

Acoustics of parental and hybridogenetic water frogs (*Pelophylax*:
Amphibia: Ranidae): a tool for monitoring an invasive species

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Supplementary material

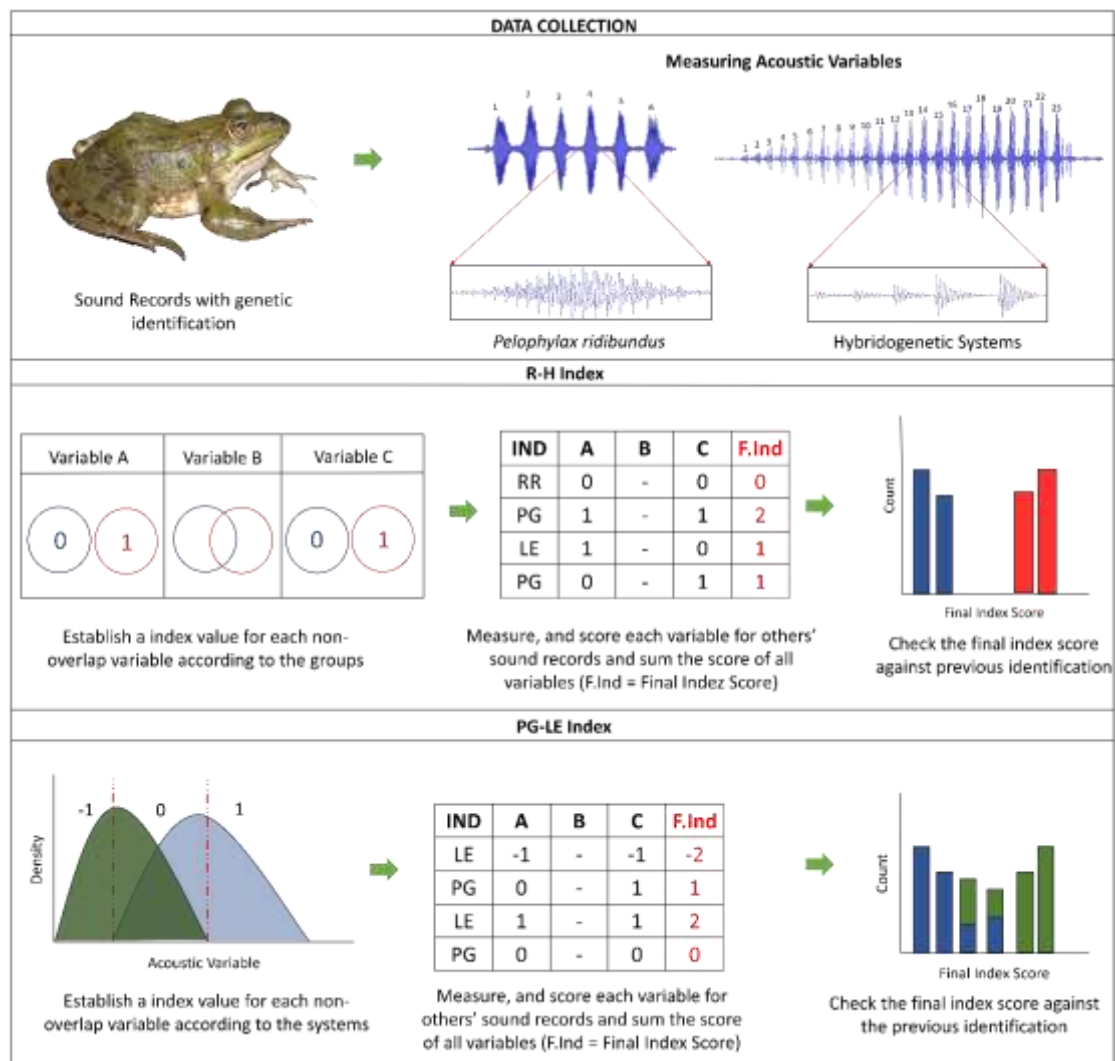


Figure S1. Workflow of the identification methods designed to identify *Pelophylax ridibundus* against LE and PG system, and PG against LE. PG = *Pelophylax perezi-grafi* system ; LE = *Pelophylax lessonae-esculentus* system.

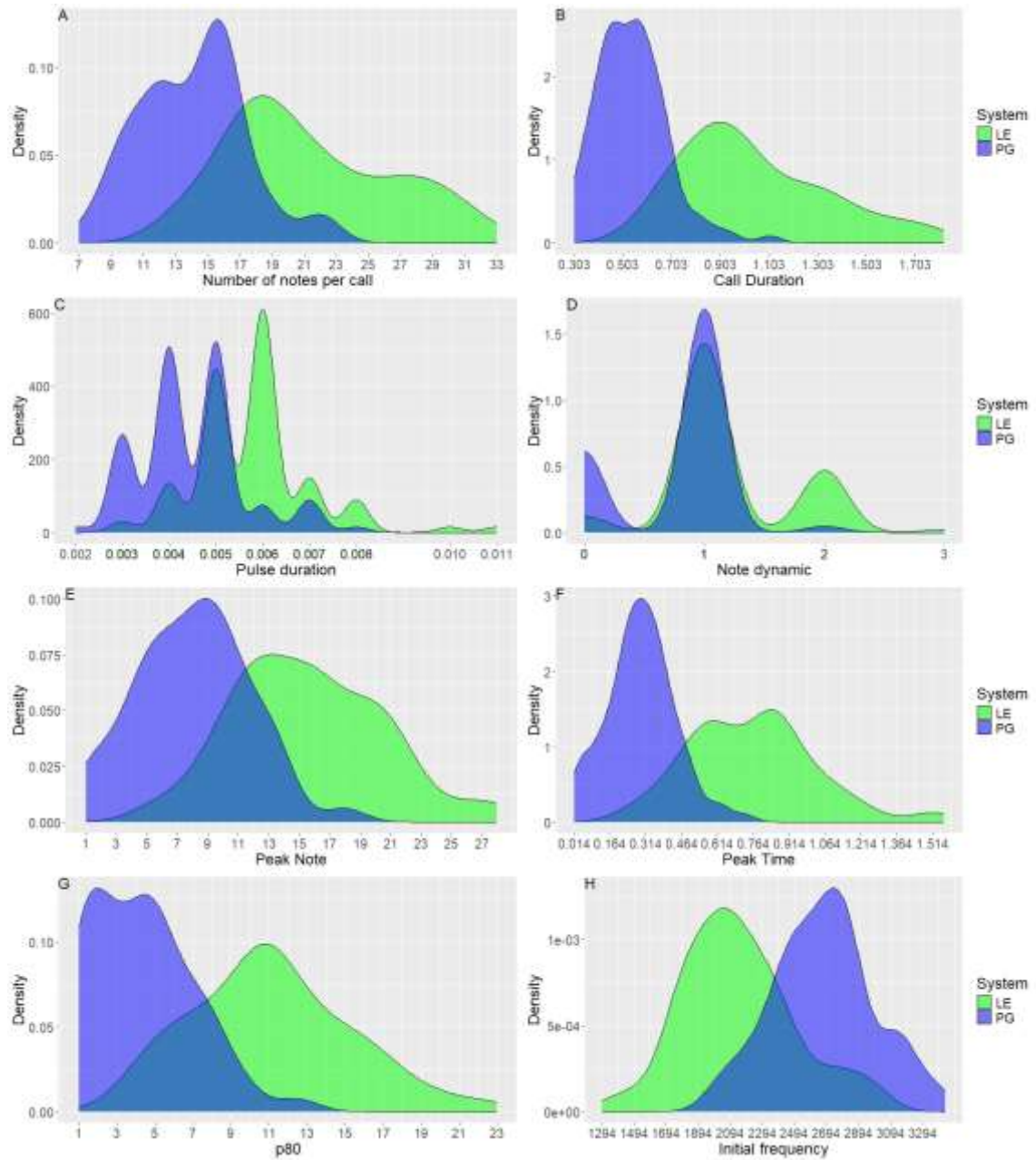


Figure S2. Density plot of acoustics variables used in PG-LE index. (A) number of notes per call. (B) Call duration (in seconds). (C) Pulse duration (in seconds). (D) Note dynamic (E) Number of notes to reach the energy peak (Peak Note). (F) Time to reach the energy peak (in seconds). (G) Number of notes to reach 80% of the highest peak note. H) Initial frequency.

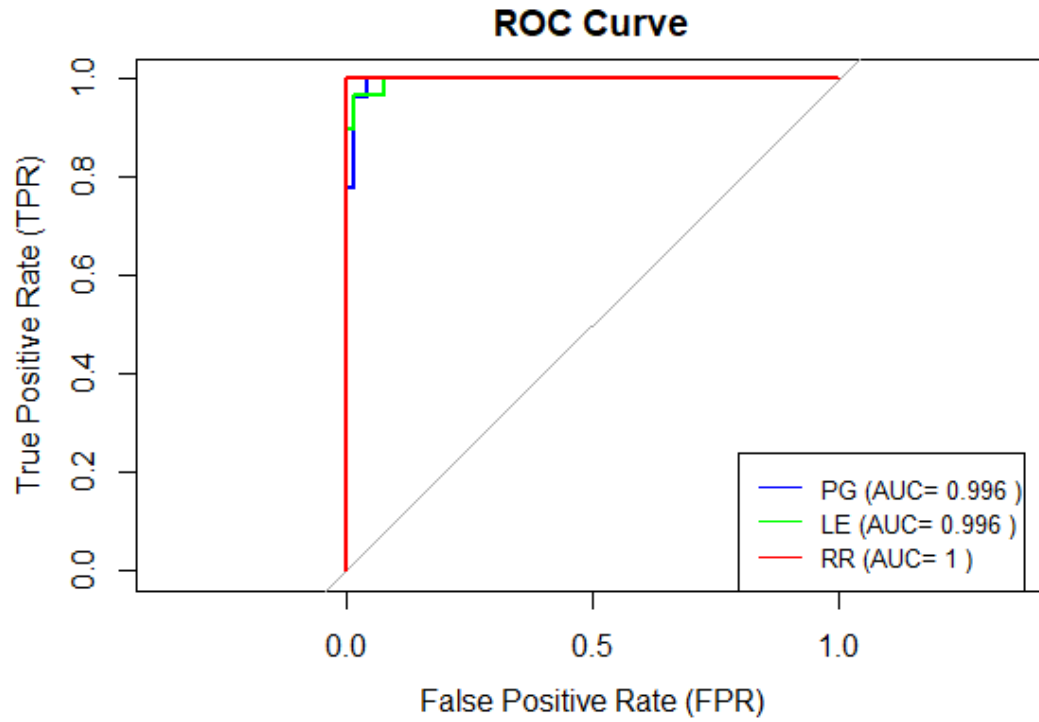


Figure S3. Density plot of the Principal Component 1 (PC1) of the Principal Component Analysis (PCA) between *Pelophylax ridibundus*, *P. perezii*, and *P. grafi*.

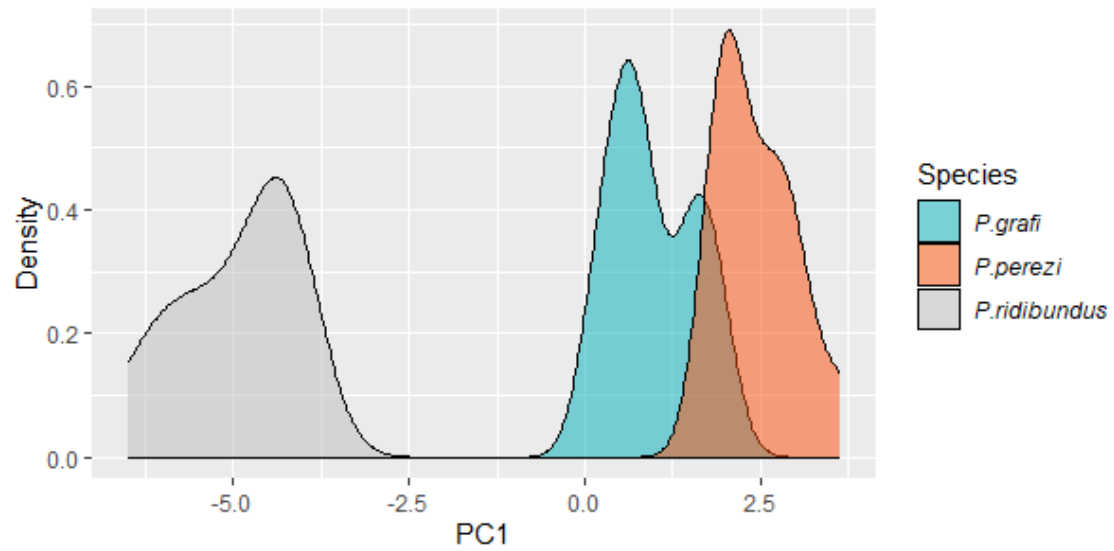


Figure S4. Receiver operating characteristic (ROC) curve and area under the curve (AUC) values for the random forest classification model. PG = *Pelophylax perez-grafi*; LE = *Pelophylax lessonae-esculentus* ; RR = *Pelophylax ridibundus*.

Text S1. Acoustic Identification Methods

Cleaning the data

Before starting your analysis of the call, it is advisable to enhance the audio quality by employing filters to improve clarity and ease of measurement. In the Audacity software, two key filters, the "high-pass filter" and "low-pass filter," can be used in this process. To access these filters, navigate to the "Effect" tab and select "EQ and Filters." The high-pass filter allows higher frequencies to pass through while attenuating lower frequencies, making it effective for eliminating low-frequency noise or rumble. The low-pass filter permits lower frequencies to pass through while attenuating higher frequencies, useful for reducing high-frequency noise or unwanted treble elements. Utilizing the spectrogram feature in Audacity, visually analyze the frequency content over time to determine appropriate cutoff frequencies for these filters. Identify areas in the spectrogram with unwanted noise or elements, and adjust the filter settings, accordingly, taking into consideration both cutoff frequencies for optimal results.

R-H index

Approach 1:

1° Step: Measure 8 acoustic variables as described below. For each variable measure at least 2 calls of the same individual and use the average between them.

Number of notes (nbn): count the number of notes in a call.

Number of pulses per note (nbp): number of pulses per note (at least 2 notes per call) measured from notes in the middle of the calls.

Note duration (nd): duration (in seconds) of each note for which the number of pulses has been measured. It is measured from the start of the first pulse to the start of the last pulse of the note.

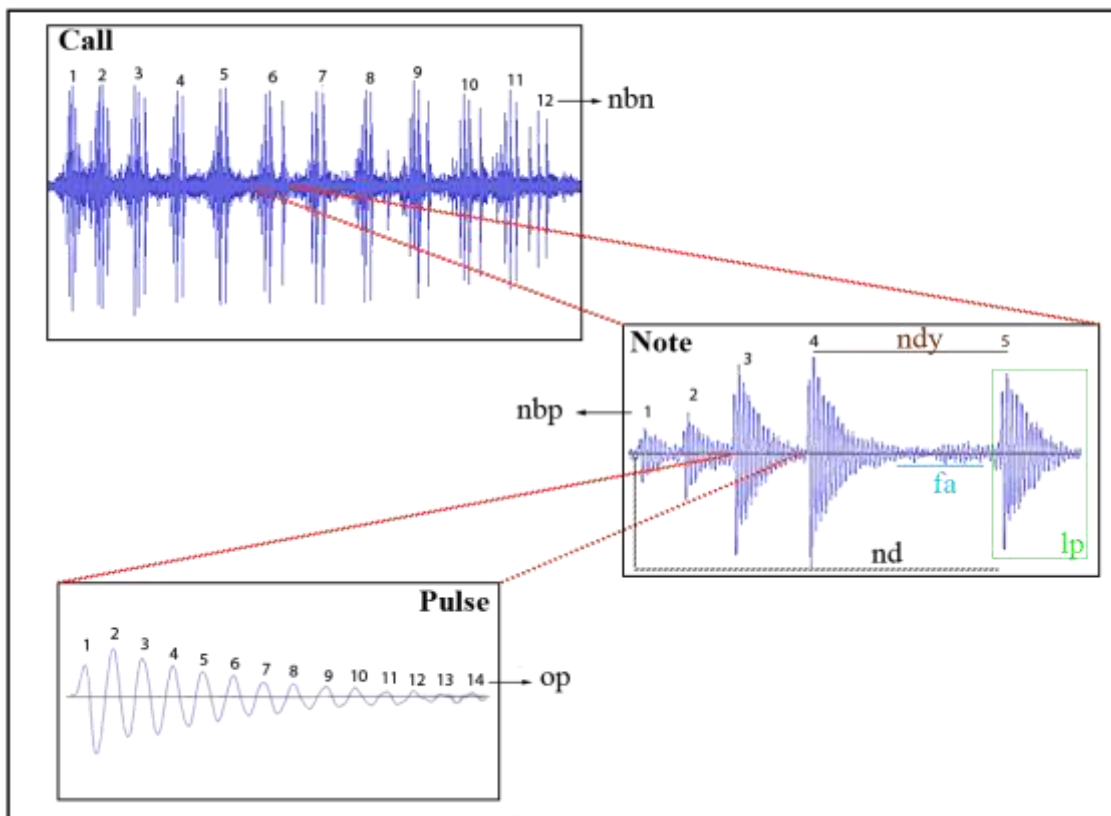
Pulse repetition rate (prp): It is calculated by $(\text{number of pulses per note} - 1) / (\text{note duration})$

Number of oscillations per pulse (op): The average of the number of oscillations per pulse by selecting 1 pulse per note, in 2 different notes, for each phrase. Exclude the first and last pulses and count any peak crossing above the line between 2 maximum oscillations.

Note dynamics (ndy): The average of the number of pulses after the most powerful within a note, taking 2 notes, on each of the selected phrases.

Last pulse (lp): Check if the last pulse in a note is separated from the rest of the note. This variable should be categorized as yes or no. In cases where it is not clear (varies according to the calls) choose "yes" if at least part of the recording presents this characteristic.

Full amortization (fa): Check if, in some part of the note, there are no oscillations, being flat the oscillogram. This variable should be categorized as yes or no. Choose "yes" if at least part of the note presents this characteristic, repeatedly in the recording.



2° Step: Score each of the acoustic variables as 0 or 1 according to the following index.

Acoustic Variables	Score as 0 if	Score as 1 if
Number of notes	Less than or equal to 9	More or equal to 10
Number of pulses per note	More or equal to 11	Less than or equal to 10
Pulse repetition rate	More or equal to 300	Less than 300
Number of oscillations per pulse	Less than or equal to 7	More or equal to 8
Note dynamics	More or equal to 3	Less than or equal to 2
Last pulse	No	Yes
Full amortization	No	Yes

3° Step: Sum the score of each variable to have a final index score.

4° Step: Find the identification according to the following instructions.

Final Index Score	Identification
0 to 2	<i>P. ridibundus</i>
5 to 7	LE or PG

PG = *Pelophylax perezi* – *grafi* system ; LE = *Pelophylax lessonae* – *esculentus* system

OBS: Final scores of 3 and 4 should be taken with attention and it would be safer to leave them unidentified.

Approach 2:

Step 1: Measure the same variables described previously and save them in an Excel file like in the next figure.

	A	B	E	F	G	H	J	K	L
1	Identification	nbn	nbp	nd	frp	op	ndy	lp	fa
2	A	8	11	0.029	344.8276	8	2	no	no
3	C	11	7	0.029	206.8966	13	1	yes	yes
4	D	13	7	0.022	272.7273	12	1	yes	yes
5	E	12	9	0.027	296.2963	11	1	yes	yes
6	F	11	6	0.021	238.0952	10	1	yes	yes
7	G	12	9	0.022	363.6364	8	0	yes	yes

Step 2: Open the acoustic identification app.

Link: https://filipegmsalmeida.shinyapps.io/PeloCall_ID/

Step 3: Input your Excel file and click on “Classify”.

Step 4: Download the table “R-H index” with the index score and identification of each individual.

OBS: If you also want an additional method of identification, you can check the “Random Forest Classification”.

PG-LE index

Approach 1

1° Step: Measure 8 acoustic variables as described below. For each variable measure at least 2 calls of the same individual and use the average between them.

Number of notes (nbn): count the number of notes in a call.

Call duration (cd): duration of call (from the middle of the first note to the middle of the last note; in seconds).

Peak note (pn): number of notes within a call to reach the highest energy peak.

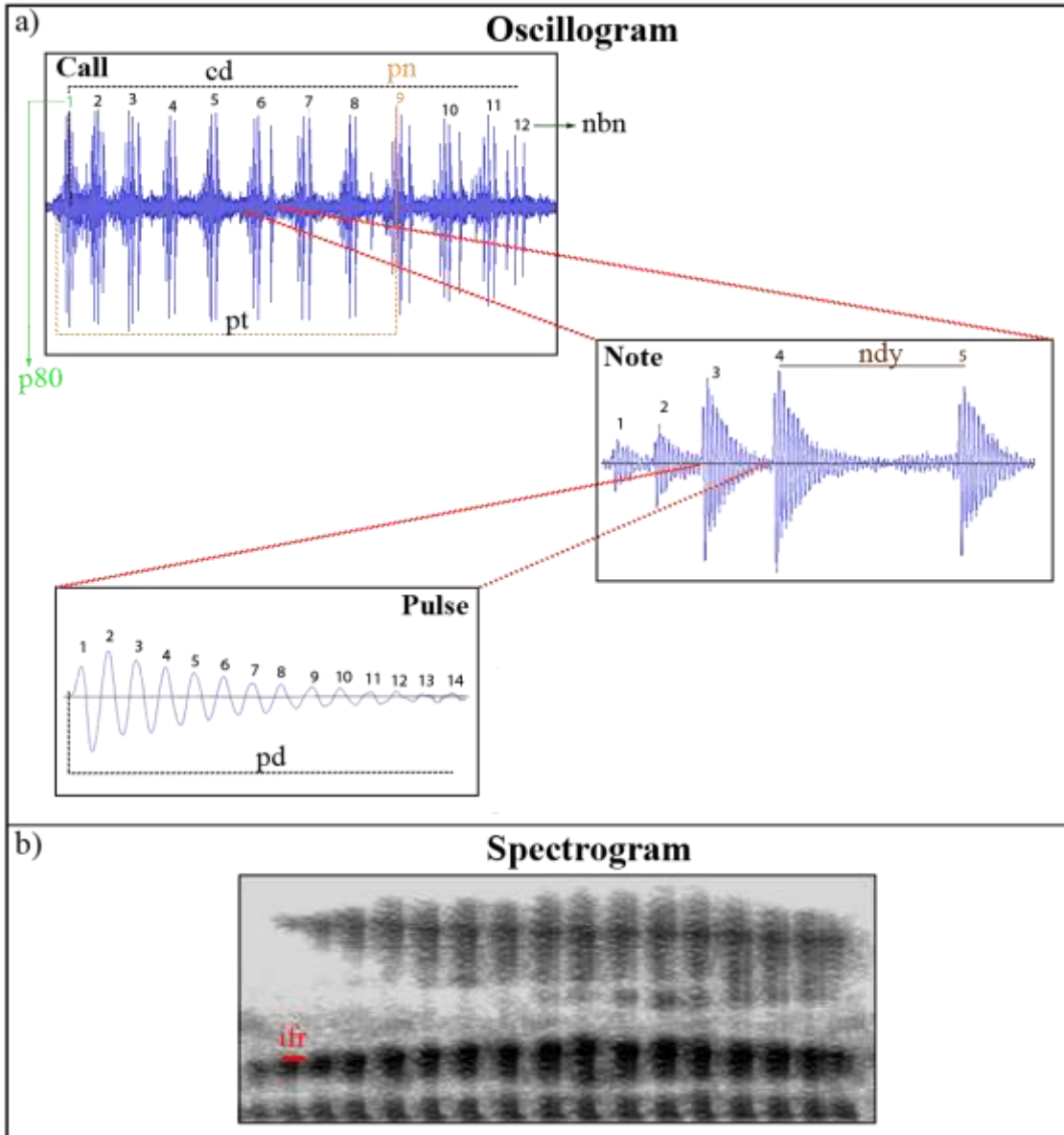
Peak time (pt): time (in seconds) from the beginning of the first note of the call to the maximum energy peak in the call.

Pulse duration (pd): average duration of one pulse (in seconds)

Note dynamics (ndy): The average of the number of pulses after the most powerful within a note, taking 2 notes, on each of the selected phrases.

Peak 80% (p80): Count how many notes are necessary to reach 80% of the high of the highest peak note.

Initial frequency (ifr): Measure the initial frequency with maximum energy of a call, looking at the spectrogram.



2° Step: Score each of the acoustic variables as 0 or 1 according to the following index.

Acoustic Variables	Score as -1 if	Score as 0 if	Score as 1 if
Number of notes	Less than or equal to 9	Between 9 and 25	More than or equal to 25
Call duration	Less than or equal to 0.4	Between 0.4 and 1	More than or equal to 1
Pulse duration	Less than or equal to 0.004	Between 0.004 and 0.0055	More or equal to 0.0055
Peak note	Less than or equal to 8	Between 8 and 16	More than or equal to 16
Peak time	Less than or equal to 0.3	Between 0.3 and 0.8	More than or equal to 0.8
Note dynamics	Equal to 0	Equal to 1	More than or equal to 2
Peak 80%	Less than or equal to 3	Between 4 and 10	More than or equal to 11
Initial Frequency	More tha or equal to 2500	Between 2001 and 2500	Less than or equal to 2000

3° Step: Sum the score of each variable to have a final index score.

4° Step: Find the identification according to the following instructions.

Final Index Score	Identification
- 6 to -3	PG
-2 to 1	LE or PG
2 to 8	LE

PG = *Pelophylax perezi* – *grafi* system ; LE = *Pelophylax lessonae* – *esculentus* system

Approach 2:

Step 1: Measure the same variables described previously and save them in an Excel file like in the next figure.

	A	B	C	I	J	M	N	O	P
1	Identification	nbn	cd	pd	ndy	pn	pt	p80	ifr
2	A	8	0.553	0.003	2	5	0.313	2	2805
3	C	11	0.558	0.005	1	6	0.278	4	3048
4	D	13	0.593	0.003	1	7	0.275	3	2414
5	E	12	0.517	0.002	1	12	0.518	10	2096
6	F	11	0.435	0.003	1	2	0.062	1	2479
7	G	12	0.461	0.003	0	9	0.437	2	2444

Step 2: Open the acoustic identification app.

Link: https://filipegmsalmeida.shinyapps.io/PeloCall_ID/

Step 3: Input your Excel file and click on “Classify”.

Step 4: Download the table “PG-LE index” with the index score and identification of each individual.

OBS: If you also want an additional method of identification, you can check the “Random Forest Identification”. However, for this, you will need to measure all 17 variables used in the paper.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R
1	Identification	nbn	cd	frn	ntbp	cd	frp	op	pd	ndy	lp	le	pn	pt	p80	ifr	mfr	ufr
2	A	8	0.553	12.65823	11	0.029	344.8276	8	0.003	2	no	no	5	0.313	2	2805	2855	2257
3	C	11	0.558	17.92115	7	0.029	206.8966	13	0.005	1	yes	yes	6	0.278	4	3048	3083	3083
4	D	13	0.593	20.23609	7	0.022	272.7273	12	0.003	1	yes	yes	7	0.275	3	2414	2934	2172
5	E	12	0.517	21.2706	9	0.027	296.2961	11	0.002	1	yes	yes	12	0.518	10	2096	2348	2213