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Rapid Sexing Hill Mynah *Gracula religiosa* by Sex Chromosomes

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Abstract: Hill Mynah *Gracula religiosa* is a monomorphic bird of which the discrimination of sex is a crucial problem in captive breeding programs. In the past ten years, the wild population of the two subspecies found in Thailand, *G. r. intermedia* and *G. r. religiosa*, have drastically decreased due to the demand of the pet market to trade them as talking birds. Sexing Hill Mynah will be an important part in breeding them in captivity. Various methods had been experimented to sex them. Best of all was to obtain sex chromosomes from feather pulp. Male is the homogametic sex (ZZ) whereas female is the heterogametic sex (ZW). To identify Z and W chromosomes in Hill Mynah is undoubtedly an easy task since Z chromosome is nearly twice the size of W chromosome. It is subtelocentric and medium in size of macrochromosomes or the same size as pairs 4, 5, 6 while W chromosome is submetacentric and smaller, the same size as pairs 7, 8, 9. The diploid chromosome number of Hill Mynah was found to be 80. The karyotype consisted of ten pairs of macrochromosomes, nine pairs of autosomes and one pair of sex chromosome and thirty pairs of microchromosomes. Captive breeding of Hill Mynahs in Thailand is no longer perplexed when sex identification is ultimately possible.

Key words: *Gracula religiosa*, sex determination

INTRODUCTION

Sex identification in monomorphic birds is very crucial especially in captive breeding programs for both economic and endangered species. Hill Mynahs have no distinctive external sexual dimorphism. The talent to mimic any sound particularly human languages in captivity led them to be ceaselessly demanded as caged pets. The wild population of both subspecies found in Thailand, *Gracula religiosa intermedia* (the northern race) and *G. r. religiosa* (the southern race) have subsequently decreased in the past ten years. Captive propagation has become paramount. One of the reasons why Hill Mynah captive breeding in Thailand hardly succeeded is that we could not differentiate between male and female birds. They look alike and cannot be identified from body side, color of eyes and legs[1], or shape of yellow wattles.

Various techniques have been tried for decades to sex monomorphic birds. Each method has its own advantages and disadvantages and depends on laboratory facilities, the experimenters' experience as well as specimens availability. Early studies pay attention to the differences of plumage color, body weight and shape of the beak[2]. Measurements of museum specimens are also the primary technique employed. Anatomical structure examinations such as cloaca, pelvic, head, wing, body size and tail length are a popular method[3]. However, applying morphological study technique to Hill Mynahs makes the sex determination rather ambiguous because of the geographical variation[4,5]. In the past, the use of sexual behavior patterns to discriminate sex is proceeded. But, sometimes, sex-differenced performances are less pronounced. Females of some species revealed the same behavior patterns as those of the males[3,6]. The uses of laparotomy method followed by various medical instruments to identify gonads such as otoscope[6,7], arthroscope[8] needlescope[3,9-10] and fibreoptic endoscopy[2,11] are reported. However, the laparotomy technique is disadvantageous in that birds needed to be properly anesthetized by veterinarians who are able to do better than others; otherwise tissue damage, hemorrhage and the death of specimens occasionally occur. Fecal or plasma steroid analysis is a reliable method but it is costly and time-consuming. A problem also arises because of seasonal fluctuations in steroid hormones[12].

Cytogenetic technique for sex determination of monomorphic birds is now well established[13]. Sexing chromosomal determination is an invasive procedure which has been accurately performed. Most avian karyotypes comprise a small number of macrochromosomes and a large number of very small chromosomes, microchromosomes[14,15]. Female is the heterogametic sex (ZW) and male is the homogametic sex (ZZ)[13,16-20]. Sex identification by sex chromosomes is

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easily accomplished because large-sized Z chromosome can be discriminated from the smaller W chromosome[19, 21-22].

In the present study, the sex determination of Hill Mynahs was based on sex chromosomes, which were prepared from feather pulp.

MATERIALS AND METHODS

Chromosomal sexing in this study was accessible from feather pulp preparation[23]. It was a direct method in which the tissue from the feather base was immediately proceeded since the specimens were taken not far away from the laboratory at Ramkhamhaeng University. Tail feathers of the northern race Hill Mynah, *G. r. intermedia*, were plucked and then waited for 14-15 days for pinfeathers growing. Since the laboratory was adjacent to the animal house where the specimens were available, I could squeeze the tissue from the growing feather at once after phosphate buffered saline containing antibiotic, penicillin 100 iu mL^{-1}, streptomycin 100 ug mL^{-1} and amphotericin B 2.5 ug mL^{-1} was added. Collagenase 200 units mL^{-1} in Hank's balanced salt solution pH 7.4 and colcemid 0.05 ug mL^{-1} were put in for a three-hour incubation at room temperature. Cells were centrifuged at 2,000 rpm for 5 mins. The supernatant was eliminated and resuspended in hypotonic solution for 15 mins at 37°C. Cells were centrifuged and the fixative was added three times and later were dropped on slides and stained with Giemsa.

Sex chromosomes were examined under the microscope; medium-sized Z chromosome was easily told apart from small-sized W chromosome, which was found only in a female bird. Hill Mynah chromosome set was also karyotyped which was the first time to be reported by this rapid technique for this bird in Family Sturnidae. To assure large number of microchromosomes, 283 metaphases were repeatedly counted for a diploid chromosome number. The characteristic nomenclature was made on the position of centromere[24].

RESULTS

The most frequent diploid chromosome number for Hill Mynahs as studied from 283 metaphases in both sexes was found to be 80 (Fig. 1 and 2). The karyotype showed nine pairs of autosomal macrochromosomes and one pair of sex chromosome (ZZ in males or ZW in females) and thirty pairs of microchromosomes. There were three sizes of macrochromosomes. Pairs 1, 2, 3 were large-sized, pairs 4, 5, 6 were medium-sized and pairs 7, 8, 9 were small-sized. The Z chromosome's size

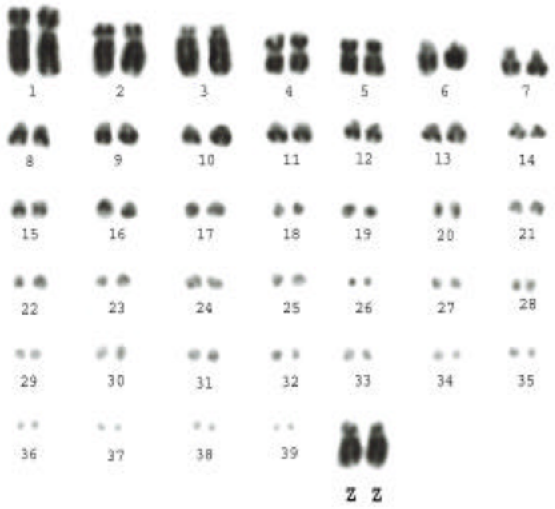

Fig. 1: The karyotype of a male Hill Mynah (ZZ sex chromosomes)

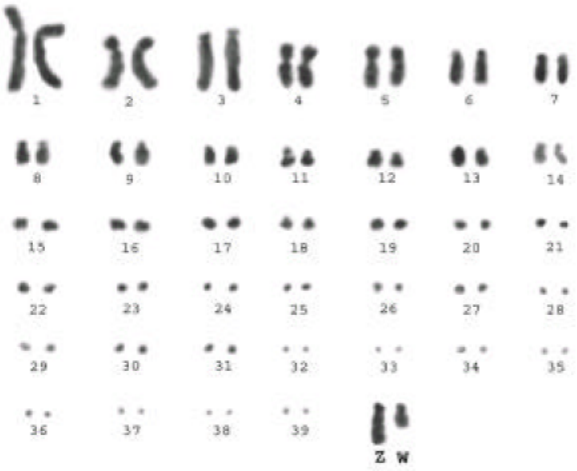

Fig. 2: The karyotype of a female Hill Mynah (ZW sex chromosomes)

was medium whereas the W chromosome was small. The first pair, the largest chromosome in the set and pairs 2 and 5 were submetacentric, while pairs 3, 6, 7, 8, 9 were subtelocentric and pair 4 was metacentric. The Z chromosome was subtelocentric and the W chromosome was submetacentric. The discrimination of sex chromosomes from the others were undoubtedly distinctive because when each karyotype of a female bird was analyzed, Z chromosome which was subtelocentric and medium-sized was left unpaired and W chromosome which was submetacentric and small-sized was left single. Meanwhile, a male bird was recognized easily by a pair of Z chromosomes left which was the fourth pair of medium-sized macrochromosomes in

addition to pairs 4, 5, 6. Therefore, sex determination in Hill Mynah was precisely discriminated by sex chromosomes.

DISCUSSION

Although avian karyotypes are relatively less studied than those of mammals and reptiles[13] and the W sex chromosome in female bird was just recognized during the 1960's, the technique in this study is one of the most efficient methods to identify sex in monomorphic birds without hurting or sacrificing specimens. Chromosome characteristics of the Sturnidae had been karyotyped[25] and reported but not the Hill Mynahs'. However, this was the first time ever that Hill Mynah was rapidly sexed by sex chromosomes. As for other bird species a diploid number of chromosome was high and composed of two sizes known as macro and microchromosomes. Most of the chromosomes set were microchromosomes and it was difficult to specify the number exactly. This problem has been solved in Hill Mynahs by analyzing large number of metaphases[26]. The macro and microchromosomes of Hill Mynahs were discriminated easily. Sex chromosomes were grouped in macrochromosomes; Z chromosome was the medium-sized chromosome whereas W chromosome was half the size of Z chromosome. Paired ZZ sex chromsomes were present in male and paired ZW sex chromosomes were obvious in heterogametic female. However, there were also studies in other birds which showed that W sex chromosome and microchromosomes were indistinguishable[18, 27-28]. Attempts to discriminate W sex chromosome had been made in various ways including C-banding technique or enlarged sex chromosome[18]. In Hill Mynahs, to identify W sex chromosome from microchromosomes was not a problem because W sex chromosome was submetacentric whereas most of microchromosomes were subtelocentric or acrocentric in the characteristic nomenclature. Therefore C-banding technique for W sex chromosome was not necessary in this study.

Several techniques were applied in this study to sexing Hill Mynahs such as laparotomy[29], leukocyte culture and bone marrow preparation to obtain chromosomal sexing. Laparotomy did hurt the birds and made them so tense during the operation procedure. One centimeter incision was made between the last and the second last of the left rib by holding birds with left hand and stretching two wings between forefinger and middle finger while the thumb pressed on the left leg. The wound was not fatal and stitches were unnecessary. The suture would eventually heal in a few days. Ovary and testis could seen clearly under the visceral organ near the kidneys especially during the breeding season (January to July) with large size gonads. Nevertheless, this technique requires a skill for handling birds, making unhurting wound and a precise determination because sex organs are under the visceral organ but above the kidneys. Kidneys are very fragile organs. If the tips of the forceps touch them accidentally, blood flushes all over the abdomen. Thus, the discrimination of testis from ovary is not possible and death occurs.

Chromosome preparations from cells in metaphase stage were obtained from numerous sources such as from leukocyte cultures[22,30-33], from bone marrow cell culture[28,34-37] or from feather pulp culture[23,37-39]. I also tried to prepare sex chromosomes by two methods. First, the leukocyte culture which was not workable in my laboratory and fairly complicated. Blood was drawn from the wing vein. The outcome of leukocyte culture was unsatisfactory. Serum from fetal calf or chicken was used. Mitogen as pokeweed or phytohemagglutinin or both were experimented. RPMI pH 6.8-8.0, temperature 37.5-42.0°C, time from 12-96 hrs for incubating were variably tested. Contamination was involved; if not, the results were still unpredictable as it was found that sometimes it grew well, but sometimes it did not. The second technique for sex chromosome preparation was from bone marrow. However, this technique was unreliable and should not be used at all especially for breeders because the specimens were sacrificed.

Ultimately, the most suitable method to access sex chromosome of Hill Mynahs was that presented in this paper. The chromosome preparation from feather pulp was a perfect technique considering all circumstances. It was the quickest and simplest method. To pluck tail feathers and wait for 14-15 days for growing feathers did not bother the birds and the experimenters. The birds were still alive, healthy and unharmed. The feather pulp preparation was advantageous to the high mitotic rate in rapidly growing pulp tissue found at the base of pinfeathers. The laboratory could be started immediately after waiting two weeks for growing feather availability and then karyotypes would be obtained in a few days.

Captive breeding of Hill Mynahs in Thailand once was not successful. The crucial obstacle was that male birds could not be discriminated from female ones. As a consequence, pairing the same sex by mistake made people thought that breeding Hill Mynahs in captivity rarely happened. It is very useful for identifying the sex of monomorphic birds especially in breeding programs[21, 39]. The findings from this research will be beneficial to the effort to increase the population of captive Hill Mynahs in Thailand since sex identification is no longer a problem.

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