Karyotype of a rare minute frog, *Oreophryne* cf. *anulata* (Anura: Microhylidae), in Agusan Marsh, Mindanao, Philippines

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ABSTRACT - The Philippine archipelago has a rich amphibian fauna but data on their karyology are scarce. A recent amphibian diversity assessment of one of the largest wetlands and a major site for biodiversity conservation in the Philippines (Agusan Marsh Wildlife Sanctuary) reported three rare endemic species of minute frogs which are suspected to be new, including two undescribed species of *Oreophryne*. Here we describe the karyotype of one of the *Oreophryne* species, which is a form of *Oreophryne anulata*. Mature frogs were obtained by acoustic and opportunistic sampling from a site bordering Terminalia forests, and then reared in an enclosure for one to three months before they were karyotyped. Analyzable metaphase spreads were routinely obtained from seven colchicine-treated frogs by squashing intestinal epithelial cells followed by vapor fixation, air-drying and staining with aceto-orcein. Chromosome analysis indicated a normal diploid karyotype of 2n=22 including four metacentric and seven submetacentric chromosome pairs, without distinguishable sex chromosomes. The karyotype differs from that of the other *Oreophryne* species that have been karyotyped so far, for example, *Oreophryne biroi* in the Australo-Papuan region. This difference has an interesting evolutionary implication, which could contribute to the understanding of the mechanisms and rates of speciation in genus *Oreophryne* and is vital to the taxonomy and conservation of the endemic *Oreophryne* species in Agusan Marsh.

INTRODUCTION

The Philippines has currently more than 100 species of amphibians and the current rate of discovery of new species is high (Almeria and Nuñeza, 2013; Diesmos et al., 2002). About 84 (78.6%) of these amphibian species are endemic but this figure is likely to increase when new species are formally described (Diesmos et al., 2002). Unfortunately, most of these species are poorly known, and the few studies are limited to species composition, diversity, endemism, abundance and threats (Almeria and Nuñeza, 2013; Alcala et al., 2012; Nuñeza et al., 2010; Relox et al., 2010; Diesmos et al., 2002; Brown et al., 2000). Despite the value of karyologic and genetic studies in formal classification of suspected new species, taxonomic revision of those that are believed to be a complex of cryptic species (Thode and Alvarez, 1983), and assessment of the variability and conservation status of many species, such studies about Philippine amphibians are scarce (Kuramoto and Yong, 1992). This lack of information is of concern due to global rapid declines of amphibian populations (Woodruff, 2010; Bickford et al., 2010). The increasing number of critically endangered, endangered and vulnerable species in the Philippines (Alcala et al., 2012) demands an urgent assessment of the status of amphibian diversity in the Philippines.

A recent species inventory of Agusan Marsh, one of the largest wetlands and one of the centres of biodiversity in the Philippines, revealed a high diversity of amphibians with 41% endemism including three rare endemic species of minute frogs suspected to be new to science (Almeria and Nuñeza, 2013). Two of these belong to the microhylid frog genus *Oreophryne* of the subfamily Astereophryine, which is widespread in the Indo-Australian archipelago between the southern Philippines and New Britain. This genus is most diverse in New Guinea and immediate adjacent islands, and is the largest component of the Papuan microhylid fauna with 54 currently named species (Frost, 2015).

There are two currently recognised species of genus *Oreophryne* in the Philippines, *O. anulata* and *O. nana*, about which little is known (Alcala, 1986). Both can be found in the southern part of the Philippines, specifically on the islands of Biliran, Camiguin and Mindanao. The two species of *Oreophryne* in Agusan Marsh, which are suspected to be new, still await further studies that could aid in their taxonomic classification and conservation.

This paper describes the karyotype of a suspected new *Oreophryne* in Agusan Marsh that is a close form of *O. anulata* (Fig. 1). To date, there is only one published report on the karyotype of a species of *Oreophryne*. *O. biroi* is an Australo-Papuan species with a diploid karyotype of 2n=26 (Mahony et al., 1992). No karyologic studies of this genus have been done in other localities where it is distributed including Indonesia and the Philippines. The present information could help clarify the evolutionary history, assess genetic diversity, and improve the conservation status of the species. Furthermore, it could contribute to the understanding of the mechanism and rates of speciation in this genus (Vences et al., 2002). Aside from supplementing the scarce karyologic data on amphibians in the Philippines, this study can also be

useful in monitoring resources of genetic diversity in its amphibians (Chulalaksananukul et al., 1998).

MATERIALS AND METHODS

Collection, identification and husbandry of frogs.
Ten mature frogs were collected by acoustic and opportunistic approaches in Sitio Kaliluan, Campo 6, Neuva Era, Bunawan, Agusan del Sur beside Magsagasang creek (08° 09.825" North latitude and 125° 58.044 East longitude) at an elevation of 27–30 masl. Collection was made in a site bordering a Terminalia forest inhabited by the amphibians of interest based on a previous survey (Almeria and Nuñeza, 2013). Samples were acclimatised and reared in an open-system customised frog enclosure at an ambient temperature of 23–25 °C following the protocol of Poole and Grow (2012) for one to three months prior to sacrifice for chromosome analysis. The enclosure was artificially lit for 12 hours daily using a lamp, and the frogs were fed every other day with captured fruit flies (*Drosophila melanogaster*). They were regularly provided with moisture by spraying rainwater at least twice a day. Waste material was drained by pouring water into the enclosure and then draining it through a hole at the bottom.

The frog samples were referred to and identified by Dr. Arvin Diesmos, curator of the Zoology division of the National Museum of the Philippines, as *Oreophryne* cf. *anulata* which exhibits distinct differences from the *O. anulata* samples that he collected from various areas of Mindanao (personal communication, 04/01/14). The samples were morphologically similar to, and were likely belonging to the same population, as the *Oreophryne* sp. 1 previously reported by Almeria and Nuñeza (2013) as a candidate new species. The frogs were identified as male on the basis of their advertisement calls and their vocal sac whereas females were identified by the presence of eggs in their coelom during dissection. All institutional and national guidelines for the care and use of laboratory animals were followed.

Chromosome analysis.
Due to the minute size of the frogs, metaphase arrest was done by keeping the frogs more or less immersed in 0.05% colchicine solution in a shallow plastic container 3–24 hours before sacrifice. They were subsequently euthanised, washed in running water, and then immediately dissected. The entire intestine from the rectum to the anterior end of the stomach was removed, cleaned, and incubated for 30 minutes in a 0.05 M KCl hypotonic solution. Initial fixation was done by soaking the intestine in a freshly prepared 3:1 ethanol-acetic acid solution for at least 10 minutes. Then, it was immersed in fresh fixative solution overnight in a refrigerator. The fixed intestine was trimmed into 2–3 mm portions, transferred to a clean glass slide with a drop of 45% acetic acid, and then stained with 1% aceto-orcein for 20 minutes. The slide was covered with a cover slip and thumb pressure was applied to squash the cells and induce chromosome spreading.

Five to twenty-two metaphase spreads from each frog sample were examined under a compound microscope with oil immersion objective, and the chromosomes in each spread were counted. At least three well-spread metaphases with the modal chromosome number were analyzed and used to prepare representative karyograms for each frog. Chromosome spreads were photographed by a CANON Power Shot A3200 IS digital camera with 14.1 megapixels. The actual sizes of the metaphase chromosomes were measured using a calibrated micrometer eyepiece. The chromosomes were cut out from the photographs and arranged in pairs according to size following the usual layout of a karyogram using the Paint tool of Microsoft XP. The lengths of the short arm and long arm, and the total length of the chromosomes, were digitally measured using the downloadable virtual actual-sized ruler application for Windows 2010. The measurements were encoded in an Excel spread sheet, and the following formulas were used to describe the features of the karyotype:

(1) Relative length = (Chromosome length / Total number of haploid genome) X 100
(2) Arm ratio = (Length of long arm / Length of short arm) X 100
(3) Centromere index = (Length of short arm / Total length of the whole chromosome) X 100
(4) Fundamental number = Total number of short arms + Total number of long arms

Based on the above measures, the chromosomes were classified according to the scheme of Levan and Tjio (1956), and an ideogram was constructed.

RESULTS

Analyzable metaphase spreads were obtained from seven out of ten frog samples: three males, three females, and one with unverified sex. The data on chromosome counts indicate that the modal chromosome number is 22 (Table 1). As shown by the representative karyotypes of the seven frog samples (Fig. 2–3), all the chromosomes are monocentric and biarmed, and there are no heteromorphic sex chromosomes. Based on the calculated relative length (RL) of the chromosomes (Table 2), the karyotype of the analyzed frogs include one large (chromosome pair 1; RL > 15%), three intermediate (chromosome pairs 2, 3 and 4; RL > 10%), and seven small pairs (chromosome pairs 5–11; RL < 10%) (Table 2, Fig. 1–2). Based on the calculated centromere indices and arm ratios (Table 2), chromosome pairs 1, 9, 10, and 11 are metacentric while chromosome pairs 2, 3, 4, 5, 6, 7, and 8 are submetacentric (Levan and Tjio, 1956). Since all of the 22 chromosomes in the diploid karyotype of the frog samples are biarmed, the fundamental number is therefore 44.

DISCUSSION

Since the frogs used in this study were minute (mean snout-vent length = 16.27 mm), colchicine treatment was carried out using the immersion technique. This was based

Figure 1. Adult female sample of *Oreophryne cf. anulata* on a Philippine one peso coin (diameter 24 mm).

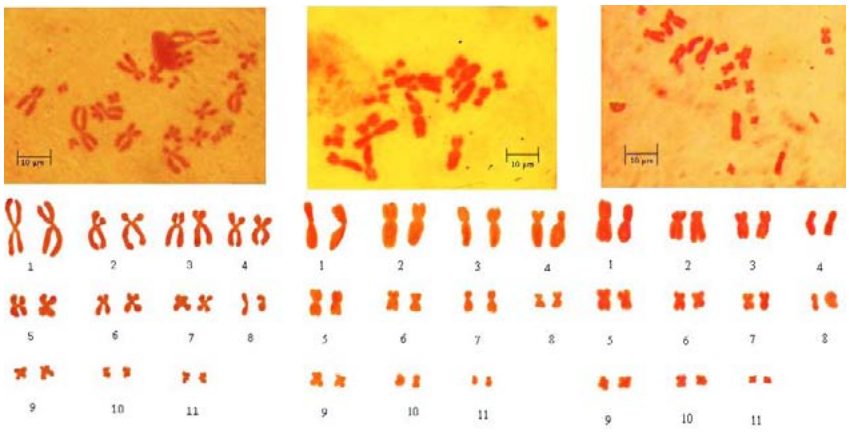

Figure 2. Representative metaphase spreads (top) and karyograms (bottom) prepared from intestinal epithelium of three *Oreophryne cf. anulata* samples from Agusan Marsh, Bunawan, Agusan del Sur, Philippines.

Frog Sample	Sex	Chromosome Numbers										
		16	17	18	19	20	21	**22**	23	24	25	26
1	Male	2	1	-	2	2	2	**12**	-	-	-	1
2	Male	-	-	-	2	3	1	**8**	-	-	-	-
3	Male	-	-	1	1	3	1	**6**	-	-	-	-
6	Female	-	-	-	-	1	1	**3**	-	-	-	-
7	Female	-	1	1	-	-	-	**4**	-	-	-	-
8	Female	-	1	-	-	1	2	**4**	-	-	-	-
9	Unverified	-	-	1	1	1	-	**8**	-	-	-	-

Table 1. Frequency distribution of chromosome numbers observed in intestinal epithelial cells of seven *Oreophryne cf. anulata* samples from Agusan Marsh, Agusan del Sur, Philippines. The modal chromosome number is 2n=22.

Chromosome Number	Mean Arm Ratio	Mean Relative Length	Mean Centromere Index	Centromere Position
1	1.21	17.01	46.75	Nearly median
2	1.66	14.01	38.26	Sub-median
3	1.78	12.49	36.41	Sub-median
4	1.72	11.45	37.58	Sub-median
5	1.56	9.77	39.51	Sub-median
6	1.44	8.19	41.54	Sub-median
7	1.33	7.34	43.41	Sub-median
8	1.34	6.48	43.08	Sub-median
9	1.17	5.4	46.38	Nearly median
10	1.15	4.33	46.71	Nearly median
11	1.1	3.6	47.58	Nearly median

Table 2. Mean arm ratios, relative lengths, centromere indices, and centromere positions of chromosomes in intestinal epithelial cells of seven *Oreophryne cf. anulata* samples from Agusan Marsh, Agusan del Sur, Philippines.

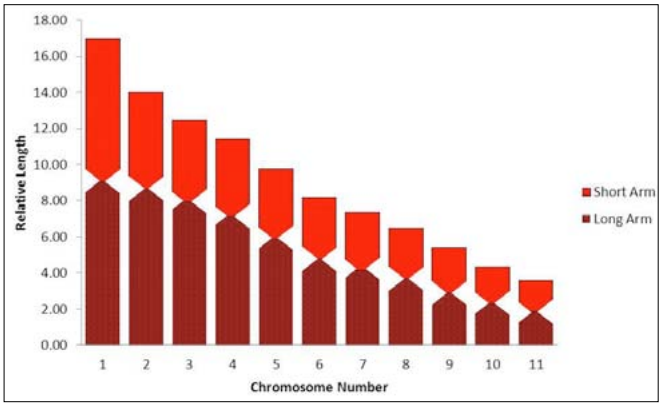

Figure 3. Ideogram of *Oreophryne cf. anulata* samples from Agusan Marsh, Bunawan, Agusan del Sur, Philippines.

on the fact that in frogs water intake is primarily through highly-vascularized "drink patches located on the posterior portion of their belly (Poole and Grow, 2012). However, the immersion technique did not yield a large number of analyzable metaphases. This could be due to a number of factors including insufficiency of colchicine that was actually absorbed through the skin, age, sex, physiological state of the amphibians, and effects of the hypotonic treatment and fixation. Although a heteromorphic pair of chromosomes was not detected, the presence of sex chromosomes cannot be discounted because the analyzed chromosomes were not differentially stained. For instance, the karyotype of *Bufo marinus* also lacks a heteromorphic pair of chromosomes but chromosome banding revealed a pair of sex chromosomes (Abramyan et al., 2009).

The karyotype of the frogs in this study differs considerably from that of the only other *Oreophryne* species that has been published, *O. biroi*, which is from the Australo-Papuan region. This has a diploid chromosome number of 2n=26 (Mahony et. al., 1992). The latter's karyotype consists of ten metacentric (1, 5-13) and three submetacentric (2-4) pairs, of which 1 is large, 3 are medium, and 9 are small pairs. This supports

previous studies of the relatively high chromosomal rearrangement in the family Microhylidae (Mahony et al., 1992; Kuramoto and Allison, 1989; Bogart and Nelson, 1976). The variations between members of the same genus may be due to several mechanisms including polyploidy and chromosomal rearrangements such as chromosome fusion, chromosome fission, and changes in composition of heterochromatin (De Mattos et al., 2014; Gruber et al., 2012; Vos et al., 2011; Wickbom, 1950). Morescalchi (1979) noted that rapid chromosomal evolution is associated with speciation in tropical habitats. On the other hand, Bogart (1981), proposed that chromosomal variability is correlated with the terrestrial habit (including parental behaviour, increased territoriality, and small clutch size).

According to Bogart and Nelson (1976) and Kuramoto and Allison (1989), the 2n=26 karyotype is "primitive" for Microhylidae. Mahony et al. (1992) suggested on the basis of the karyological data that the standard karyotype of the microhylid subfamily Astereophryinae (which includes the genus *Oreophryne*) found in New Guinea and Australia has 2n=26 chromosomes consisting of 5 large and 8 small pairs. The results of the present study show that a species of genus *Oreophryne* in the Philippines has a smaller diploid number of 2n=22. All microhylids with 2n=22 chromosomes are said to be New World microhyline members of the Microhylidae (Bogart and Nelson, 1976).

Based on molecular studies, Kurabayashi et al. (2011) suggested that the colonisation route of *asterophryine microhylids* where the genus *Oreophryne* belongs was via Indo-Eurasia. According to their hypothesis, the family Microhylidae split into the subfamilies Asterophryinae, Microhylinae and Dyscophinae in India around 70 Ma. Microhylinae and Asterophryinae entered Eurasia while Dyscophinae proceeded to Madagascar. Asterophryinae split to Gastrophrynoidae in Southeast Asia around 48 Ma while the remaining Asterophryinae proceeded to New Guinea and Australia at around 25 Ma. However, the data of Mahony et al. (1992) and Kuramoto and Allison (1989) on the karyotype of genus *Oreophryne* in the Indo-Australian archipelago, and the karyological data on *O. cf. anulata* in the present study, do not support the suggested colonisation route of subfamily Asterophryinae via Indo-Eurasia. Instead, the data suggest that the origin of the Southeast Asian Oreophryne is from the Australo-Papuan population. This is illustrated by a reduced diploid chromosome number of 22 in *O. cf. anulata* from the Philippines as compared to the primitive diploid chromosome number of 26 in the Australo-Papuan Oreophryne species. The reduction in chromosome number from 2n=26 to 2n=22 might have resulted from chromosomal rearrangements such as fusion of smaller chromosomes in the "primitive" karyotype into larger chromosomes (De Mattos et al., 2014; Vos et al., 2011).

Since the karyological data presented in this study is the only report so far on an *Oreophryne* species, which is not from an Australo-Papuan form, further investigations of the karyology and molecular studies of the *Oreophryne* species present in the Philippines and the other Southeast Asian representatives are urgently needed to validate these initial findings.

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