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# Near infrared spectroscopy for the identification of live anurans: Towards rapid and automated identification of species in the field

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**Abstract**

In megadiverse regions, such as the Amazon, the identification of species generally requires specialists that are often not available. Therefore, the use of new species-recognition tools is necessary to streamline surveys and avoid errors in species identification that lead to ineffective decision-making. Near infrared spectroscopy is a quick and non-destructive tool that has been widely used in the recognition of biodiversity. In addition to being used as an indicator group, anurans have species with high morphological diversity, which make them the focus of studies and application of new tools that help in the identification and recognition at the species level. In this study, the viability of recognition of species of live Amazonian frogs under field conditions using the near infrared technique and portable equipment was examined. The performance of classification models based on a linear discriminant analysis, built using spectra obtained from the dorsal and ventral surfaces of four pairs of phylogenetically-close and morphologically-similar species was evaluated. It was possible to distinguish the species of live anurans in five of the eight species studied with hit rates above 80% when using only one spectral reading per individual. The overall mean of correct prediction of the models was below that of previous studies that tested the method with anurans, which are likely to be due to particularities in the acquisition of spectra under field conditions and live species. Therefore, suggestions are made to improve the predictive capacity of the techniques.

**Keywords**

Amazon, diversity, frogs, species recognition, anuran

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## Introduction

"Know to conserve" has been the most used justification for studies dealing with biodiversity.[1] Classifying and naming an organism are the most efficient ways to communicate between different study areas and plan conservation actions.[2] Mistakes in species identification can result in sampling errors that can cause inefficient, and even harmful, management and decision-making.[3] However, in megadiverse regions, such as the Amazon,[4,5] the identification and recognition of species are challenges that generally require the action of specialists.

Amphibians, especially anurans, are organisms commonly used as environmental indicators, due to their sensitivity to the different environments they occupy during their life stages (egg stage, larval stage and post-metamorphosis).[6–10] However, because Anura is a diverse group (estimated at 7354 species worldwide,[11] 329 in the Amazon[12]), it has species with high morphological diversity, including similarities attributed to complexes of cryptic species,[13] making the group a challenge in fauna studies. This morphological diversity includes different patterns of colors and spots for camouflage that can be found in individuals of the same species (e.g. genus *Adenomera*[14]). This makes it difficult to identify individuals only through visible patterns, especially in the field and by non-specialists.

The number of specialists in taxonomic identification has been steadily decreasing over the past 30 years due to a dwindling number of career opportunities in this field. Furthermore, most specialists tend to work in regions outside the Amazon biome.[15] Added to the already known

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megadiversity of the biome, a large number of new species are being discovered every year.[5] Therefore, the use of new techniques and tools is necessary to carry out surveys and recognize species[16] by non-specialists in the Amazon.

Near infrared spectroscopy is a fast and non-destructive tool, which allows accessing the composition of a sample based on the interaction of the vibrations of the bonds of atoms or groups of atoms, which constitute the chemical compounds present in a sample, with electromagnetic radiation.[17,18] When combined with multivariate data analysis, the technique allows the characterization of organic samples, which can be from whole organisms[19] or from parts of organisms.[20] The technique produces spectra with reflectance or absorbance values according to the type of sample and equipment used,[21] giving a molecular signature.[22]

NIR spectroscopy has been relatively efficient in species identification and recognition tests for plants[23–27] and animals,[19,20,28] including frogs.[29,30] These results are promising, but the technique is not yet as consolidated as others already used in the identification of biodiversity, such as the use of genetic data. Therefore, there is a need for future spectral and taxonomic databases, standardized and available in open-access.[31]

Anurans have been the focus for proposals for new tools that help in the identification and recognition at the species level. In a previous study using a bench-top Fourier-transform near infrared spectrometer (FT-NIR), a high classification efficiency was found for anurans fixed in formalin and kept in ethyl alcohol from a zoological collection.[30] These results indicated a new field of study that has a high potential to resolve numerous taxonomic problems found in collections and which are sources for reviewing species around the world.[32]

The use of spectroscopy in studies and conservation of anurans, especially in situ, would be useful for several reasons. Environmental surveys and licensing require data from organisms sampled and identified in the field (e.g environmental-impact studies), often resulting in inaccurate and/or contradictory information.[33,34] Difficulties of identifying young specimens or specimens with similar characteristics to another species (cryptic species), can be solved through genetic or molecular tests, which are expensive. NIR spectroscopic techniques can potentially complement[30] or replace[28] the usual methods providing more objective results which can be attained faster and at lower cost. For example, for individuals with similarities in color and spots, the biochemical signature of species obtained in NIR spectra could complement the morphological analysis at the time of identification.

However, knowledge about the use of spectroscopy in the recognition of biodiversity is limited[19] and different applications need to be tested. The present study evaluated the performance of spectroscopy using a portable NIR spectrophotometer in the identification of live anurans collected in situ in the Amazon. To meet this objective, discriminant analyses were carried out to better understand the spectral responses of live anurans from four pairs of morphologically similar species, which are often difficult to distinguish.

## Methods

In this study, spectra of live whole anuran specimens collected using a Analytical Spectral Devices Field Spec 3 portable spectrophotometer (Analytical Spectral Devices - now Panalytical, Cambridge, UK) with contact probe were evaluated. The equipment is hyperspectral, capable of monitoring from the visible region to the near infrared (VIS-NIR). Each spectrum obtained consists of 2151 reflectance values measured in the wavelength region of 350–2500 nm. The nominal spectral resolution is 3 nm for the visible region (350–700 nm) and 10 nm for the NIR and Short-wave infrared regions (700–2100 nm).[35]

Only adult individuals with different sizes, different reproductive modes, and habitat use were studied (Figure 1). It was not possible to determine the sex of the individuals collected in the field, even when after locating the collection site by male calls. This is because no individuals were collected during calling and presence/absence of vocal sacs was not recorded.

Four pairs of morphologically-similar and phylogenetically-close anuran species, that could be found in the same site, were chosen to test the spectral models constructed using NIR data, simulating the encounter of cryptic species that can be confused at the time of identification in the field (Figure 1). This allowed classification models based on spectral data to be evaluated under a realistic scenario, considering the similarities between the studied species. Common species with well-defined taxonomic identification were used to test the method. Classification models based on spectral data for each species were constructed with a minimum of nine specimens (Table 1). With the exception of *Phyllomedusa tarsius* and *Callimedusa tomopterna*, the other pairs of species used were morphologically similar, which make them difficult to differentiate by non-experts. All individuals were collected and their identification confirmed using local photographic guides[36] and consultations with at least two specialists.

### *Obtaining spectra of live anurans*

Live anurans were captured manually between January and May 2020 in four tropical forest areas in the state of Amazonas, Brazil (Table S1 - Supplementary Material). Of these areas, three correspond to fragments of native forest in the urban center of the municipality of Manaus, mainly composed of small areas of disturbed and secondary forest, with a few open areas on the Campus of the University of Amazonas (Campus UFAM),[37,38] the Adolpho Ducke Reserve (Ducke) and forest in the Amazon Museum (MUSA).[36] Collection was also undertaken at the UFAM Experimental Farm (FEX-UFAM) which corresponds to an area of continuous dryland forest, with a mostly closed canopy and a low understory[39,40] (Figure S1 – Supplementary Material).

A total of 85 adult anuran specimens belonging to 8 species were captured (Table 1). The frogs were cleaned with water and an absorbent cloth (reusable cloth - Scott Duramax®) to remove soil, excreta and urine that could be present after capture. Then, one spectral reading was taken on the dorsal surface and one on the ventral surface of each specimen[30] with the portable equipment in the field. To


**Figure 1.** Ecological and morphological information for the four pairs of phylogenetically-close and morphologically-similar anuran species used for NIR spectral collection. *General information on the species taken from Lima* et al. *(2006); Photographs provided by Dr. Albertina Lima.*

protect the equipment and avoid possible contamination between the frog readings, the spectral window of the equipment was wrapped with insulfilm® plastic made of PVC polymer.[29] For each specimen read (Figure 2(a) and (b)), the plastic was replaced and the reference reading, which is equivalent to 100% reflectance in the entire spectral region monitored by the equipment, was taken. For species smaller than the equipment's circular window, the reading area was

restricted with a black rubber disk (ethyl-vinyl acetate - EVA) containing a small central hole (Figure 2(c)), simulating an accessory known as an iris or fish eye.

Approximately three specimens of each species were sacrificed after the reading with the use of topical lidocaine, fixed by injection of 10% formalin and preserved in 70% ethanol. The remainder of the specimens were released at their capture site. All procedures followed the capture, collection and release protocols approved under license SISBIO 70834-3 and ethics committee - n° 004/2020, SEI 01280.000146/2020-71.

## Data analysis

The collected spectra were initially inspected visually to detect anomalies. The spectra collected in reflectance mode were transformed into absorbance by the formula - $\log_{10}(1/R)$, where R is the reflectance. Two transients (or breaks) in the spectral range resulting from the technology employed by the equipment were detected. In addition, noisier spectral regions were found at the ends of the monitored spectral range (Figure 3(a)). Therefore, only the central part of the spectrum between 1002 and 1825 $cm^{-1}$ (Figure 3(b)) was employed. Additionally, the Savitzky-Golay filter algorithm was used to pre-process the spectra with the second derivative, $2^{nd}$ degree polynomial and 31-point window (15 points for each side), in order to enhance the chemical information present in the spectra and simultaneously reduce non-informative sources of variability, such as radiation scattering and instrumental noise. These procedures were undertaken with The Unscrambler® program, version 11.0 (Camo Software, Oslo, Norway. Now Aspen Technolgy, Inc., Bedford, MA, USA). The spectra set employed in this study is available as an additional file of Research Data.

The performance of two models for species recognition of live anurans was evaluated: a model built with spectra obtained from the dorsal surface and a model with spectra from the ventral surface. Principal component analysis (PCA) was used to reduce the dimensionality of the spectral data and to verify the occurrence of groups of samples according to species. The analysis used the scores referring to the first 6 PCs that captured 92% and 89% of the total variance of the spectra taken on the ventral and dorsal surfaces, respectively. Thus, the correlations among the spectral variables were eliminated.

The PC scores were used to construct classification models based on linear discriminant analysis (LDA) using holdout validation, which returns prediction values in percentage. The models were built using 70% of the data of each species for calibration and 30% for validation

**Table 1.** Number of live specimens of each species collected in the field for spectra acquisition.

| | Samples | | |
|---|---|---|---|
| Species | Total | Calibration (70%) | Validation (30%) |
| *Adenomera andreae* (Müller, 1923) | 11 | 8 | 3 |
| *Adenomera hylaedactyla* (Cope, 1868) | 11 | 8 | 3 |
| *Callimedusa tomopterna* (Cope, 1868) | 11 | 8 | 3 |
| *Leptodactylus knudseni* Heyer, 1972 | 9 | 6 | 3 |
| *Leptodactylus pentadactylus* (Laurenti, 1768) | 13 | 9 | 4 |
| *Phyllomedusa tarsius* (Cope, 1868) | 9 | 6 | 3 |
| *Pristimantis fenestratus* (Steindachner, 1864) | 10 | 7 | 3 |
| *Pristimantis zimmermanae* (Heyer and Hardy, 1991) | 11 | 8 | 3 |
| **Total samples** | **85** | **60** | **25** |

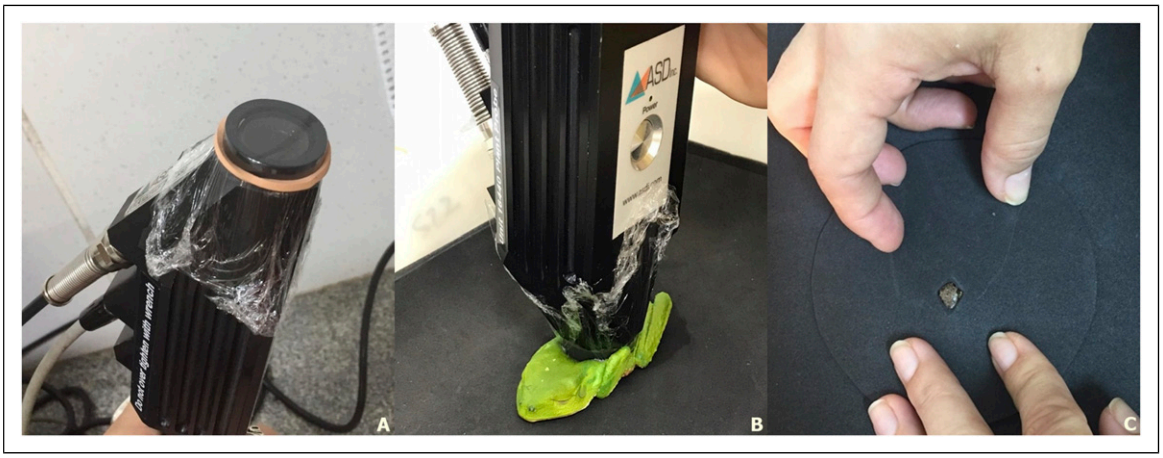


**Figure 2.** Detailed images of the method used for spectral readings with the VIS-NIR equipment. (A) Reading window wrapped in PVC film; (B) Spectral acquisition of a live anuran specimen; (C) Spectral acquisition of live small anuran specimen with the help of an accessory to restrict the reading area.

(Table 1). Selections of the calibration and validation sets were used individually on each species subset. Classification probabilities were based on 100 randomized selections, and results were expressed as the mean.

Cross-validation was performed with the leave-one-out (LOO) method, which tests the model's performance sample-by-sample, removing one at a time and testing its classification with the model generated with *n*-1 samples, where *n* is the total number samples used in the study. The predictions resulting from the leave-one-out cross-validation were shown in a confusion matrix (NIRtools R-package).[41] This validation procedure was preferred over the split subsets because the total number of individuals of each species is small, and the removal of a significant number to build an external test set can compromise the representativeness of the remaining samples.

Statistical analyses were undertaken using the R statistical program[42] with the support of the packages MASS,[43] klaR,[44] caret,[45] dplyr,[46] lattice[47] e ggpubr,[48] with adaptations of command scripts already used in data processing and spectral analysis of botanical samples (available at: https://www.botanicaamazonica.wiki.br/labotam/doku.php?id=analises:nir:inicio).

## Results

The graphs of PCA scores for the first two PCs permit the identification of groups corresponding to some species (e.g *C. tomopterna*, *L. knudseni* and *P. fenestratus*), with these components capturing 70 and 76% of the spectral variance in the sets of pretreated spectra obtained from the dorsal and ventral surfaces of the specimens, respectively (Figure 4). Grouping associated with other species (e.g *L. pentadactylus* e *P. zimmermanae*) is more evident in the higher-order components (Figure S2 - Supplementary Material). Six principal components, resulting from the analysis of the entire spectral dataset, captured 89% of the original variance for the data collected on the dorsal surface and 92% on the ventral surface.

With linear discriminant analysis using validation holdout, average relative correctness values of 67.5% (C.I (0.96%) = 65.7%–69.2%) and 68.3% (C.I (0.96%) = 50.0%–90.9%) were obtained for readings on the dorsal and ventral surfaces, respectively. Leave-one-out cross-validation gave correct-classification hits of 47% for the dorsal readings and 60% for the ventral readings. Both preliminary validation procedures indicate a better performance of the technique with spectra collected from the ventral surface of the specimens.

The probability of correct species identification in the confusion matrices generated from the leave-one-out cross-validation obtained higher rates of correct hits in both dorsal and ventral positions (Figure 5). *Callimedusa tomopterna*, *L. knudseni* and *L. pentadactylus* with ventral reading obtained correct predictions in 82, 89 and 92% of cases, respectively, while *P. tarsius* had 89% of correct identifications in both dorsal and ventral reading positions, and *P. zimmermanae* had 82% success for dorsal readings. *Adenomera andreae* and *A. hylaedactyla* also showed the highest correct predictions with the dorsal readings; between 64 and 73%. *Pristimantis fenestratus* had intermediate success in the two spectral reading points (60% on the dorsal surface and 40% on the ventral surface). The individual errors of each sample (off-diagonal values) did not show a specific pattern of errors among most of the phylogenetically close pairs.

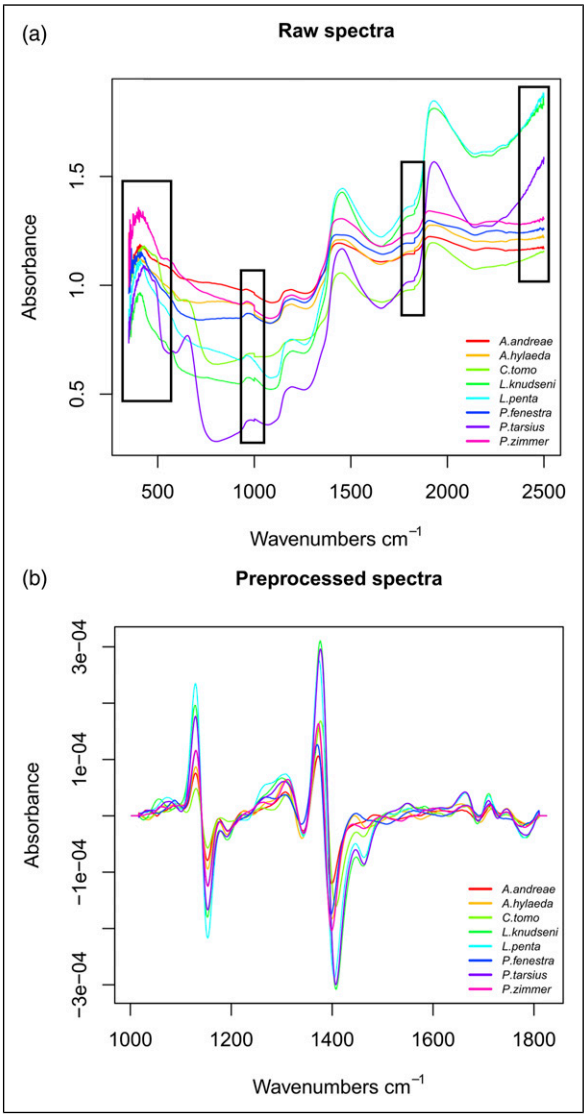


**Figure 3.** Species spectra collected from the dorsal and ventral surfaces of live anurans: (a) original VIS-NIR spectra with emphasis (rectangles) indicating the noisier regions and transients; (b) spectra pre-processed by the 2$^{nd}$ derivative, using the region without noise or transients (between 1002 and 1825 cm$^{-1}$) and with Savitzky-Golay filter. In the caption: **A.andreae =** *Adenomera andreae*, **A.hylaeda =** *Adenomera hylaedactyla*, **C.tomo =** *Callimedusa tomopterna,* **L.knudseni =** *Leptodactylus knudseni*, **L.penta =** *Leptodactylus pentadactylus,* **P.fenestra =** *Pristimantis fenestratus*, **P.tarsius =** *Phyllomedusa tarsius,* **P.zimmer =** *Pristimantis zimmermanae*.

## Discussion

It was possible to recognize the species of live anurans in five of the eight species tested with hit rates above 80% when using only one spectral reading per individual. The overall mean of correct prediction of the models was below that of other studies that tested the method with anurans.[29,30] However, it is proposed that

these data do not necessarily reflect the true potential of the approach. The grouping of species by means of the scores of the first principal components (PCA – Figure 4) is a strong indication that the NIR technique with portable equipment is promising for the recognition of live anuran species.

The phylogenetically closely-related species in the genus *Leptodactylus* could be distinguished using the models. The results for the pair of species *L. knudseni* and *L. pentadactylus* indicate that, for this genus, with ventral-reading spectra, it is possible to separate species using spectra of living organisms (only one individual incorrectly predicted – Figure 5). These species have large body size, reaching up to 155 mm in length, live on the forest floor where they breed in foam nests (Figure 1) and have brown spot patterns used for camouflage in the environment that can be different between individuals. These species have frequently been confounded in past field studies.[49]

For *Adenomera andreae* and *A. hylaedactyla* species, in which intermediate hit rates were obtained in the two sampling positions, eight of the ten individuals were assigned wrongly in the ventral model and three of the seven individuals assigned wrongly in the dorsal model (Figure 5). This pair of species belongs to the same family as the genus *Leptodactylus*, presenting the two species difficult to distinguish by morphological, behavioral and spatial distributional characteristics, except when calling. Other prediction errors were between non-congener species, indicating that the prediction errors were not determined only by the phylogenetic closeness of the pairs. Also, unlike a previous study with preserved anuran specimens,[30] models constructed using the dorsal or ventral spectra (separately) did

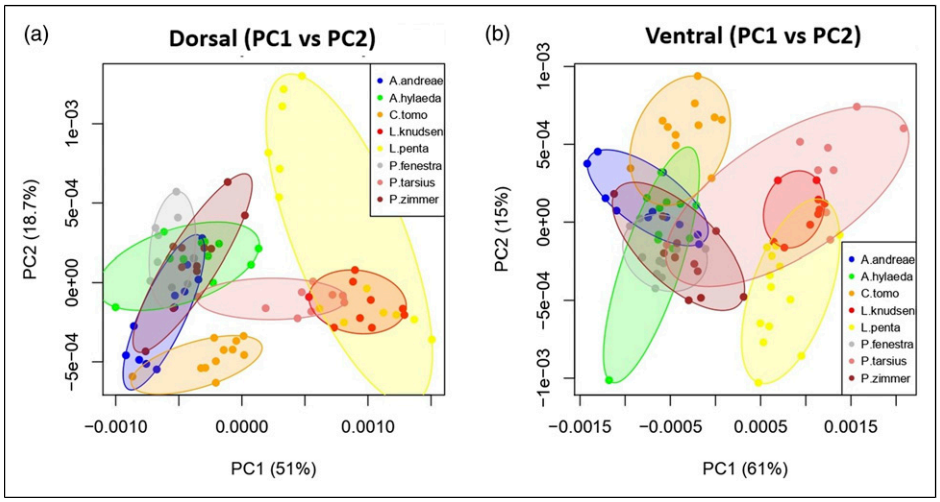


**Figure 4.** Two-dimensional plots of the first two principal component (PCA) scores showing spectral groupings of live anuran species measured on (a) dorsal and (b) ventral surfaces. In the caption: **A.andreae =** *Adenomera andreae*, **A.hylaeda =** *Adenomera hylaedactyla*, **C.tomo =** *Callimedusa tomopterna,* **L.knudseni =** *Leptodactylus knudseni*, **L.penta =** *Leptodactylus pentadactylus,* **P.fenestra =** *Pristimantis fenestratus*, **P.tarsius =** *Phyllomedusa tarsius,* **P.zimmer =** *Pristimantis zimmermanae*.

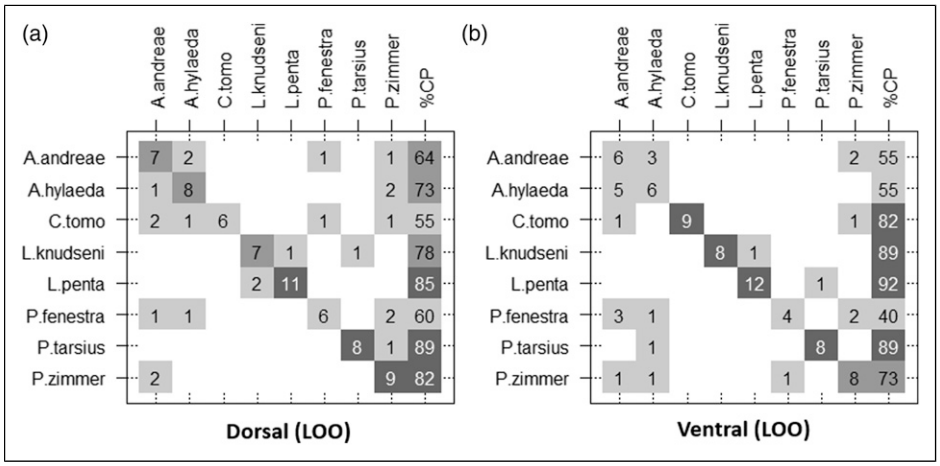


**Figure 5.** Confusion matrix resulting from LDA-LOO classification for recognition of live anuran samples with NIR spectra collected on (a) dorsal and (b) ventral surfaces. The species names in the calibration are given in rows, while predicted names are given in columns. Diagonal values are correct predictions and off-diagonal values are incorrect predictions. The number inside the squares refers to the number of samples used in the models for each species. The last column shows the correct prediction (CP) rate by species in percent **A.andreae =** *Adenomera andreae*, **A.hylaeda =** *Adenomera hylaedactyla*, **C.tomo =** *Callimedusa tomopterna,* **L.knudseni =** *Leptodactylus knudseni*, **L.penta =** *Leptodactylus pentadactylus,* **P.fenestra =** *Pristimantis fenestratus*, **P.tarsius =** *Phyllomedusa tarsius,* **P.zimmer =** *Pristimantis zimmermanae*.

not show a significant difference in distinguishing live anuran species. The contrasting results obtained for preserved and live anurans reinforce the importance of always obtaining spectral readings at both positions to generate spectral models, especially in exploratory studies.

In the present study, several tools available for spectral pre-processing were used to extract relevant information and minimize non-informative variability. The use of derivatives was important to eliminate unimportant baseline shifts, and digital smoothing algorithms were used to reduce instrument noise.[17,50] Also, selecting spectral ranges that have more consistent information and excluding regions with large instrumental noise helped capture the spectral information needed for species classification. Therefore, the pre-processing steps were valuable in order to reduce noise and non-informative sources of variability, making the results more consistent.[17]

Considering that light can pass through superficial tissues, information about internal organs, stomach and intestine contents and the presence of pregnant females[51] may be present in the spectral signatures. These sources of physiological variation were not controlled in the spectrum of each individual (even if of the same species), which may be a limitation of our study. Therefore, it is suggested that further studies use the technique controlling these variations to test the limitations to the efficiency of the technique.

The results obtained in the present study are consistent and gave promising results for most species studied. Developing high-tech tools that help to recognize Amazonian biodiversity is important to improve species recognition and add to traditional techniques such as the use of photographic and audio guides in the field. Thus, including the spectral signatures of species as an additional perspective in classical taxonomy,[30,52] as suggested by the integrative taxonomy approach.[16,53]

Technology that helps professionals in field or after collecting vouchers to more accurately identify numerous species would facilitate communication between researchers and zoological collections. However, there are still several steps that need to be taken to improve the applicability of NIR in the field. As well as optimizing the field techniques, it will be necessary to create a robust bank of reference spectra to feed libraries for portable equipment. With this, we believe there is great potential for using NIR to assist monitoring and management actions, and the development of public policies for the conservation of biodiversity, especially where there is a lack of taxonomic specialists available for field work.

## Authors' note

Andresa Viana helped in the financial logistics of collections. Igor Yuri, Alexandre Mônaco, André de Lima, Jonas Gonçalves, Thiago Bicudo, Natália Kinap and Marcelo Gordo helped in the search for anuran specimens. Flávio Costa and Caroline Vasconcelos helped with the R programming.

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## Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Supplemental Material

Supplemental material for this article is available online.

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