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# Some molecular cytogenetic markers and classical chromosomal features of *Spilopelia chinensis* (Scopoli, 1786) and *Tachybaptus ruficollis* (Pallas, 1764) in Thailand

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**Abstract.** This study analyzed the karyological features of two bird species – *Spilopelia chinensis* and *Tachybaptus ruficollis* – from Northeastern Thailand. Mitotic chromosomes were indirectly prepared by fibroblast cell culture. The chromosomes were stained by conventional Giemsa staining and microsatellite repeat of fluorescence *in situ* hybridization techniques. Giemsa staining showed that the diploid chromosome number of *S. chinensis* was 2*n*=70 and *T. ruficollis* was 60. The types of chromosomes observed in *S. chinensis* were 4 large metacentric, 2 medium acrocentric, 2 small metacentric, 2 small submetacentric, 2 sex chromosomes and 58 microchromosomes; the karyotype of *T. ruficollis* comprised 2 large metacentric, 2 large submetacentric, 2 large acrocentric, 8 small metacentric, 4 small submetacentric, ZW sex chromosomes and 40 microchromosomes. The molecular cytogenetical features that were exhibited only on the male *T. ruficollis* chromosome included two microsatellites and telomeric sequences: two signals of $d(CA)_{15}$ on two microchromosomes, one signal of $d(GC)_{15}$ on one of the first pair, and signals of $AGGGTT_n$ sequences on each telomeric region of all macro- and microchromosomes. The karyotype formula was deduced as: 2*n* (70) = $L^m_4$ + $M^a_2$ + $S^m_2$ + $S^{sm}_2$ + 2 sex chromosomes ($S^m_1/S^{sm}_1$) + 58 microchromosomes for *S. chinensis* and 2*n* (60) = $L^m_2$ +$L^{sm}_2$ + $L^a_2$ + $S^m_8$ + $S^{sm}_4$ + Z ($M^{sm}_1$) W ($S^{sm}_1$) + 40 microchromosomes for *T. ruficollis*.

**Keywords:** *Spilopelia chinensis*, *Tachybaptus ruficollis*, Bird chromosome, Bird karyotype.

## INTRODUCTION

Birds, also known as avian dinosaurs, are a group of endothermic vertebrates, characterized by many features. *Spilopelia chinensis* (Figure 1a), or spotted dove, is a small pigeon that is a common local breeding bird throughout its native range on the Indian Subcontinent and in Southeast Asia. The species belongs to the genus *Spilopelia*, subfamily Columbinae, family Columbidae, order Columbiformes, clade Columbimorphae and class Aves (Gibbs *et al.* 2001). *Tachybaptus ruficollis* (Figure 1b) or little grebe, is native to Europe, Africa and Asia. *Tachybaptus ruficollis* is one of six grebe species in the genus *Tachybaptus*, family Podicipedidae, order Podicipediformes, clade Phoenicopterimorphae and class Aves (BirdLife International 2020). Twenty-eight species of Columbidae and three species of Podicipedidae have been reported in Thailand, which the genus *Spilopelia* comprises three species (*S. orientalis, S. chinensis* and *S. tranquebarica*) and the genus *Tachybaptus* has only one species (*T. ruficollis*) (Pratumthong *et al.* 2011).

Columbiformes, one of three orders in the Columbimorphae clade, and Podicipediformes, one of two orders in the Phoenicopterimorphae clade, are both classified to the same Columbea group by genome analyses. Paleobiology and molecular biology suggest that neoavians and placental mammals originated about 66 million years ago during the late Cretaceous to early Paleogene period. The evolutionary lines of Columbimorphae, including mesites, sandgrouse and doves, and Phoenicopterimorphae, comprising flamingos and grebes, divided about 70 million years ago during the late Cretaceous period (Pacheco *et al.* 2011; Ksepka and Boyd 2012; Yuri *et al.* 2013; Jarvis *et al.* 2014).

Few avian chromosomal data studies have been reported, because of their difficulty compared to other vertebrates, as avian chromosomes are highly conserved compared to other vertebrate groups. At present, about 10% of total 10,857 bird species that have been reported karyotypic study. Approximately half the number of karyotyped birds (≈50.7%) have diploid number of 78 and 82 chromosomes, and about 21.7% have 2n=80. Extraordinary diversity of bird chromosome ranges from 2n=40 in *Falco columbarius* (Falconiformes) to 2n=142 in *Corythaixoides concolor* (Musophagiformes). The number of chromosomes, karyotypic features and sex chromosomes have been preserved in the avian genome on the chromosomal level and shared across all avian species (Degrandi *et al.* 2020). The diploid number of 2n=80 was proposed to the presumptive ancestral bird chromosome, which can be used to explain the chromosomal evolution of birds well (Griffin *et al.* 2007).

This classic chromosomal study of Thailand populations of *S. chinensis* and *T. ruficollis* species is the new recorded; in addition, we are the first to report on the molecular cytogenetic features of the *T. ruficollis* species.

## MATERIALS AND METHODS

### *Sample collection*

*S. chinensis* tissue samples were derived from whole embryo tissue from two eggs; *T. ruficollis* tissues samples were derived from the feather coat. The *S. chinensis* eggs were collected from Ban Hauyrai (15°51'23.1"N, 102°50'06.1"E), Wang Muang Sub-district, Paui Noi District, Khon Kaen Province, Thailand. The *T. ruficollis* samples were collected from a nesting area at the wastewater treatment plant of Khon Kaen University, Khon Kaen Province, Thailand. Chromosomes were prepared from the tissue samples using fibroblast cell culture.

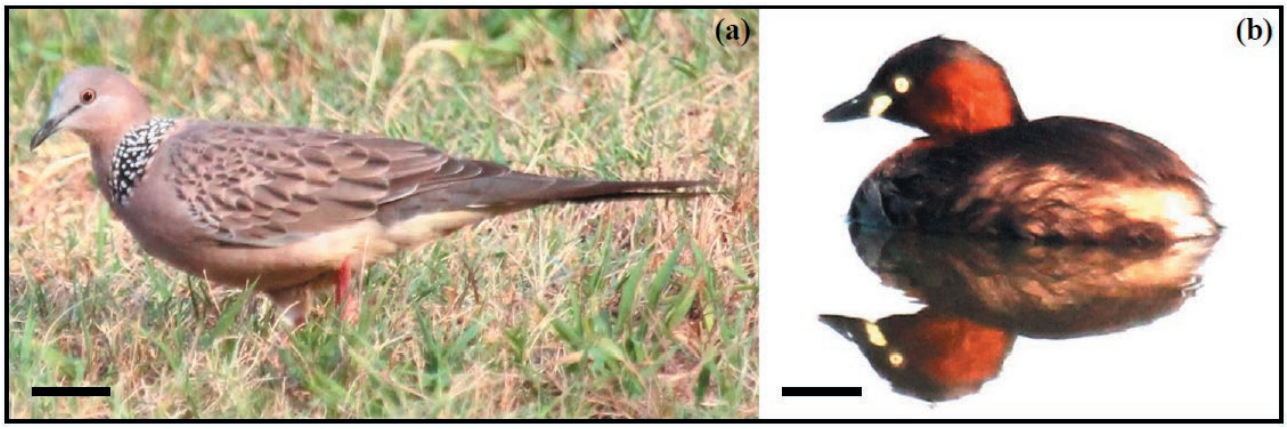


**Figure 1.** General characteristics of *Spilopelia chinensis* (a) and *Tachybaptus ruficollis* (b); scale bars = 5 centimeter.

*Fibroblast cell culture and chromosome preparation*

The chromosomes were prepared in three steps. First, the half-period old of eggs life cycle of *S. chinensis* and feather coat tissue of *T. ruficollis* used in this research were collected from bird nests as noted in the section above. Second, the embryos and feather coat tissue were isolated and washed three times with phosphate buffered saline (PBS). The tissue samples were then chopped into pieces of 1 $mm^3$ and placed onto the surface of a tissue culture flask at 41°C in a humidified air atmosphere containing 5% of $CO_2$ for 3-4 h. Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum was added into the inverted flask and cultured overnight. The medium was refreshed after 2-3 d. Finally, colchicine was introduced and mixed for further incubation of 30 min. The cells were harvested at 80-90% confluence using 0.25% trypsin (m/v) solution; they were separated into culture flasks in ratios of 1:2 or 1:3. The cell mixtures were centrifuged at 3,000 rpm for 10 min. After discarding the supernatant, the cells were treated with 10 mL of hypotonic solution (0.075 M KCl) and incubated at room temperature for 30 min. The cells were centrifuged and the supernatant discarded. The cells were fixed by gradually adding fresh cool fixative (3 methanol: 1 acetic acid) up to 8 ml. After centrifuging, the cells were repeatedly fixed until the supernatant was clear. The cells were added to 1 ml fixative by dropping onto a clean cold slide and then air dried (Bai *et al.* 2011; Phimphan *et al.* 2015).

*Chromosome staining*

The chromosomes were conventionally stained using a 20%-Giemsa working solution for 30 minutes (Patawang *et al.* 2017). $d(CA)_{15}$ and $d(GC)_{15}$ microsatellites and telomeric $(TTAGGG)_n$ sequence were used as probes. These probes were generated by PCR (PCR DIG-Probe Synthesis Kit, Roche) in the absence of a DNA template. Fluorescence *in situ* hybridization (FISH) was performed under highly stringent conditions on mitotic chromosome spreads. Metaphase chromosomes and non-metaphase cells on slides were incubated with RNAse (40 lg/ml) for 1.5 h at 37 $^{O}$C. After the chromosomal DNA was denatured for 4 min in 70 % formamide/29 SSC at pH 7.0 and 70 $^{O}$C, the hybridization mixture (2.5 ng/ll probes, 2 lg/ll salmon sperm DNA, 50 % deionized formamide, and 10 % dextran sulphate) was dropped on the slides and the hybridization was performed for 14 h at 37 $^{O}$C in a moist chamber containing 29 SSC. The first post-hybridization wash was performed with 29 SSC for 5 min at 65 $^{O}$C, and a final washing was performed at room temperature in 19 SSC for 5 min. The microsatellite repeats and telomeric probe were detected using Anti-digoxigenin-FITC. Finally, the slides were counterstained with DAPI and mounted in an antifade solution (Getlekha *et al.* 2016).

*Chromosome checking and classifying*

The lengths of short arm (Ls) and long arm (Ll) chromosomes were measured to calculate the length of the total arm chromosome (LT, LT = Ls + Ll). Relative length (RL) and centromeric index (CI) were estimated. CI was also computed to classify the types of chromosomes according to Chaiyasut (1989). All parameters were used in karyotyping and idiograming.

## RESULTS AND DISCUSSION

*Karyological characteristics of* S. chinensis

Both embryo samples of *S. chinensis* showed a diploid number of 70. The two embryos exhibited the same type of sex chromosome – Z and W, which lead to presumed female embryo. The autosome comprised of 10 macrochromosomes –v4 large metacentric, 2 medium acrocentric, 2 small metacentric, and 2 small submetacentric – and 58 microchromosomes (Table 1 and Figures 2a-b).

The diploid number found here differed from previous reports in the genus *Spilopelia*: 2*n*=80 in *S. chinensis* (You-Sheng *et al.* 2008), 2*n*=66 in *S. risoria* (Tange and Nakahara 1938-1939), 2*n*=78; 10 macrochromosomes + two sex-chromosomes (ZZ/ZW) + 66 microchromosomes and 2*n*=76; 16 macrochromosomes + 60 microchromosomes in *S. decaocto* (Srivastava and Misra 1971), and 2*n*=76; 16 macrochromosomes + 60 microchromosomes in *S. orientalis orientalis* (Makino *et al.* 1956).

*Chromosomal features of* T. ruficollis

*T. ruficollis* had a diploid number of 60 and fundamental number of 80 in both male and female (Figures 3a-b). The karyotype comprised of 20 macrochromosomes –2 large metacentric, 2 large submetacentric, 2 large acrocentric, 8 small metacentric, 4 small submetacentric and two sex chromosomes – and 40 microchromosomes. The sex chromosomes of *T. ruficollis* were classified to the ZZ/ZW system; Z was a medium submetacentric chromosome and W was a small submetacentric chromosome (Table 2 and Figures 3a-b). Also,

**Table 1.** Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) of RL, CI from 20 metaphase cells of two female individuals spotted dove (*Spilopelia chinensis*), 2*n*=70.

| Ch.p | Ls | Ll | LT | RL±SD | CL±SD | Ch.s | Ch.t |
|---|---|---|---|---|---|---|---|
| 1 | 3.630 | 5.190 | 8.820 | 0.242±0.012 | 0.588±0.024 | L | m |
| 2 | 2.690 | 3.920 | 6.610 | 0.181±0.010 | 0.593±0.030 | L | m |
| 3 | 1.180 | 4.320 | 5.500 | 0.151±0.008 | 0.785±0.026 | M | a |
| 4 | 1.770 | 2.260 | 4.030 | 0.110±0.008 | 0.561±0.032 | S | m |
| 5 | 1.450 | 2.210 | 3.660 | 0.100±0.009 | 0.604±0.028 | S | sm |
| 1st Sex chro. | 1.850 | 2.220 | 4.070 | 0.111±0.008 | 0.545±0.024 | S | m |
| 2nd Sex chro. | 1.340 | 2.490 | 3.830 | 0.105±0.010 | 0.650±0.030 | S | sm |
| 7-35 | - | - | - | - | - | Microchromosomes | |

Abbreviations: *Ch.p*, chromosome pair; *Ch.s*, chromosome size; *Ch.t*, chromosome type; *L*, large size; *M*, medium size; *S*, small size; *m*, metacentric; *sm*, submetacentric; *a*, acrocentric.

the karyotype showed the gradually series size of the 11th to 30th pairs of microchromosomes. Our result differed from Ebied *et al.* (2005), who found a diploid number of 58 in *T. ruficollis* from an Egyptian population. However, many of the karyotypic features of these two populations of *T. ruficollis* were the same, including the number of macrochromosomes (18) and sex chromosomes (2), and the type and size of each.

The molecular cytogenetical features in this report that exhibited only on the male *T. ruficollis* chromosome included two microsatellites and telomeric sequences. First, signals of $d(CA)_{15}$ microsatellites showed two signals on two microchromosomes; these presented alike in interphase (Figure 4a), prophase (Figure 4b) and metaphase (Figure 4c) cells. Next, microsatellite $d(GC)_{15}$ appeared on the sub-centromeric region of the long arm of one chromosome of the first pair macrochromosome (Figure 4d), shown in the idiogram as pair 1a and 1b (Figure 4e), which is same only one signal of both non-metaphase and metaphase cells. Finally, $AGGGTT_n$ sequence signals showed on each telomeric region of all macro- and microchromosomes, which appeared as green signals on interphase, prophase and metaphase cells as shown in Figures 4(f-h). Ours is the first study of these markers in this species, and is one of only a few avian chromosomal reports.

Microsatellites, simple sequence repeats (SSR), short tandem repeats (STR) and simple sequence length polymorphisms (SSLP) are found in prokaryotes and eukaryotes. They are widely dispersed in the genome, especially in the euchromatin of eukaryotes, and coding and noncoding nuclear and organellar DNA (Vieira *et al.* 2016; Kumar 2018). The signals of $d(CA)_{15}$ microsatellites on two microchromosomes of male *T. ruficollis* showed the one functional that was needed to find the answer in the future study. The signal of the $d(GC)_{15}$ microsatellite that exhibited on only one chromosome of the 1st pair is another issue that needs to be addressed. We used $AGGGTT_n$ sequence probes to investigate the feature of the male *T. ruficollis* chromosome. $AGGGTT_n$ are repeated sequences on the terminal end of the chromosome arm of general vertebrates, for example humans, mice and the *Xenopus* frog (Ichikawa *et al.* 2015). The $AGGGTT_n$ signals that appeared on the interphase, prophase and metaphase cells of the male *T. ruficollis* showed the existence of this sequence in this species (Figures 4f-h).

*Overview of avian chromosome*

In birds, females are the heterogametic sex with Z and W sex chromosomes; males are the homogametic sex, with ZZ sex chromosomes. Studies of sex chromosome evolution in birds and other systems with female heterogamety are important, because they offer independent replication of observations from X–Y species. We observed the heterogametic ZW sex chromosomes in female *T. ruficollis* (Figure 5a) and found heterogametic chromosomes in two embryonic *S. chinensis* samples (Figure 5b) in this study; this agreed with other avian sex chromosome studies (Ellegren 2000; Shibusawa *et al.* 2004).

Most avian chromosome studies have shown conserved characteristics on three macrochromosome pairs, including the 1st (metacentric, *m*), 2nd (submetacentric, *sm*) and 3rd (acrocentric, *a*) pairs. In addition, the 4th pair (metacentric or submetacentric) have been shown to exhibit the semi-conserved characteristic typical of many avian species. These characteristics have been

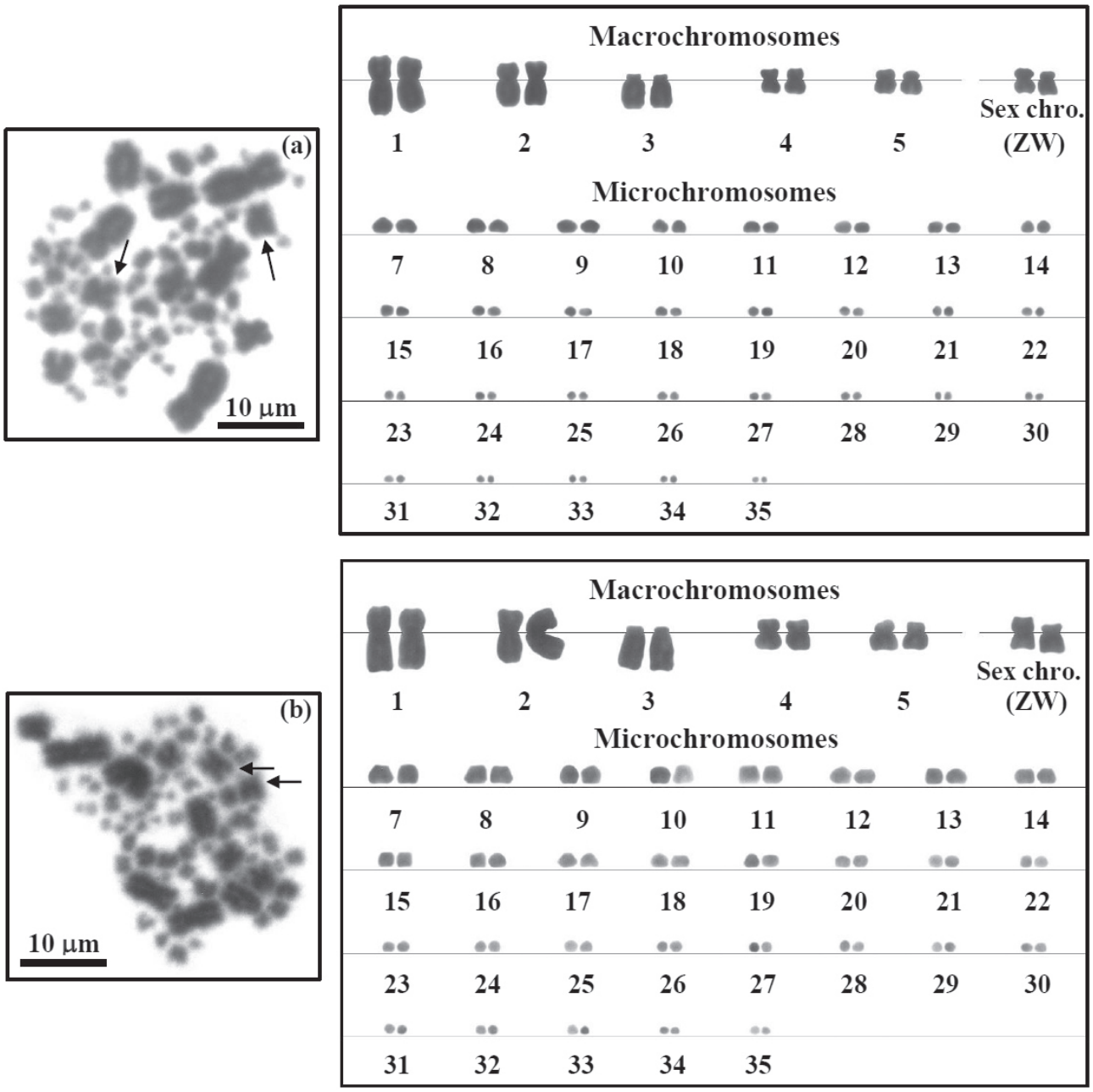


**Figure 2.** Metaphase chromosome plates and standardized karyotypes of embryonic individual 1 (a) and embryonic individual 2 (b) *Spilopelia chinensis*, 2*n*=70 by conventional staining.

observed in many species, for example *Agelaius phoeniceus* [2*n*=76] (Cox and James 1984), *Anas platyrhynchos* [2*n*=80] (Skinner *et al.* 2009), *Rupornis magnirostris* [2*n*=68], *Buteogallus meridionallis* [2*n*=68], and *Asturina nitida* [2*n*=68] (de Oliveira *et al.* 2013), *Lonchura punctulata* [2*n*=72] (Kaewmad *et al.* 2013), *Ara macao* [2*n*=62-64] (Seabury *et al.* 2013), *Turdus rufiventris* [2*n*=78], *T. albicollis* [2*n*=78] (Kretschmer *et al.* 2014), *Gallus gallus* [2*n*=78] (Phimphan *et al.* 2015); with the 1st pair *m*, 2nd *sm*, 3rd *a* and 4th *m*/*sm*. We found the same conserved chromosome pair characteristics in the two species in our study as in these other avian reports.

In addition, the microchromosome is one of many characteristics that has been conserved in the genome of all avian and many reptilian species. The archetypal avian chromosome comprises about 40 chromosome pairs

**Table 2.** Mean length of short arm chromosome (Ls), long arm chromosome (Ll), total arm chromosome (LT), relative length (RL), centromeric index (CI), and standard deviation (SD) of RL, CI from 20 metaphase cells of male and female little grebe (*Tachybaptus ruficollis*), 2*n*=60.

| Ch.p | Ls | Ll | LT | RL±SD | CL±SD | Ch.s | Ch.t |
|---|---|---|---|---|---|---|---|
| 1 | 4.410 | 5.920 | 10.330 | 0.185±0.004 | 0.573±0.020 | L | m |
| 2 | 3.150 | 5.750 | 8.900 | 0.159±0.005 | 0.646±0.032 | L | sm |
| 3 | 0.900 | 5.900 | 6.800 | 0.122±0.004 | 0.868±0.015 | L | a |
| 4 | 1.800 | 2.530 | 4.330 | 0.078±0.006 | 0.584±0.025 | S | m |
| 5 | 1.450 | 2.560 | 4.010 | 0.072±0.005 | 0.638±0.040 | S | sm |
| 6 | 1.500 | 2.340 | 3.840 | 0.069±0.004 | 0.609±0.035 | S | sm |
| 7 | 1.340 | 1.760 | 3.100 | 0.056±0.005 | 0.568±0.042 | S | m |
| 8 | 1.300 | 1.450 | 2.750 | 0.049±0.007 | 0.527±0.045 | S | m |
| 9 | 1.250 | 1.400 | 2.650 | 0.047±0.003 | 0.528±0.038 | S | m |
| Z | 2.030 | 3.450 | 5.480 | 0.098±0.004 | 0.630±0.042 | M | sm |
| W | 1.320 | 2.290 | 3.610 | 0.065±0.003 | 0.634±0.036 | S | sm |
| 11-30 | - | - | - | - | - | Microchromosomes | |

Abbreviations: *Ch.p*, chromosome pair; *Ch.s*, chromosome size; *Ch.t*, chromosome type; *L*, large size; *M*, medium size; *S*, small size; *m*, metacentric; *sm*, submetacentric; *a*, acrocentric.

and usually 30 small to tiny microchromosome pairs. This karyotypic feature perhaps evolved 100-250 million years ago (Burt 2002). The *S. chinensis* and *T. ruficollis* in this study had a microchromosome number of 58 and 40, respectively, indicating the close evolutionary lines between these two species and other avian species.

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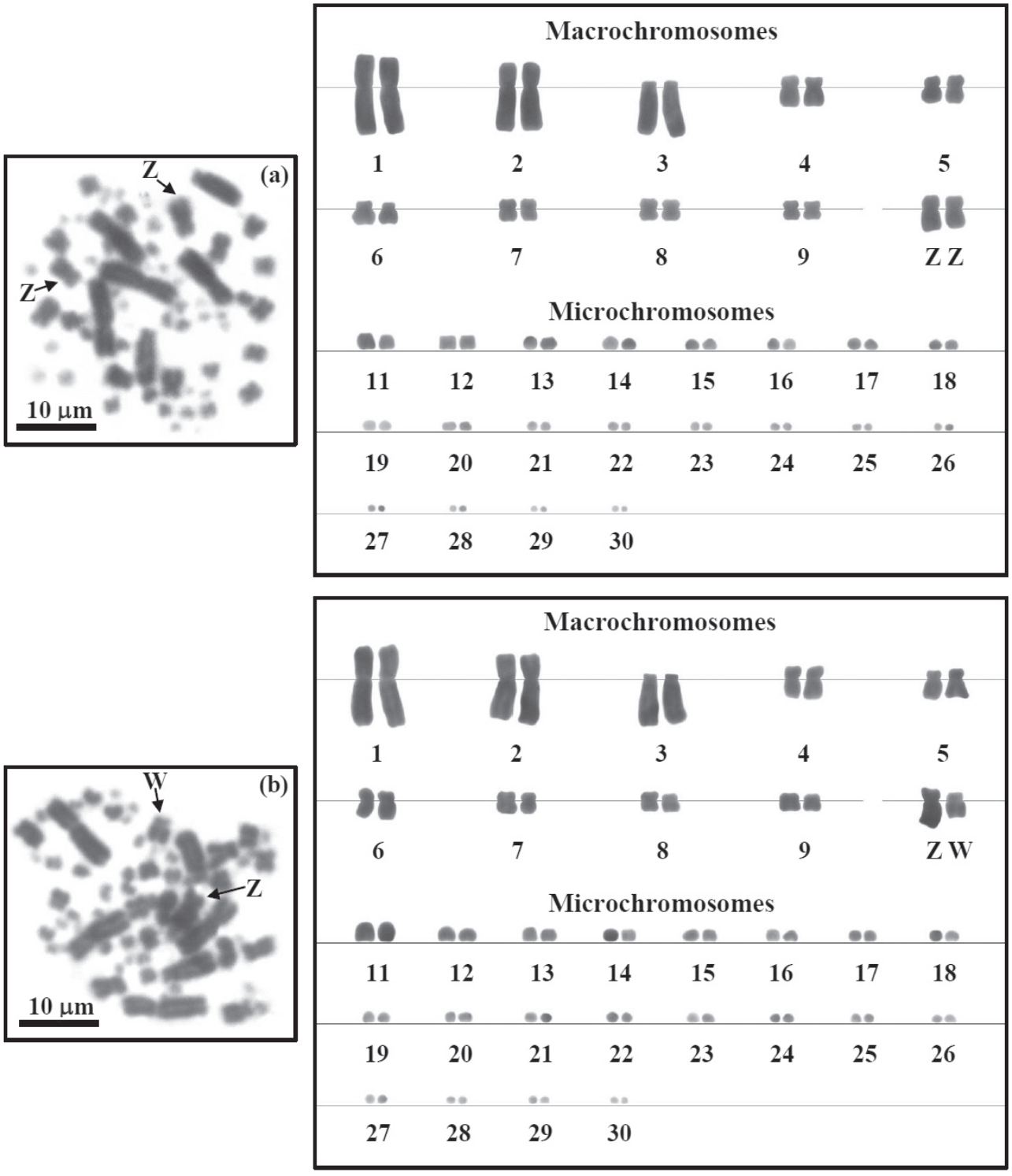


**Figure 3.** Metaphase chromosome plates and standardized karyotypes of male (a) and female (b) *Tachybaptus ruficollis*, 2*n*=60 by conventional staining.

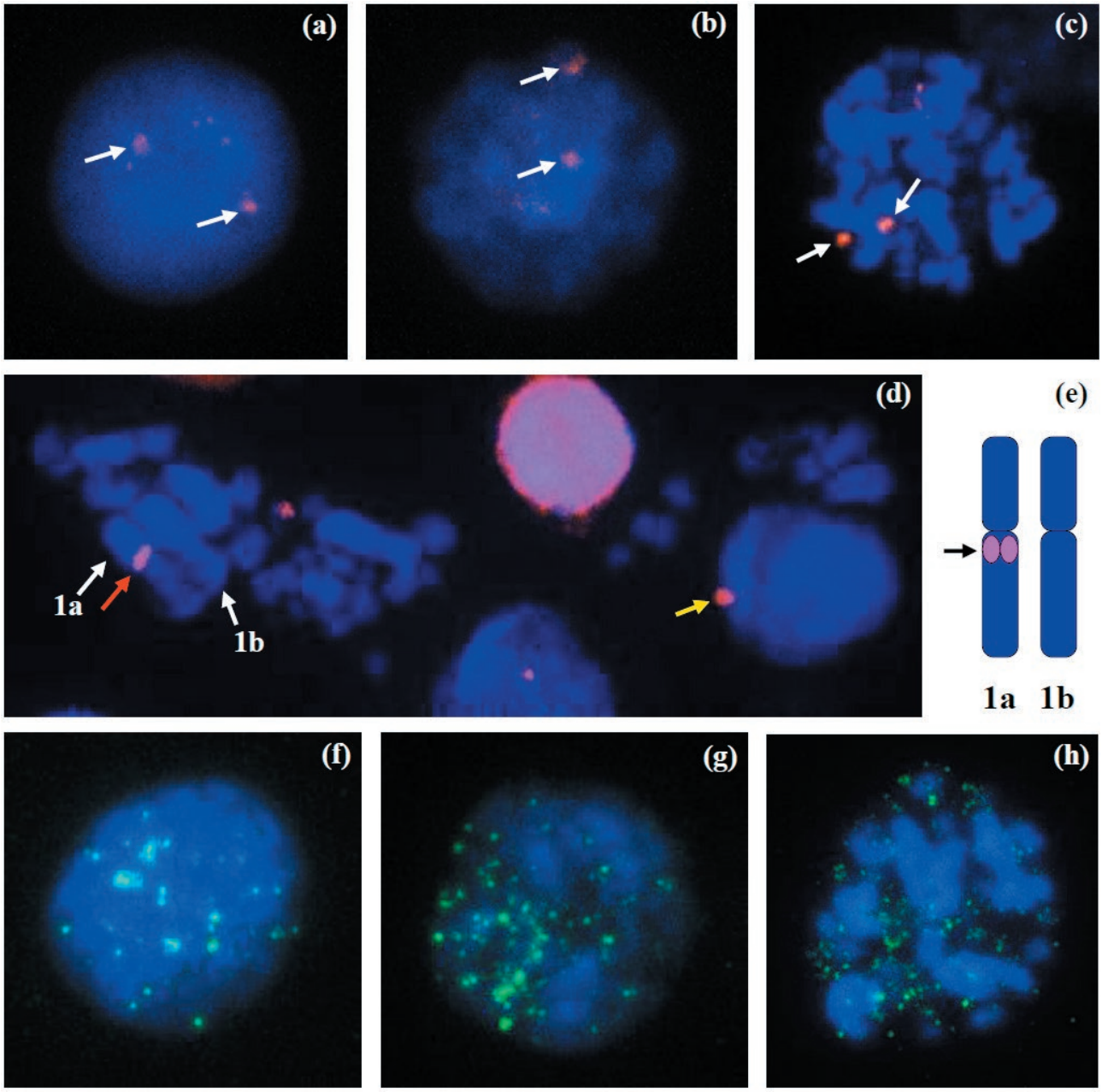


**Figure 4.** The molecular cytogenetical features of male *Tachybaptus ruficollis*, including: d(CA)$_{15}$ microsatellite signals on two microchromosomes of interphase (a) prophase (b) and metaphase (c); d(GC)$_{15}$ microsatellite signals on only one chromosome of the 1$^{st}$ pair of metaphase (d, red arrow), interphase (d, yellow arrow) and the position of this signal on idiogram (e); and AGGGTT$_n$ telomeric sequences on interphase (f), prophase (g) and metaphase (h).

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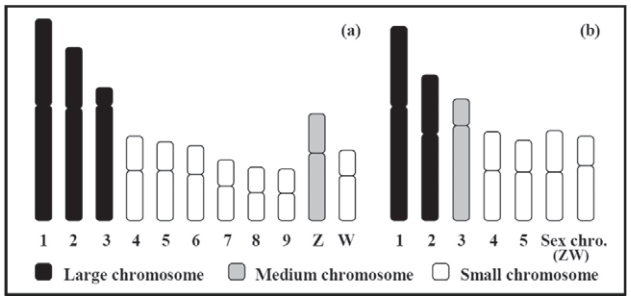


**Figure 5.** Standardized macro-chromosomal idiogram of *Tachybaptus ruficollis*, 2*n*=60 (a) and *Spilopelia chinensis*, 2*n*=70 (b) by conventional staining.

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