.

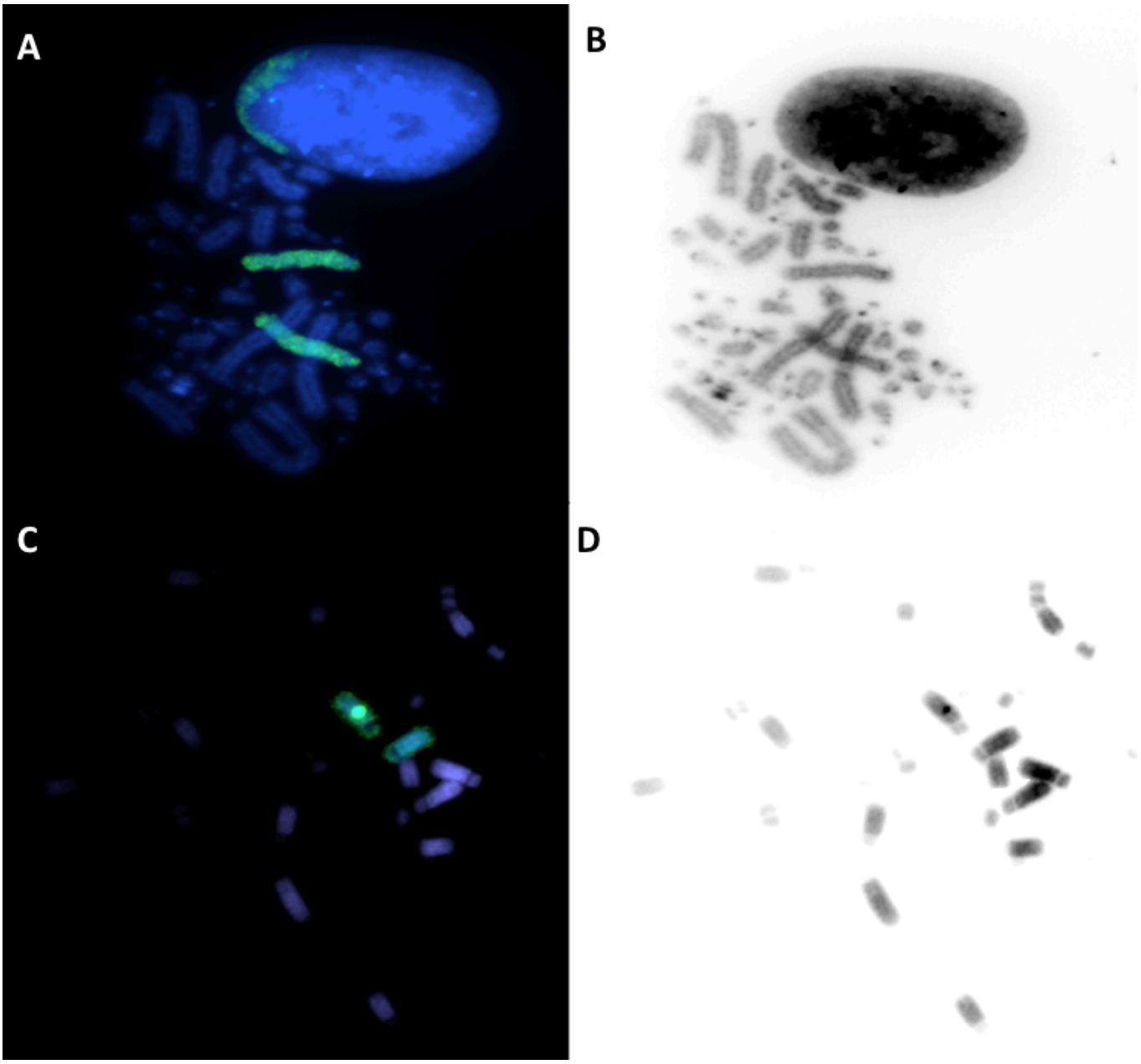

Figure 10. Chicken (GGA) whole chromosome paint 3 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

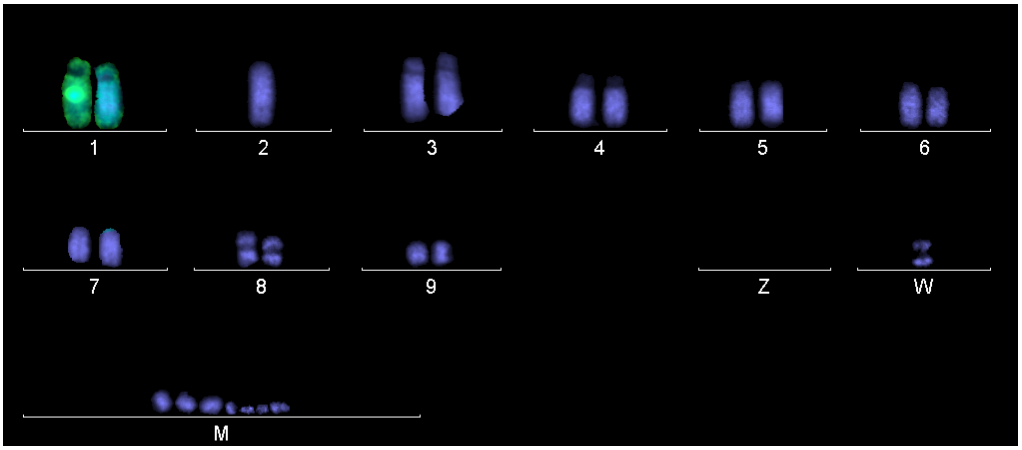

Figure 11. *A. vittata*'s karyotype result of GGA whole chromosome 3 Zoo-FISH.

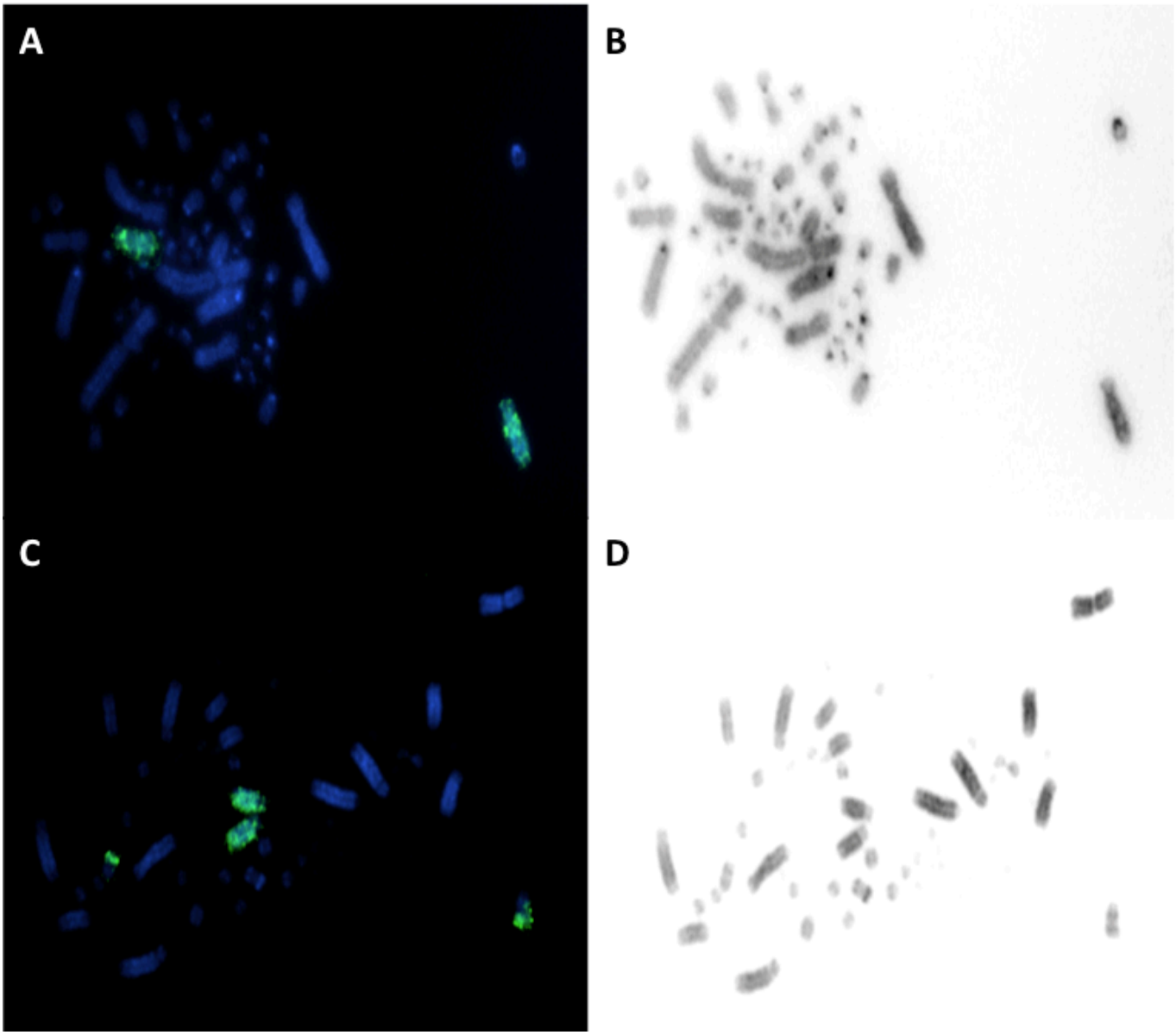

Figure 12. Chicken (GGA) whole chromosome paint 4 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

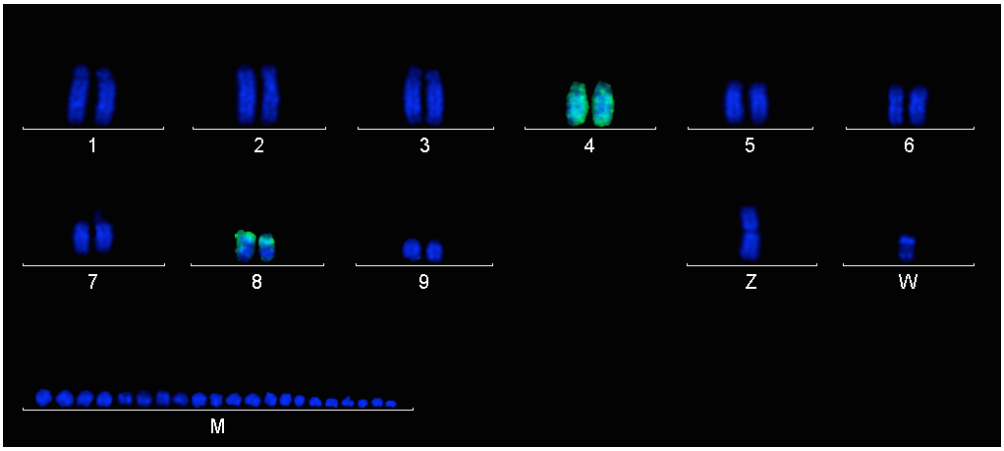

Figure 13. *A. vittata*'s karyotype result of GGA whole chromosome 4 Zoo-FISH.

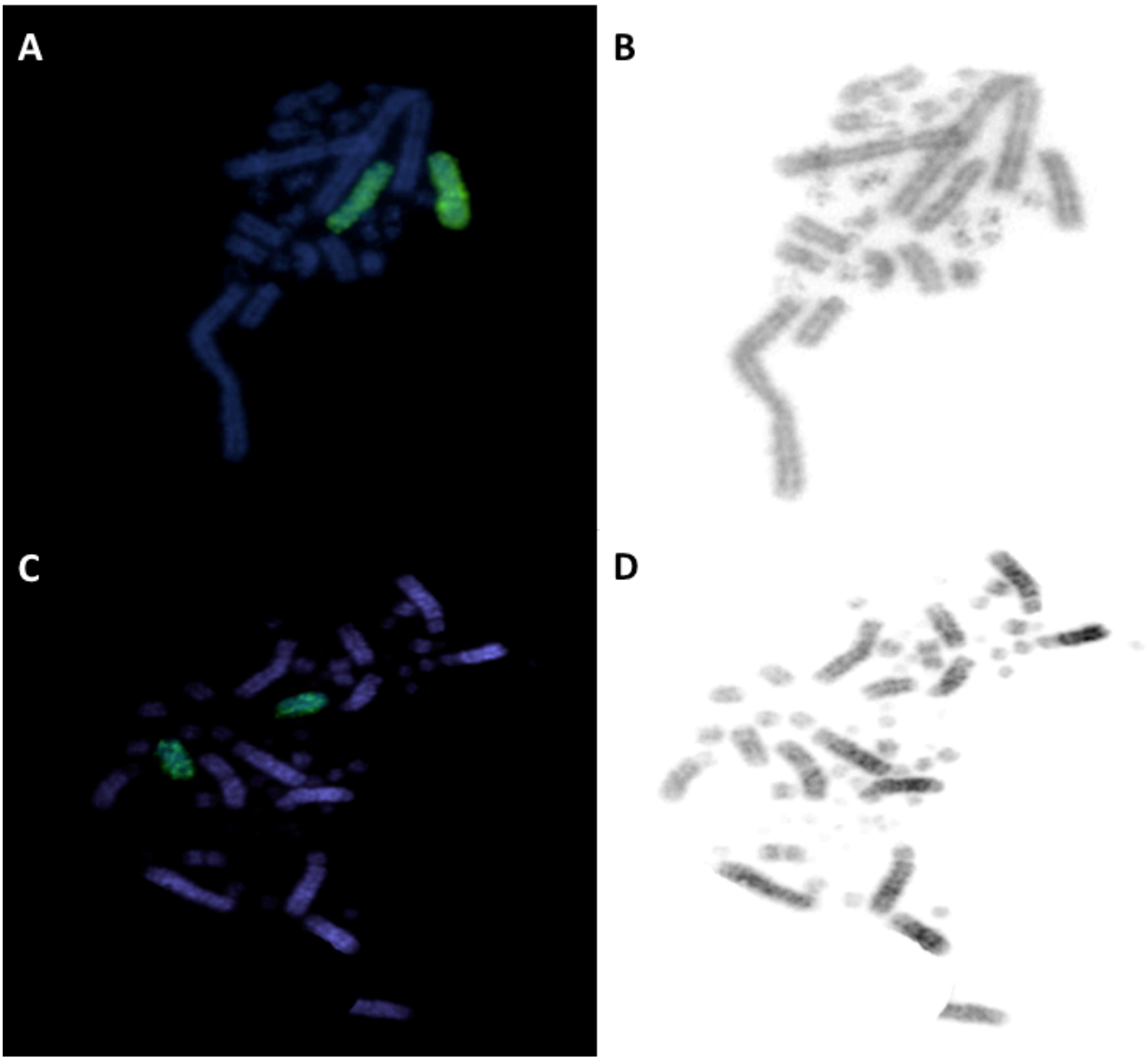

Figure 14. Chicken (GGA) whole chromosome paint 5 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

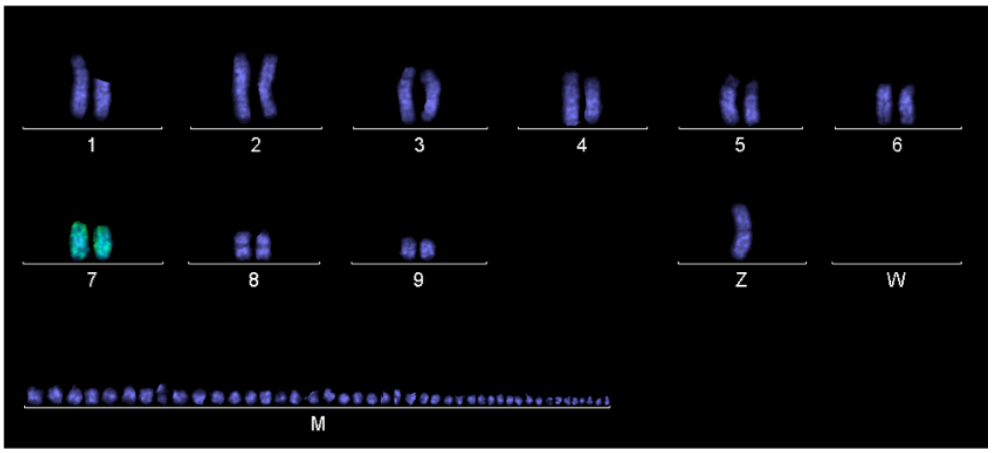

Figure 15. *A. vittata*'s karyotype result of GGA whole chromosome 5 Zoo-FISH.

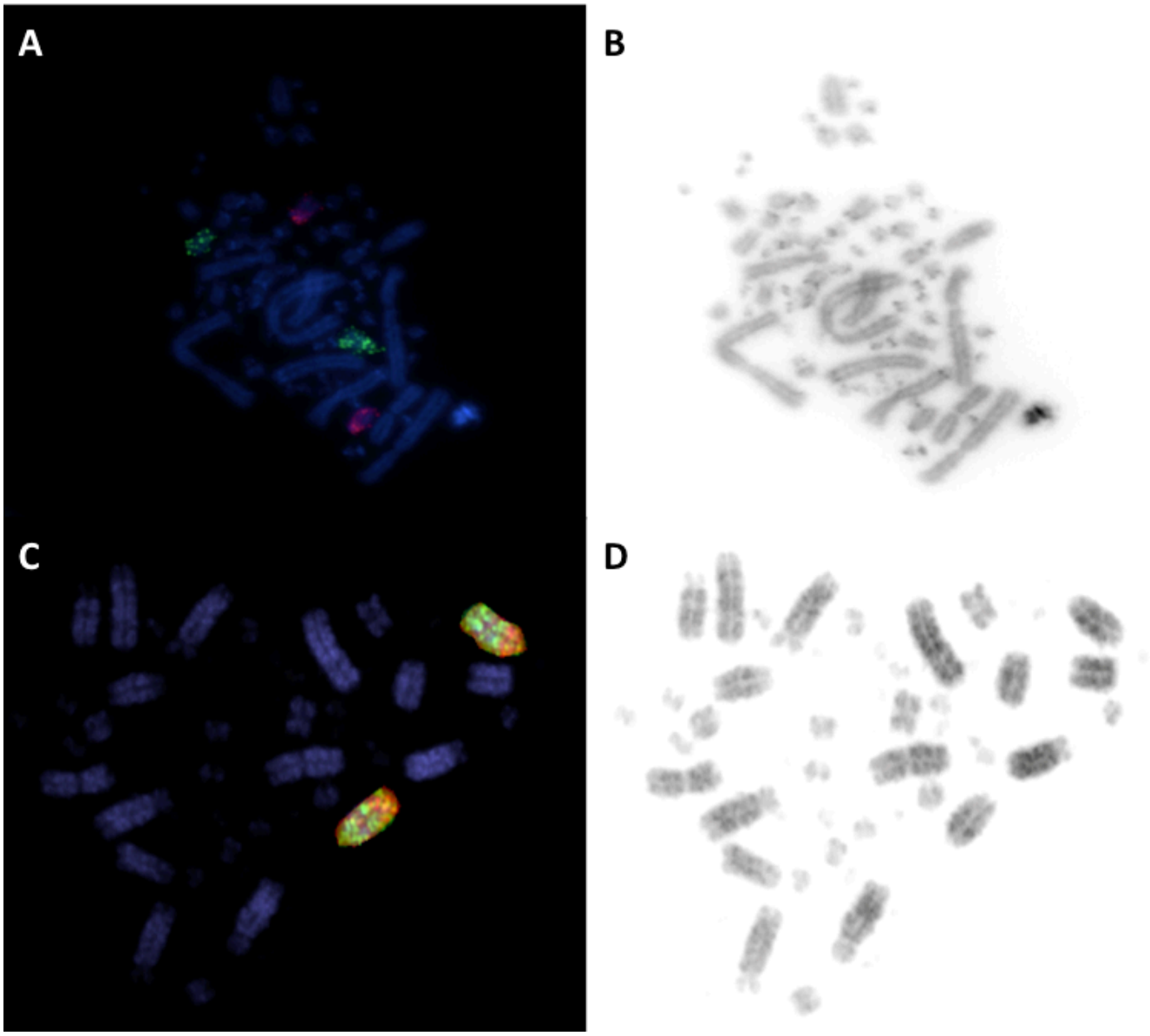

Figure 16. Chicken (GGA) whole chromosome paint 6 (red) and 7 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

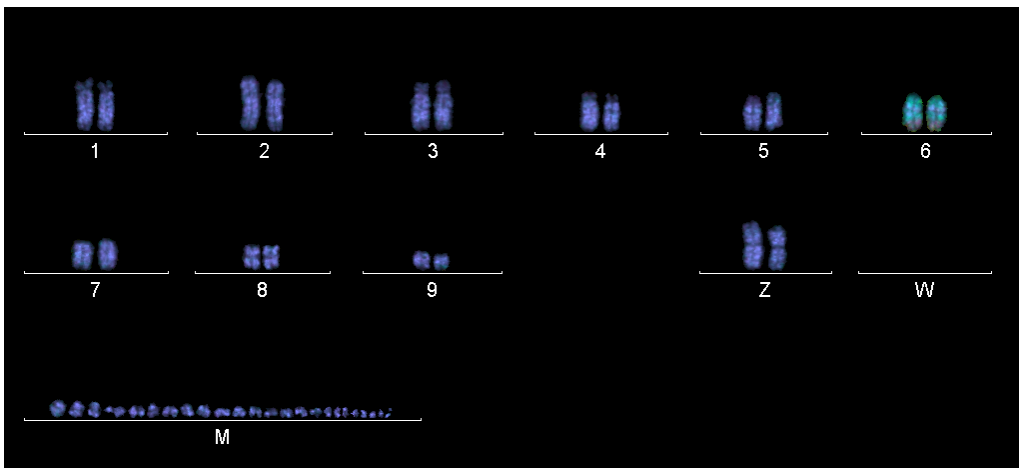

Figure 17. *A. vittata*'s karyotype result of GGA whole chromosome 6 (red) and 7 (green) Zoo-FISH.

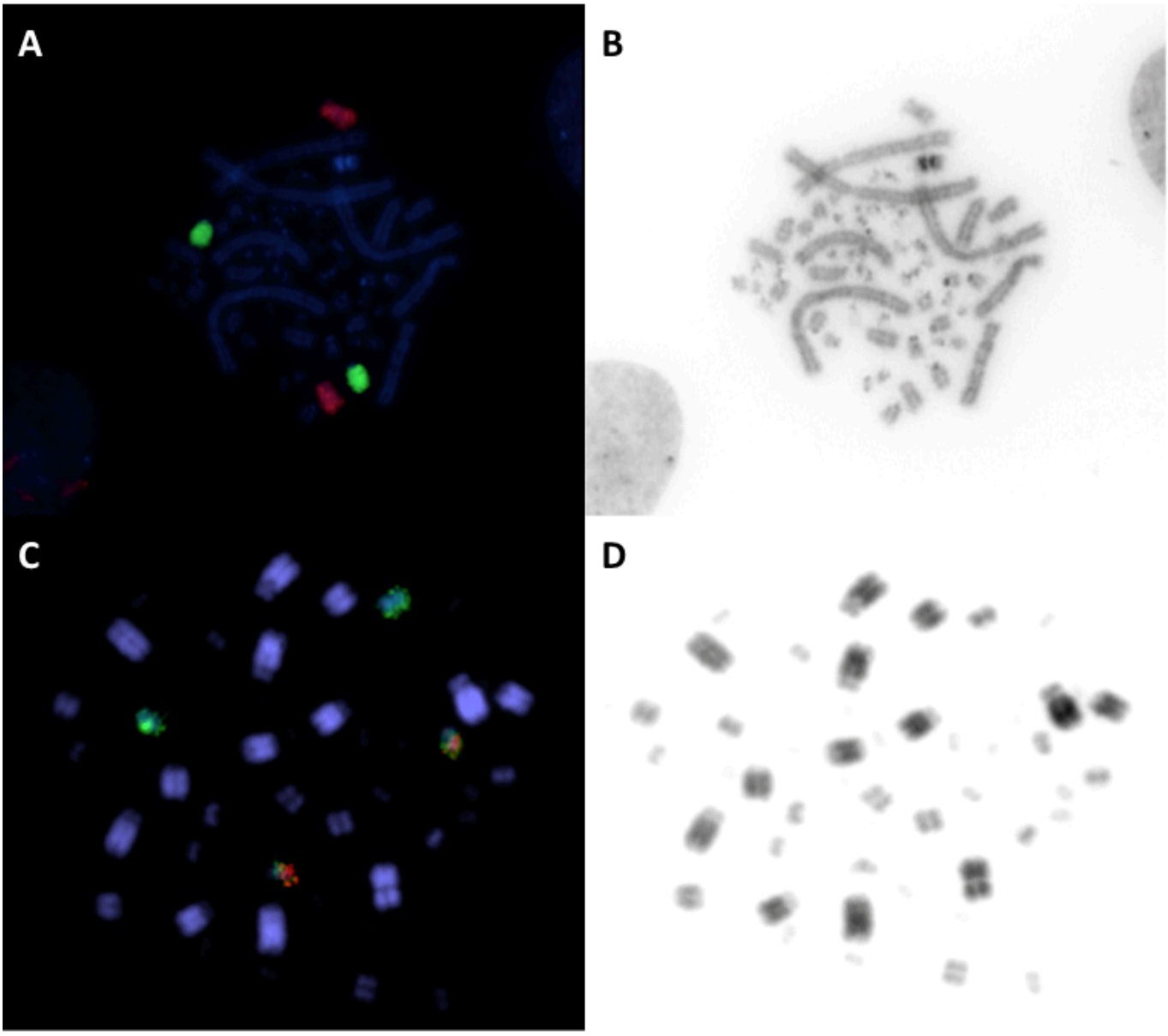

Figure 18. Chicken (GGA) whole chromosome paint 8 (red) and 9 (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

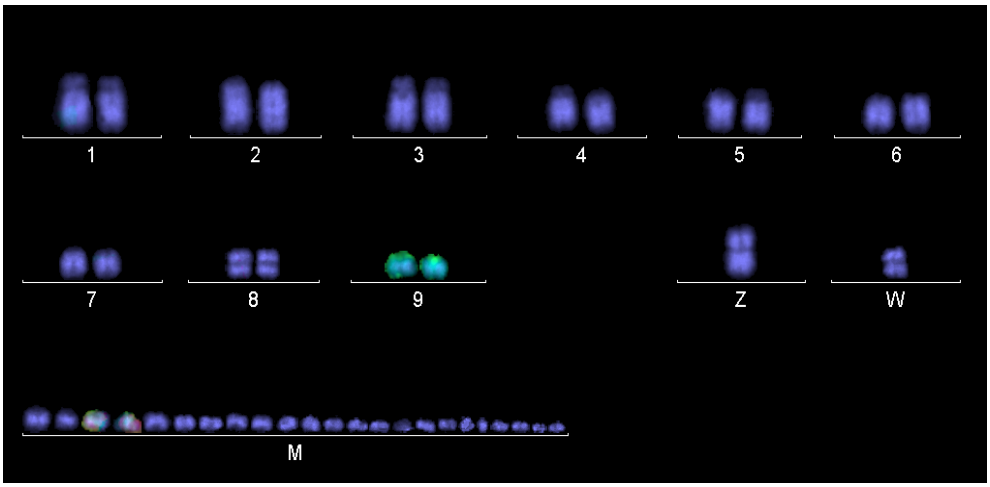

Figure 19. *A. vittata*'s karyotype result of GGA whole chromosome 8 (red) and 9 (green) Zoo-FISH.

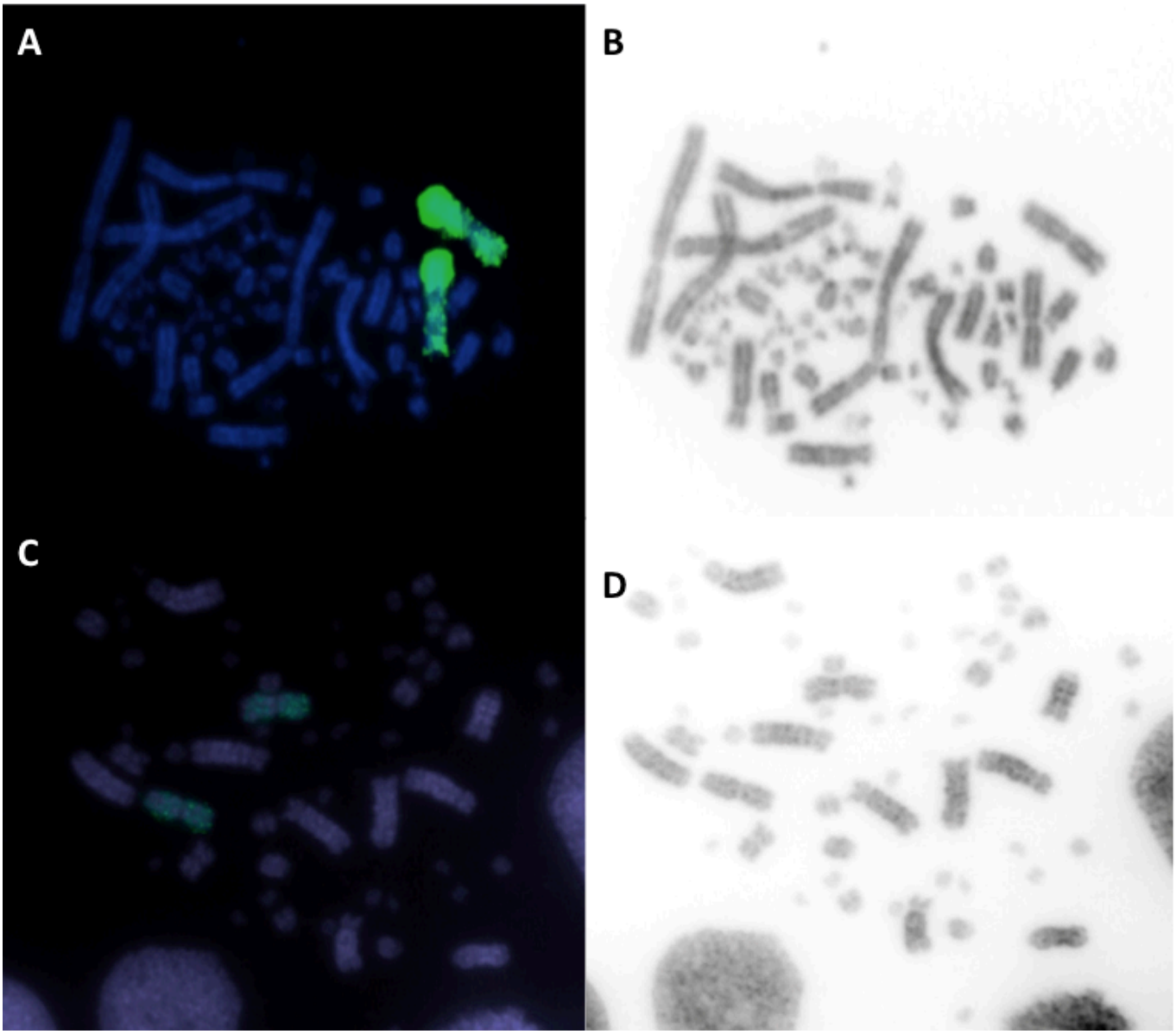

Figure 20. Chicken (GGA) whole chromosome paint Z (green). (A) FISH on chicken (control). (B) DAPI-inverted picture of (A). (C) Zoo-FISH on *A. vittata* chromosomes. (D) DAPI-inverted picture of (C).

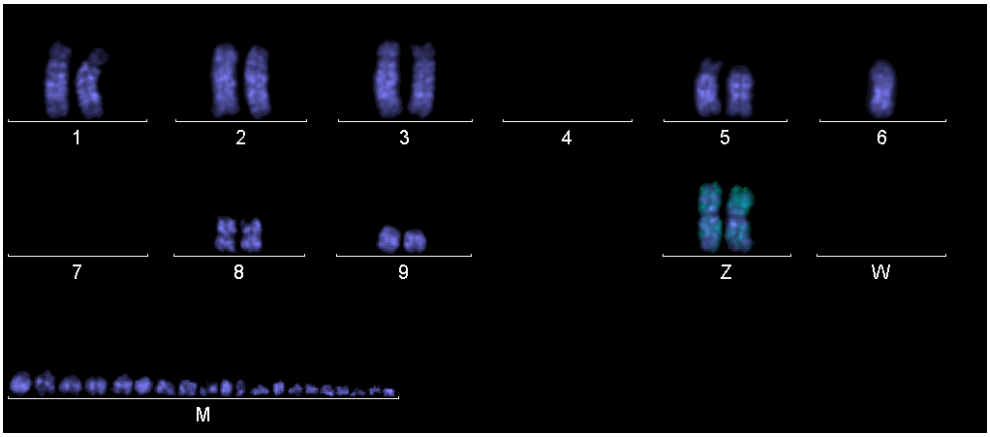

Figure 21. *A. vittata*'s karyotype result of GGA whole chromosome Z (green) Zoo-FISH.

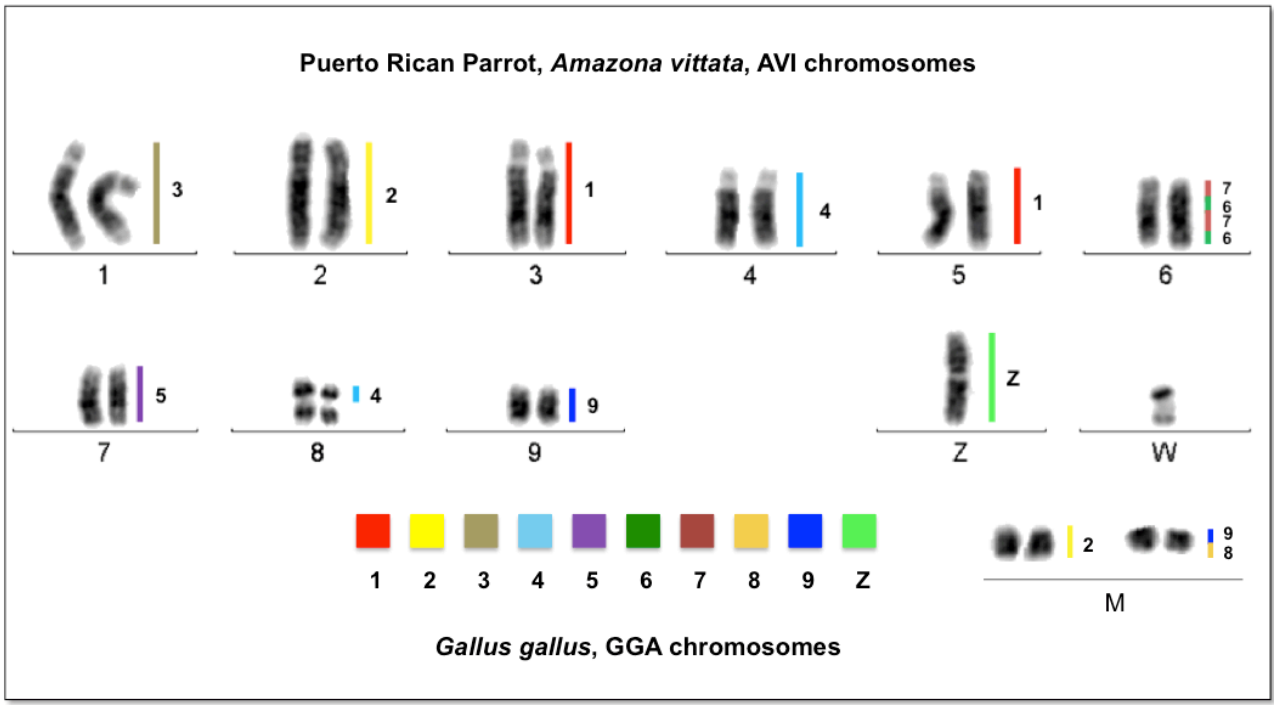

Figure 22. *Amazona vittata*'s Zoo-FISH summarized results. Color bars in the right side of each chromosome pair define the hybridization signals obtained in the experiments. Each chicken whole -chromosome paint is given a different color as shown on the key on the bottom of the figure.

One Zoo-FISH example each per chicken (GGA) whole-chromosome paints 1-9 and Z on the Puerto Rican parrot (AVI) chromosomes are shown in Figures 6 to 21, and summarized in Figure 22. Figures 6 to 21 also show chicken FISH images that show exclusive hybridization of each probe to each corresponding chicken chromosome.

All ten chicken macrochromosome paints hybridized successfully on *A. vittata* chromosomes. Altogether 16 homologous segments showed hybridization signals with the GGA paints. GGA1, 2, 3, 4, 5, 9 and Z paints hybridized to AVI whole chromosomes. Particularly, GGA1 completely painted AVI3 and AVI5. Interestingly, GGA2 and GGA4 painted the same chromosome equivalents in AVI, painting whole chromosome AVI2 and AVI4 respectively. However, GGA2 painted a pair of microchromosomes in addition to AVI2, and GGA4 hybridized one of the arms of metacentric AVI 8 in addition to AVI4. The biggest chromosome for the parrot (AVI1) showed whole chromosome hybridization signal with GGA3 probe. In addition, GGA5 painted AVI7 and GGAZ painted same sex chromosome AVIZ.

In order to confirm using FISH those chromosomal rearrangements previously validated through PCR (types 6 &7 and 8 & 9), dual-color probes where utilized simultaneously. GGA6 in red and GGA7 in green showed syntenic alternated hybridization signals in AVI6, in a green-red-green-

red manner. Likewise, GGA8 in red and GGA9 in green showed split hybridization in a same pair of microchromosomes. GGA9 also hybridized to AVI9 completely.

Zebra finch BACs were properly labeled but not tested on *A. vittata* chromosomes since all chicken whole-chromosome paints hybridized effectively. The labeled zf-BAC probes were stored at -20°C in Dr. Raudsepp's laboratory in case of another collaboration happening in the future.

Discussion

Karyotype

The avian genome is widely described as having a large number of chromosomes. In 1990, Christidis stated that 63% of birds have a diploid chromosome number between 74 to 86; while 24% have from 66 to 74 chromosomes, based on karyotype findings. Likewise, avian karyotypes have portrayed numerous amounts of dot-like, almost indistinguishable, microchromosomes and a low medium sized macrochromosome number that ranges from 6-10 pairs in most species (Guttembach et al., 2004; Nie et al., 2009). The karyotype of the Puerto Rican parrot was not the exception with a diploid chromosome number of 76, 10 pairs of macrochromosomes (including Z and W) and 56 microchromosomes.

Some sex chromosomes (Z and W) are mostly found as metacentric, but can also found with submeta-, acro- or telocentric morphology, and always W is smaller than the Z chromosome (Guttembach et al., 2003). Puerto Rican parrot Z chromosome was described as a large metacentric chromosome, as well as for the W chromosome although it is much more smaller in size. Griffin et al. (2007), based on the findings of Christidis in 1990, stated that the parrot family (Psittacidae) "are rare examples of where clear differentiation between macro- and micro-chromosomes can be seen (including Z and W)". However, our results demonstrate that for the Puerto Rican parrot that is not the case, since between AVI9 and the first pair of dot-like chromosomes there is not that much size difference after all.

Zoo-FISH

While the avian genome is thought to be highly conserved, multiple macrochromosome rearrangements have been observed, especially in chicken chromosome 4 when compared with birds of several orders (Guttembach et al., 2003; Stapley et al., 2008; Volker et al., 2010). Nevertheless, rearrangements among microchromosomes and macrochromosomes are rare and thus only a few and sometimes no interchromosomal rearrangements are observed between bird

species (Shetty et al., 1999; Schmid et al., 2000; Grützner et al., 2001; Shibusawa et al., 2002; Guttembach et al., 2003; Hansmann et al., 2009).

Zoo-FISH results for *Amazona vittata* exhibited 8 events of chromosomal rearrangements, including the ones involving microchromosomes. Chicken chromosome 1 appears to have split into 2 chromosomes, AVI3 and AVI5 specifically. In consistency, GGA1 fission was also described for the peach-faced lovebird (*Agapornis roseicollis*), the budgerigar (*Melopsittacus undulatus*) and, a member of the Cacatuidae family, the cockatiel (*Nymphus hollandicus*) (Nanda et al., 2007). However, this event is different from what has been seen in one of the remainder members of the Psittacidae family that have been studied using the same technique, the scarlet macaw, in which GGA1 have appeared to have undergone a fusion with GGA4 to form the first chromosome pair in this species' karyotype (Figure 23) (Seabury et al., 2013). This is outstanding as it suggests a major chromosomal rearrangement occurring in a relatively short period of evolutionary time within the Psittaciformes order (circa 117.3 million years - www.timetree.org), and differentiating parrot taxa from one another.

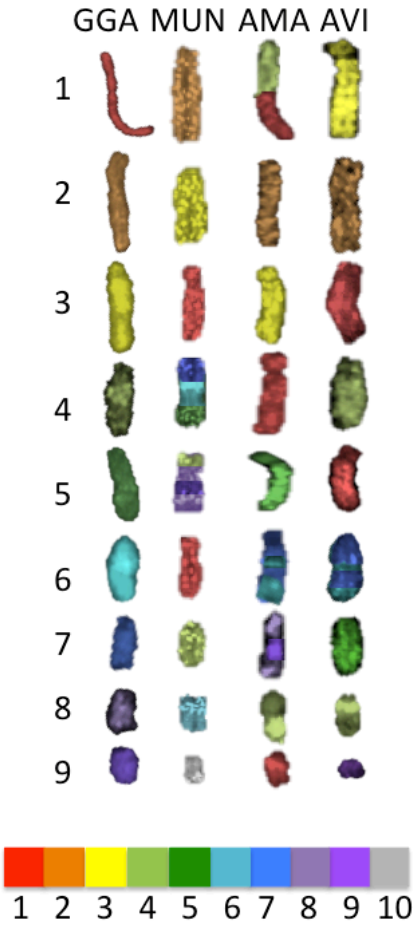

Figure 23. FISH signal outline of chicken (GGA), Budgerigar (MUN), Macaw (AMA) and Puerto Rican parrot (AVI) chromosomes. Chromosomes were cut from FISH results of Nanda et al., 2007, Seabury et al.. 2013 and our own.

The rearrangements that the Puerto Rican parrot does share with the macaw are the peculiar GGA6 and GGA7 alternated synteny in the corresponding chromosome 6 of each parrot species (Figure 24). Interestingly, this rearrangement is also present in the lovebird in their chromosome pair number 6 as well, and thus it is present in all Psittacidae members studied with the exception of the budgerigar, which has a single GGA6&7 fusion (Table 1) (Nanda et al., 2007). Therefore, we suggest this rearrangement happened later on after the budgerigar divergence. Nanda et al.

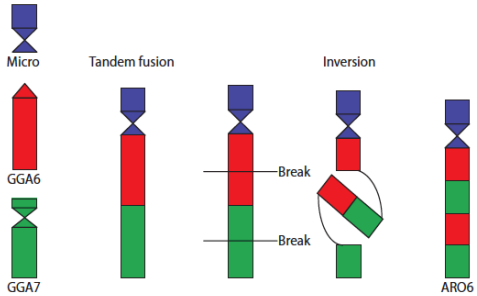

Figure 24. Schematic representation for chromosome 6 and 7 rearrangement mechanism in *Agapornis roseicollis* as proposed by Nanda et al., 2007. Retrieved from Nanda et al., 2007.

(2007) described a possible mechanism for which this alternated arrangement of chromosomes 6 & 7 would have developed. As proposed, the steps to this parrot-specific peculiar arrangement, involves the fusion of GGA6 and GGA7 following a break and an inversion (Figure 24) (Nanda et al., 2007).

In addition to the latter rearrangement, chicken whole-chromosome paints 8 & 9 have also shown an event of fusion in the Puerto Rican parrot. This type of chromosome rearrangement between 8 and 9 is compatible with what has been found in representatives of the parrot family and the closely related family Cacatuidae (Table 1). In the scarlet macaw and in the budgerigar genomes, GGA9 seems to have introduced itself inside chromosome 8; while in the lovebird and the cockatiel it appears a fusion of both chromosomes (Nanda et al., 2007; Seabury et al., 2013). However, in all of the birds mentioned above this rearrangement occurred in a macrochromosome and the Puerto Rican parrot is the first one to have this fusion located in a microchromosome. As the 8-9 PCR validation showed the same fusion for all Psittacidae species assayed (Figure 25), a possible explanation would be that the rearranged chromosome has been broken into smaller chromosomes in Amazon parrots. Hence, the 8-9 microchromosome of *A. vittata* may be homologous to one of the arms of metacentric chromosome AMA7. In addition, it is also unusual how in *Amazona vittata*, GGA2 was found to have broken into AVI2 and a pair of microchromosomes.

PCR validation

The PCR amplifications of chromosome joint regions confirmed three chromosomal rearrangements that have been described previously by Zoo-FISH methods (6-7(2) & 8-9) and two others that have been not (2-15 & 3-9). It can be assumed that visualization under a microscope sometimes is not enough to recognize a signal in tiny dot-like chromosome. Thus, it may be that the 3-9 fusion occurred in of the smallest microchromosomes, making it impossible to detect by comparative chromosome painting methods. In addition, the 2-15 fusion could not be detected because no paint for chromosome 15 was used. This approach due to its inexpensive nature could be used for many birds if DNA is available. After unraveling the chromosomal rearrangements along several Psittacidae species, it was possible to identify where in the evolutionary tree these occurred (Figure 25).

The results show how 2-15, 6-7 and 8-9 fusions occurred before the divergence of the African grey parrot and how 3-9 was Amazon parrot specific. There was a 6-7 type of

rearrangement that was amplified only for the Amazon parrots in this study. However, it is known from Zoo-FISH studies that two 6-7 fusions in an alternated fashion are present in the scarlet macaw and in the African grey parrot (unpublished data – Dr. Terje Raudsepp). Therefore, one possible explanation might be that, although conserved in the chicken and the zebra finch, one of the primer sequences was not sufficiently conserved in the scarlet macaw. Alternatively, it should be noted that the amplicon is by far the largest of all amplicons synthesized (Figure 5), and it is probable that the distance between the primers in the scarlet macaw exceeded that which could be amplified by our methods. Another possible exclamation is that a different chromosomal rearrangement may be present in only in the Amazon parrots. As these rearrangements presumably involved inversions (Nanda et al., 2007), and inversions are very common in chromosome evolution, different inversion events could have occurred in the different lineages. Nevertheless, this represents the first PCR-based validation to unraveling chromosomal rearrangements between different species.

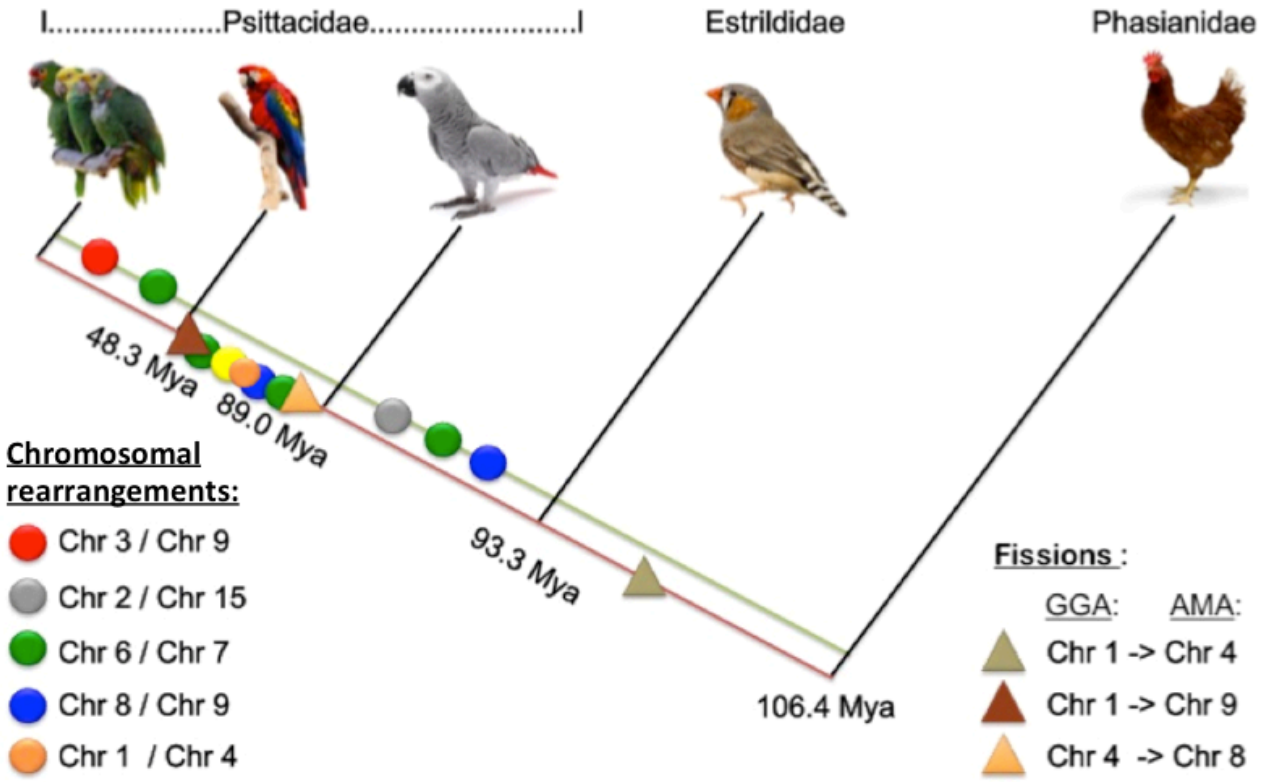

Figure 25. Phylogram of chromosomal rearrangements and fissions in bird lineage. Our studied rearrangements are placed at the earliest evolutionary times confirmed. Time of species divergence was calculated using TimeTree. The green line represents our study and the red line: Seabury et al., 2013.

Conclusions

The Puerto Rican parrot is the first among Amazon parrots to have its karyotype completed. With a chromosome diploid number of 76, it portrays a typical avian karyotype according to the descriptions suggested by Christidis (1990). With our *de novo* approach, using *in silico* work and PCR, we uncovered two rearrangements that had never been described for parrots before (2-15 & 3-9). Thus, with the improvement of sequencing platforms and bioinformatics tools and more bird species getting their genome sequenced and analyzed, this type of analysis could be used in the future as it represents a less expensive and less time-consuming approach to study chromosomal rearrangements in a group of species. In terms of interchromosomal rearrangements detected by Zoo-FISH, although it possesses rearrangements, they are characteristic of the Psittacidae family and thus high synteny and conservation is implied. However, Volker et al. (2010), by studying avian copy number variants (CNVs), demonstrated that the avian genome is more plastic that what is perceived through comparative cytogenetic work; being intrachromosomal, rather than interchromosomal, rearrangements the source of avian structural genomic evolutionary rearrangements.

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Supporting information

Supplemental Table 1. Putative chromosomal rearrangements resulted from alignments of the chicken to *Amazona vittata*'s ALLPATH 2012 scaffolds. N=164.

Score Seg 1:	Scaffold:	Chr 1:	Chr 2:	Score Seg 2:	Scaffold size:
2263	3	1	14	2092	9406901
7243	8	9	3	3583	8095756
4273	9	1	15	3962	7566741
2076	18	2	3,1	3935	6137548
5766	31	8	2	5303	4528924
low	41	3	2	low	4006141
303	43	2	26	5084	3974232
6503	57	2	11	5017	3456683
1875	59	9	2	3737	3420564
6136	77	6	7	4909	3096808
low	79	2	8	low	3068785
7405	83	1	3	5108	2977528
low	91	1	3	low	2831270
low	93	1	2	low	2760552
2869	94	1	4	5068	2769435
low	97	2	6	low	2688676
5277	99	9	8	5318	2632485
7217	109	2	3	2070	2498176
3012	110	17	1	207	2490167
3040	125	1	5	3201	2329123
1430	127	1	2	2416	2310534
6787	133	7	6	3653	2205159
2997	143	1	2	407	2085803
1090	144	7	6	2026	2089787
3169	151	9	8	2338	2026016
1512	167	6	9	5997	1836990
321	177	4	2	3826	1729123
1085	191	8	13	3391	1638771
5467	194	14	23	4370	1594063
3012	208	18	4	2826	1502315
4068	220	9	8	1403	1402669
4398	240	3	7	1738	1316228
1405	304	7	6	1759	996700
2053	339	6	4	1046	843703
1919	385	4	8	6338	693002
3649	422	1	2	1131	579920
3411	450	13	20	3786	535837
1449	458	1	3	4889	519973
2540	475	9	8	2419	485510
5279	483	Z	1	3008	486781
2306	494	1	Z	4817	471049
3677	499	1	2	4380	449784
2420	505	11	Z	2201	446644

1085	525	5	9	3502	426230
3771	529	3	5	4349	417733
3359	570	27	5	6758	372855
2009	640	4	Z	2250	301918
2770	678	25	28	1010	277808
572	776	3	1	562	210340
885	781	1	2	163	214705
200	806	2	1	428	195648
336	906	1	4	1532	167180
176	913	5	1	382	162426
2772	937	11	1	202	156627
1970	939	18	3	335	154285
925	965	4	1	327	145874
204	972	5	1	434	144124
603	1020	W	4	189	131881
151	1024	Z	3	118	131569
2646	1042	Z	W	3869	128326
162	1059	5	3	212	127867
232	1063	3	1	393	120418
172	1065	2	Z	84	122798
2429	1079	11	1	512	121367
555	1086	3	28	283	120880
5996	1089	25	19	3333	112717
127	1092	2	Z	593	118221
342	1097	3	4	910	116176
240	1109	1	11	520	111102
77	1117	3	Z	2058	113090
2131	1127	2	W	832	109462
820	1135	2	4	1303	103551
3094	1137	3	25	2033	109633
72	1152	1	Z	441	102192
1812	1154	Z	1	208	105057
1988	1157	27	10	292	103889
3842	1158	27	1	404	105392
1043	1180	2	1	217	100073
195	1191	2	W	315	96050
267	1211	1	Z	2407	96180
317	1240	Z	1	1005	90775
1253	1244	1	2	209	94708
273	1255	1	Z	838	88671
2493	1290	4	2	201	82605
762	1296	2	1	786	80537
61	1312	1	Z	93	76056
128	1324	2	Z	224	77707
206	1330	4	1	320	76491
433	1333	Z	2	107	73390
448	1337	Z	1	212	75711
306	1338	2	1	173	76947
252	1351	6	3	247	70021

182	1353	Z	25	1365	73512
2237	1376	2	4	290	71448
1426	1380	3	1	352	71087
119	1402	2	18	67	64702
411	1403	1	4	701	63831
1586	1415	Z	3	263	68152
107	1418	1	Z	218	64103
211	1427	1	Z	95	64615
155	1439	9	16	652	68249
2457	1442	11	11	2491	63173
164	1449	2	6	122	62346
388	1451	5	Z	472	61891
3716	1458	5	1	80	54725
427	1459	Z	2	101	62031
2514	1462	Z	2	26	61549
1502	1467	Z	1	108	60605
452	1468	3	11	968	60172
54	1471	2	2	60	61046
1201	1477	Z	1	242	57396
153	1478	3	Z	1094	59411
5053	1481	11	13	63	55330
422	1485	3	1	62	56982
1167	1489	Z	2	65	58198
95	1490	3	8	28	57993
1324	1516	Z	2	32	55330
69	1524	1	Z	172	52212
647	1549	3	5	485	52714
1771	1553	Z	3	87	50186
77	1562	1	9	111	51844
5653	1568	8	1	34	50930
277	1572	2	5	3413	51223
101	1575	5	Z	278	49946
151	1576	Z	1	32	47826
1163	1584	Z	4	63	51372
410	1605	1	5	1014	48913
164	1609	2	1	362	47593
914	1610	Z	1	169	46424
274	1614	Z	1	108	47867
1250	1616	11	Z	2727	46465
169	1621	1	Z	1253	46973
1863	1633	Z	1	145	43858
65	1636	1	4	35	45455
179	1642	1	Z	161	45005
147	1643	2	1	483	44424
152	1649	2	4	61	44450
1731	1676	Z	2	132	41796
274	1693	3	1	135	40509
187	1702	12	1	122	38070
111	1703	2	11	32	41646

159	1707	Z	1	106	41744
489	1712	2	5	438	36743
220	1718	Z	12	307	39827
220	1719	Z	12	307	40389
313	1720	Z	3	87	40753
1838	1727	11	1	252	36980
365	1739	3	1	225	38456
215	1744	2	1	324	38488
83	1745	1	Z	208	39098
307	1746	2	3	135	39238
351	1756	1	Z	377	37788
227	1766	4	1	236	40327
247	1767	2	Z	565	38003
157	1771	3	Z	640	34622
197	1775	1	11	557	31058
484	1788	Z	1	237	30440
460	1791	Z	1	102	34862
398	1809	Z	1	105	33673
151	1855	1	2	161	31180
209	1871	1	Z	196	30269
100	1890	3	Z	408	27837
279	1899	Z	1	147	30576
165	1943	1	2	117	35773

low = below 1000

Supplemental Table 2. Scaffolds containing putative chromosomal rearrangements with BLAT alignments scores greater than 1000.

		First 25 kb					Last 25 kb		
Scaffold:	Scaffold size:	Start chicken:	End chicken:	Score Seg 1:	Chr 1:	Chr 2:	Start chicken:	End chicken:	Score Seg 2:
3	9406901	8130	23233	2263	1	14	232	24801	2092
8	8095756	307	24560	7243	9	3	277	17231	3583
9	7566741	2305	2305	4273	2	15	63	23995	3962
31	4528924	61	24382	5766	8	2	1	24395	5303
57	3456683	1071	24893	6503	2	11	182	22881	5017
59	3420564	9312	23490	1875	9	2	16241	16923	3737
77	3096808	2312	24944	6136	6	7	393	24083	4909
83	2977528	41	24552	7405	1	3	1048	24336	5108
99	2632485	17779	18391	5277	9	8	1060	24430	5318
109	2498176	37624966	37649574	7217	2	3	84265102	84282860	2070
125	2329123	69802326	69824238	3040	1	5	10442504	10456656	3201
133	2205159	20189487	20208216	6787	7	6	14829201	14845124	3653
144	2089787	3521189	3527986	1090	7	6	8904820	8920622	2026
151	2026016	5924584	5941736	3169	9	8	2646152	2668970	2338
167	1836990	6560471	6566539	1512	6	9	12630262	12648815	5997
191	1638771	2938486	2945270	1085	8	13	5761864	5778877	3391
208	1502315	9541345	9564379	3012	18	4	2585068	2612769	2826
304	996700	35735094	35744937	1405	7	6	9175781	9185512	1759
339	843703	3626486	3638243	2053	6	4	18516884	18838064	1046
385	693002	71670596	71693170	1919	4	8	19214855	19232540	6338
450	535837	5785038	5808685	3411	13	20	5879342	5897085	3786
525	426230	41809063	41814479	1085	5	9	23217987	23228415	3502
570	372855	1535518	1550240	3359	27	5	18796929	18817476	6758

*In blue are the corresponding segments which were taken from the middle part of the scaffold and not the last 25 kb.

Supplemental Table 3. *Amazona vittata*'s putative inter-chromosomal rearrangement primer sets

Scaffold	Start in scaffold	End in scaffold	Forward primer	Tm	Size	Reverse primer	Tm	Size	Amplicon size	Rearrangement	Annealing Temp °C
3	8,627,040	8,631,431	ATGCAGCCACCACATGACTTA	64	21 bp	AGTGGTAACAGAAAGGGTATGAACT	64	25 bp	4,391	chr 1A / chr 14	55°
8	99,588	99,602	CTATGCTAGCACTGACAGGAC	62	21 bp	TTCGTTAAGCTACAGTTGATTGC	62	23 bp	1,151	chr 9 / chr3	52°
9	1,210,321	1,211,429	ACTTCAACTGTCCAGCTCTTAAA	62	23 bp	CACAGGAGCGATCAACTCATAG	62	22 bp	1,120	chr 2 / chr 15	53°
31	14,134	14,153	TTGTATGTTGCCTTCCACTGC	63	21 bp	GCTGAAGAGTTCCAGCTTTCC	63	21 bp	1,476	chr 8 / chr 2	55°
57	41,939	41,958	AGGCCTCAATTGTTAACCCTTAC	63	23 bp	CTGAATGCAGCCTTCCTGTAATA	63	23 bp	1,405	chr 2 / chr 11	54°
59	38,670	38,729	CTCATGAATGCATCAGGGTAGT	62	22 bp	GGCTATTGGCAAGACTAGCATA	62	22 bp	4,770	chr 9 / chr 8	53°
77	608,459	611,385	GTCCGAAGTCTGCAGTCTAATCC	62	23 bp	GCCATCCTTACTCTTGCTTCCTC	62	23 bp	2,948	chr 6 / chr 7	57°
83	1,132,545	1,134,023	GTGAAAGAACCTGTGGTTTCTAATG	62	25 bp	GAGATGCCAGGGTGAAAGAATA	62	22 bp	1,500	chr 3 / chr 1	53°
109	1,756,864	1,758,523	GCAGAACTCGCATTTACAAAGTAT	62	24 bp	GAACTCCAGTATTCGTTCAGAGG	62	23 bp	1,682	chr 2 / chr 3	52°
133	1,354,431	1,355,781	TTGTCAGGGTGATGTTCTTGTGAG	61	24 bp	AGTGAGTGGAGTAACAGCATTTGG	61	24 bp	1,351	chr 7 / chr 6	56°
144	1,948,570	1,952,748	CAACAAGTCGTGTGCCATCAGTTAC	59.7	25 bp	TACATCGAAGGGGCCACTCTTG	58.9	22 bp	4,179	chr 7 / chr 6	58°
167	1,775,553	1,778,670	AGCAGGACTTCCATCATGATTAC	62	23bp	CCTAGGTCTGCCTTATATTTCTTGTAT	62	27bp	3,200	chr 6 / chr 9	53°
191	967,602	968,394	AATTAGATACAGGCCCAACCAGG	62	23 bp	AGACACCTGGAATTCACAAAGAC	62	23 bp	793	chr 8 / chr 9	54°
191	1,587,254	1,592,719	GCCATGCTTGTGTTACAATAGTT	62	23 bp	GCACAAGTATTGGTTGGTAGTTG	62	23 bp	5,466	chr 9 / chr 13	53°
208	13,075	13,129	GTTTGCTTTGTGTTAGGTTGTTTG	62	24 bp	TCATCTTCTGCTTCCAGTTGTT	62	22 bp	4,400	chr 18 / chr 4A	52°
304	712,749	716,875	CTCTGTAAAGAGCGCAGTATGTTG	57.2	24 bp	TGAAGACTTCCCATTTCCCATCTC	58.1	24 bp	4,127	chr 7 / chr 6	55°
339	426,240	429,505	GAATAAAGGTGTCCCTAAAGAAAGG	62	25 bp	GACTATGACAAATACGAGCCAAAC	62	24 bp	3,265	chr 6 / chr 4A	52°
385	87,924	91,642	GGGCCTCAGAGTAGTTTCATCCAT	62	24 bp	CCAAAGGATTCCCAAAGTGCTGTT	62	24 bp	3,719	chr 4 / chr 8	57°
525	213,450	216,168	AACTGACAAGACTCCACCAAAAC	62	23 bp	GTGCAATAACTGGGAAGAGGAGC	62	23 bp	2,719	chr 5 / chr 9	54°

Highlighted in light blue are the scaffolds with confirmed rearrangements.

Supplemental Table 4. Zebra finch BAC selection according to chromosomal rearrangement location in the TGU genome

Chromosomal Rearrangement		Genome location Chr A	BAC ID 1	BAC genome location	Genome location Chr B	BAC ID 2	BAC genome location
A	B						
Chr 9	Chr 3	chr9:27,008,394-27,009,279(+)	TGMCBa-213F19	chr9:26,997,521-27,124,917	chr3:102,964,496-102,964,734(+)	TGMCBa-367F12	chr3:102,897,646-103,016,350
Chr 15	Chr 15	chr2:12,709,832-12,710,074(+)	TGMCBa-314M5	chr2:12,675,550-12,808,151	chr15:7,924,083-7,924,364(-)	TGMCBa-67J14	chr15:7,858,362-7,989,443
Chr 6	Chr 7	chr6:14,929,777-14,930,221(-)	TGMCBa-4P17	chr6:14,867,517-14,998,116	chr7:11,079,595-11,079,936(-)	TGMCBa-132A5	chr7:11,046,248-11,217,328
Chr 7	Chr 6	chr7:11,090,971-11,091,219(-)	TGMCBa-132A5	chr7:11,046,248-11,217,328	chr6:14,928,355-14,928,867(-)	TGMCBa-4P17	chr6:14,867,517-14,998,116
Chr 8_random	Chr 9	chr8_rand:299,557-300,174(+)	Not found	N/A	chr9:7,730,285-7,730,605(+)	TGMCBa-333B22	chr9:7,675,857-7,825,234