Pulse variations of the advertisement calls of slender toads, genus *Ansonia* (Amphibia: Anura: Bufonidae) species complex in the Philippines

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Abstract. The study was undertaken with the notion that pulse, as the smallest undividable unit of calling frogs/toads and a static variable, is a relevant parameter for species recognition, particularly pulse rates and number of pulses per call. Thus, would help shed light in the reassessment of the identity of the *Ansonia* species complex. The pulse characteristics of the advertisement calls of *Ansonia* species recorded from the eight rivers in Mindanao, Philippines were described and differentiated in relation to their call features, body sizes, and the ecological attributes of the rivers. The number of pulses per call, pulse rate, pulse duration, and interval were taken into account and analyzed for bioacoustics using Raven Pro 1.4 for Windows (2011). Five one-minute-call slices served as replicates per individual toad recording using a Tascam DR-1 Portable Solid State Recorder and a uni-directional external microphone. Results revealed variations in these pulse characteristics. These pulse features showed highly significant variations except for pulse interval. In general, *Ansonia* sp.1 of Maragang River stood as one cluster from the rest of the toads. These features also exhibited high correlations with other call parameters, peak call frequency, body sizes, and ecological features.

1. Introduction
The vocalization of *Ansonia* species is characterized by pulsed calls for advertisement. As species identification and delimitation by advertisement calls need total evidence, the smallest undividable unit of sound energy of calling frogs warrants assessment. Pulses are defined as the smallest undividable unit, a single burst of sound energy, not further subdivided into subunits and separated by strong amplitude modulation from other pulses. Pulse features include pulse duration, pulse interval, pulse rate and number of pulses per call. These are considered temporal variables of frog calls, together with the call parameters which are relevant in species recognition [5].

In the search of establishing the identity of *Ansonia* species complex in the Philippines, all possible evidences must be gathered. Thus, this study looked into the pulse properties of the advertisement calls of the *Ansonia* species in relation to their call properties, their body sizes, and the ecological features of the rivers. Pulse properties include pulse duration, pulse interval, pulse rate (number of pulses per second) and number of pulses per call. Recent phylogenetic-based classification Sanguila *et al.* [8] was used in this study. Results would possibly provide additional input for the reassessment of the identity of this species complex.
2. Method

2.1. Study sites

The recording of the advertisement calls of Ansonia species was done on the eight rivers of Mindanao Island, Philippines (Fig. 1), namely: Inobulan River near Mt. Balatukan Range in Gingoog City, Misamis Oriental; Clarin River at the eastern portion of Mt. Malindang Range in Ozamis City, Misamis Occidental; Kabangahan River nearby Mt. Gabunan Range in Iligan City, Lanao del Norte; Kirondong River at the foot of Mt. Apo Range, and Mt. Linanggasan in Kirondong, Magpet, North Cotabato; Maragang River at the lower elevation of Mt. Timolan Range in Tigbao, Zamboanga del Sur; Moradje River at the western portion of Mt. Malindang Range in Josefina, Zamboanga del Sur; Murias River at the foot of Mt. Murias in Lourdes, Pagadian City; and Kanloslo River at the vicinity of Mt. Busa Range in Kanloslo, Kiamba of Sarangani Province.

2.2. Data collection and recording

Each river was trekked at around 1600 to 2000 hours where Ansonia spp. were identified and observed until a call is made. Each call was recorded to a maximum of 15 minutes (where applicable) at distances of 0.5 to 2.0 meters. This recording was done using a Tascam DR-1 Portable Solid State Recorder with a WAV recording resolution of 44100Hz. A unidirectional external microphone was also used to minimize recording of the other ambient sounds. After or simultaneous to recording, the ambient and water temperatures, relative humidity, elevation, and coordinates were noted. The height from which the toad perched during the call was also measured using a ruler.

2.3 Pulse variables measurement

From the recorded call, a maximum of 5-minute call was selected per individual toad. This was then sliced and further dissected into a one-minute-call slice for bioacoustics analysis using Raven Pro 1.4 for Windows (2011) in wave sound file format with a sample rate of 44100Hz, encoding 24-bit. Five one-minute call slices served as replicates per individual toad where pulse duration, pulse interval, pulse...
rate, and the number of pulses per call were measured. Fig. 2 shows waveform or oscillogram of *Ansonia* spp. calls and pulses.

2.4 Statistical analysis
Univariate statistics was used in determining means, standard deviations, and ranges of the pulse characteristics. Cluster analysis of paired group using Euclidean for similarity of measure was also employed. One-way analysis of variance (ANOVA) was used to determine significant variations between and within group means of the pulse features with Tukey’s pairwise comparisons to identify where the significant variations lie. Significant differences on the body weight and SVL were ascertained using two-tailed tests, while relationships between ecological factors and the body measurements were determined using R-squared test. Statistical Package for the Social Sciences (SPSS) version 21 and PAleontological Statistics (PAST) version 2.17 programs were used in these statistical analyses.
3. Result and discussion

Table 1 shows the variations of the pulse features of the advertisement calls of *Ansonia* species as recorded from the eight rivers on Mindanao. Overall, the number of pulses per one-minute-call recorded a mean of approximately 40 pulses with mean pulse duration of around 0.026s. These pulses were generated at a mean rate of 13.48 pulses per second with a mean interval of about 0.150s.

3.1. Number of pulses per call

The pulses of the advertisement calls of *Ansonia* species ranged from 5.96 -191.78 pulses per call. The least extent was recorded from the calls of *A. mcgregori/A. sp. 1* in Murias River (24.14±14.09) and highest number was from *A. sp. 1* in Maragang River (105.80±55.13) which had a wide variance from the lowest and the rest of the rivers. The lowest mean was in close proximity with those from the other rivers like *A. muelleri* in Kanloslo (27.74±21.88), Kabangahan (28.42±15.91), Kirondong (30.94±19.34) and Inobulan (32.75±40.85). *A. mcgregori* in Moradje (33.10±28.44) and *A. muelleri* in Clarin (37.71±17.19).

Table 1. Pulse characteristics of the advertisement call of *Ansonia* species in the study

| SAMPLING SITES | N  | NUMBER OF PULSES | PULSE DURATION | PULSE INTERVAL | PULSE RATE |
|----------------|----|------------------|----------------|----------------|------------|
| Inobulan River | 6  | 32.75 ± 40.85    | 0.027 ± 0.0021 | 0.111 ± 0.009  | 7.30 ± 0.564 |
| A. muelleri    | 9.40 - 114.04 | 0.024 - 0.03    | 0.096 - 0.124  | 6.59 - 8.23   |
| Clarin River   | 10 | 37.71 ± 17.19    | 0.023 ± 0.003  | 0.057 ± 0.006  | 12.55 ± 0.784 |
| A. muelleri    | 16.50 - 64.90 | 0.016 - 0.026   | 0.045 - 0.067  | 11.34 - 14.13 |
| Kanloslo River | 3  | 27.74 ± 21.88    | 0.054 ± 0.024  | 0.071 ± 0.0058 | 10.436 ± 0.704 |
| A. muelleri    | 13.04 - 52.88 | 0.026 - 0.069   | 0.067 - 0.078  | 9.64 - 10.99  |
| Kirondong River | 7  | 30.94 ± 19.34    | 0.026 ± 0.0025 | 0.093 ± 0.010  | 8.545 ± 0.722 |
| A. muelleri    | 8.40 - 59.09  | 0.022 - 0.029   | 0.077 - 0.109  | 7.56 - 9.78   |
| Moradje River  | 8  | 33.10 ± 28.44    | 0.016 ± 0.0021 | 0.050 ± 0.006  | 15.204 ± 1.173 |
| A. mcgregori   | 10.92 - 89.00 | 0.013 - 0.021   | 0.043 - 0.062  | 13.18 - 16.99 |
| Murias River   | 6  | 24.14 ± 14.09    | 0.029 ± 0.0038 | 0.751 ± 1.683  | 10.99 ± 0.797 |
| A. mcgregori/A. sp. 1 | 5.96 - 42.20 | 0.025 - 0.034   | 0.052 - 0.188  | 9.93 - 11.67  |
| Kabangahan River | 5  | 28.42 ± 15.91    | 0.018 ± 0.0016 | 0.042 ± 0.0037 | 16.80 ± 1.19  |
| A. muelleri    | 17.40 - 55.80 | 0.016 - 0.020   | 0.036 - 0.046  | 15.58 - 18.69 |
| Maragang River | 9  | 105.80 ± 55.13   | 0.012 ± 0.0009 | 0.027 ± 0.002  | 26.01 ± 1.33  |
| A. sp. 1       | 44.04 - 191.78 | 0.010 - 0.014   | 0.024 - 0.031  | 24.21 - 27.34 |
| Mean           | 8  | 40.08±26.87      | 0.026±0.013    | 0.150±0.24     | 13.48±5.98   |

When these means were subjected to ANOVA (Table 2), the variations showed highly significant difference between and within group means (F (7, 46) = 5.982, p < 0.01).

Table 2. Analysis of variance for the mean number of pulses per call of *Ansonia* spp.

|                      | Sum of Squares | Df | Mean square | F      | p (same)   |
|----------------------|----------------|----|-------------|--------|------------|
| Between groups       | 42048.4        | 7  | 6006.92     | 5.982  | 5.519E-05  |
| Within groups        | 46191.6        | 46 | 1004.16     |        |            |
| Total                | 88240          | 53 |             |        |            |

Highly significant differences lie between those mean number of pulses from *A. sp. 1* in Maragang with *A. muelleri* in Inobulan (p=0.006), Clarin (p=0.012), Kabangahan (p=0.0028), Kanloslo (p=0.0025), and Kirondong (p=0.004). The same is true to *A. sp. 1* in Maragang with *A. mcgregori* in Moradje (p=0.006) and *A. mcgregori/A. sp. 1* in Murias (p=0.001). These relationships showed that *A.*
There existed no significant variation of these pulse durations (R-squared test) against all means of the call parameters and of peak call frequency. However, this was significantly correlated with elevation in Kanloslo ($R^2=0.998$, $p=0.028$), RH% in Kirondong ($R^2=0.722$, $p=0.016$), water temperature in
Inobulan (R^2=*, p=0.00), and perch height in Murias (R^2=0.991, p=0.00). Moreover, this was also correlated with SVL of the calling toads in Kabangahan (R^2=0.778, p=0.048).

Pulse duration was said to decrease with temperature [10] but positively correlated with water temperature in Inobulan as in the findings of similar studies [5, 6, and 12]. While duration was significantly associated with peak call (dominant) frequency [7], in this study it was not significantly associated which is parallel to that of previous study [3]. The correlation of pulse duration with SVL is in agreement with another research finding [6].

3.3 Pulse interval (s)
The pulse interval of the advertisement calls of *Ansonia* spp. recorded the shortest mean call interval from *A. sp. 1* in Maragang (0.027±0.002s) and the longest from *A. mcgregori/A. sp. 1* in Murias (0.751±1.683s). In close proximity to the shortest interval was noted from *A. muelleri* in Kabangahan (0.042±0.0037s), *A. mcgregori* in Moradje (0.050±0.006s), and *A. muelleri* in Clarin (0.057±0.006s). Of longer intervals were from *A. muelleri* in Kanloslo (0.071±0.0058s), Kirondong (0.093±0.010s), and Inobulan (0.111±0.009s).

With ANOVA (Table 4), these mean variations of pulse interval were found not significant (F (7, 46) =1.193, p=0.3256). Pulse interval was not influenced by all the call parameters, body sizes and ecological parameters, except temperature. Pulse interval decreases with temperature [10].

| Table 4. Analysis of variance for the mean pulse interval of *Ansonia* spp. |
|-----------------|----------------|-------------|-------|-------|
| Sum of Squares  | df  | Mean square | F     | p(same) |
| Between groups  | 2.57345 | 7 | 0.367636 | 1.193 | 0.3256 |
| Within groups   | 14.1735 | 46 | 0.30812 |       |      |
| Total           | 16.747 | 53 |          |       |      |

3.4 Pulse rate (pulses/s)
The total number of pulses per second (pulse rate) in the advertisement calls of *Ansonia* spp. ranged from 6.59 of *A. muelleri* in Inobulan River to 27.34 of *A. sp. 1* in Maragang River with the least (7.30±0.564) and the highest (26.01±1.33) means, respectively. The pulse rates from *A. muelleri* in Inobulan (7.3±0.564s) was similar to those from Kirondong (8.545±0.722s). This was also noted between *A. muelleri* in Kanloslo (10.436±0.704s) and *A. mcgregori/A. sp. 1* in Murias (10.99±0.797s), as well as from *A. muelleri* in Clarin (12.55±0.784s), *A. mcgregori* in Moradje (15.204±1.173s), and *A. muelleri* in Kabangahan (16.8±1.19).

There existed a highly significant difference (F (7, 46) =*, p<0.01) between and within the means of pulse rates (Table 5). The significant variations were noted between the pulse rates of the following:

a. *A. muelleri* from Inobulan and the rest of the rivers (p<0.01), except those *A. muelleri* from Kirondong (p=0.369);

b. *A. muelleri* of Clarin and the other rivers (p<0.05), except those *A. mcgregori/A. sp. 1* from Murias (p=0.1315);

c. *A. muelleri* of Kabangahan and the other rivers (p<0.01), except those *A. mcgregori* from Moradje (p=0.1149);

d. *A. muelleri* in Kanloslo and the other rivers (p<0.05), except those *A. mcgregori/A. sp. 1* in Murias (p=0.975);

e. *A. sp. 1* from Maragang, *A. mcgregori* of Moradje and *A. mcgregori/A. sp. 1* of Murias with *A. muelleri* of Kirondong (p<0.01);

f. *A. sp. 1* in Maragang with *A. mcgregori* of Moradje and *A. mcgregori/A. sp. 1* of Murias (p<0.01) and

g. *A. mcgregori* from Moradje and *A. mcgregori/A. sp. 1* of Murias (p<0.01).
Table 5. Analysis of variance for the mean pulse rate of Ansonia spp.

| Sum of Squares | Df  | Mean Square | F     | p(same) |
|----------------|-----|-------------|-------|---------|
| Between groups | 1938.78 | 7           | 276.969 | 290.7   | 7.266E-36 |
| Within groups  | 43.8222 | 46          | 0.952656 |         |          |
| Total          | 1982.6 | 53          |        |         |          |

The pulse rate of A. muelleri in Kabangahan was highly correlated ($R^2=0.908$, $p=0.012$) with call interval. Also, peak call frequency was found to be correlated with pulse rates of A. muelleri from Clarin ($R^2=0.466$, $p=0.030$) and Kabangahan ($R^2=0.780$, $p=0.047$) rivers. This correlation extent was also observed with RH% in A. muelleri from Kanloslo ($R^2=0.996$, $p=0.039$) and A. mcgregori/A. sp. 1 in Murias ($R^2=0.770$, $p=0.022$). Moreover, pulse rate was found highly correlated with water temperature in Inobulan ($R^2=*$, $p=0.00$), but not correlated with body size.

Pulse rate was found affected by temperature [5, 6, 10, and 12] and SVL [6]. A study [3] is in accordance with the findings of this study that pulse rate was not influenced by SVL in any of the rivers. It is also recognized that the correlation of pulse rate and temperature was only true in some cases [5]. Being a comparatively static variable made this feature an important trait for taxonomy and is considered to represent the most important call property in mate recognition in some species.

3.5 Relationship among pulse features

Considering all pulse features from all rivers, in totality (Fig. 3), A. sp. 1 from Maragang River was a cluster of its own in contrast with those toads from the rest of the rivers, as another big cluster. This big cluster was characterized by sub-clusters exhibiting similar pulse features such as those from A. muelleri of Kanloslo and A. mcgregori/A. sp. 1 of Murias; A. muelleri of Kirondong and Inobulan; and A. muelleri of Kabangahan - A. mcgregori of Moradje - A. muelleri of Clarin.
Figure 3. Dendrogram of the overall pulse characteristics of *Ansonia* spp.

There existed no significant difference (Table 6) between and within group means of the pulse features of the toads considering all the rivers (F (7, 24) =0.5091, p=0.8185).

**Table 6.** Analysis of variance for the mean pulse features of *Ansonia* spp. populations.

|                  | Sum of squares | Df | Mean square | F     | p(same) |
|------------------|----------------|----|-------------|-------|---------|
| Between groups   | 1788.35        | 7  | 255.479     | 0.5091| 0.8185  |
| Within groups    | 12043.4        | 24 | 501.807     |       |         |
| Total            | 13831.7        | 31 |             |       |         |

However, Table 7 showed highly significant variations (F (3, 28) =0.229, p=0.000005157) of the mean pulse characteristics (duration, interval, number of pulses per call and pulse rate).

**Table 7.** Analysis of variance for the overall mean pulse characteristics of *Ansonia* spp.

|                  | Sum of squares | df | Mean square | F     | p(same)   |
|------------------|----------------|----|-------------|-------|-----------|
| Between groups   | 8528.04        | 3  | 2842.68     | 15.01 | 5.157E-06 |
| Within groups    | 5303.68        | 28 | 189.417     |       |           |
| Total            | 13831.7        | 31 |             |       |           |

The highly significant variations were found between pulse duration and number of pulses (p=0.0001758), pulse interval and number of pulses (p=0.0001765), and pulse rate and number of pulses.
per call ($p=0.003299$). As observed in the study, particularly in the case of $A$. sp. 1 in Maragang River, the higher the number of pulses per call, the shorter the pulse duration and interval and the higher the pulse rate.

4. Conclusion

There existed variations between/among the pulse features of the advertisement calls of *Ansonia* species recorded from the eight rivers on Mindanao Island, Philippines. $A$. sp. 1 from Maragang River recorded the highest number of pulses per call with the shortest pulse duration and interval and the highest pulse rate. The pulse features, $A$. sp. 1 from Maragang greatly differed from the toads of the rest of the rivers. *A. muelleri* in Kanloslo and *A. mcgregori/A*. sp. 1 in Murias, *A. muelleri* of Kirondong and Inobulan had similar pulse features. Also *A. muelleri* in Kabangahan and Clarin were comparable with *A. mcgregori* in Moradje. The number of pulses was associated with the pulse duration, interval, and rate.

The number of pulses per call was found associated with SVL, weight, water and ambient temperatures, peak call frequency, call duration and interval, and calling rate. Pulse duration was affected by SVL, elevation, RH%, water temperature and perch height. Pulse interval was influenced by water temperature alone. The pulse rate (number of pulses per second) was correlated with RH%, water temperature, peak call frequency, and call interval.

These findings need to be carefully examined, especially with variations in species similarly grouped, and in relation to other bioacoustics properties, and to be verified with more studies to serve as basis for the establishment of the real identity of the *Ansonia* species in the Philippines.

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