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Real-time DNA barcoding in a rainforest using nanopore sequencing: opportunities for rapid biodiversity assessments and local capacity building

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Abstract:	Background Advancements in portable scientific instruments provide promising avenues to expedite field work in order to understand the diverse array of organisms that inhabit our planet. Here we tested the feasibility for in situ molecular analyses of endemic fauna using a portable laboratory fitting within a single backpack, in one of the world's most imperiled biodiversity hotspots: the Ecuadorian Chocó rainforest. We utilized portable equipment, including the MinION nanopore sequencer (Oxford Nanopore Technologies) and the miniPCR (miniPCR), to perform DNA extraction, PCR amplification and real-time DNA barcoding of reptile specimens in the field. Findings We demonstrate that nanopore sequencing can be implemented in a remote tropical forest to quickly and accurately identify species using DNA barcoding, as we generated consensus sequences for species resolution with an accuracy of >99% in less than 24 hours after collecting specimens. The flexibility of our mobile laboratory further allowed us to generate sequence information at Universidad Tecnológica Indoamérica in Quito for recently collected rare, endangered, and undescribed specimens. This includes the recently re-discovered Jambato toad, which was thought to be extinct for 28 years. Sequences generated on the MinION required as little as 30 reads to achieve high accuracy relative to Sanger sequencing and with further multiplexing of samples, nanopore sequencing can become a cost-effective approach for rapid and portable DNA barcoding. Conclusions Overall, we establish how mobile laboratories and nanopore sequencing can help to accelerate species identification in remote areas to aid in conservation efforts and be applied to research facilities in developing countries. This opens up possibilities for biodiversity research by promoting local research capacity building, teaching laymen and students about the environment, tackling wildlife crime or by promoting conservation via research focused eco-tourism.
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Real-time DNA barcoding in a rainforest using nanopore sequencing: opportunities for rapid biodiversity assessments and local capacity building

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Abstract

Background

Advancements in portable scientific instruments provide promising avenues to expedite field work in order to understand the diverse array of organisms that inhabit our planet. Here we tested the feasibility for *in situ* molecular analyses of endemic fauna using a portable laboratory fitting within a single backpack, in one of the world's most imperiled biodiversity hotspots: the Ecuadorian Chocó rainforest. We utilized portable equipment, including the MinION nanopore sequencer (Oxford Nanopore Technologies) and the miniPCR (miniPCR), to perform DNA extraction, PCR amplification and real-time DNA barcoding of reptile specimens in the field.

Findings

We demonstrate that nanopore sequencing can be implemented in a remote tropical forest to quickly and accurately identify species using DNA barcoding, as we generated consensus sequences for species resolution with an accuracy of >99% in less than 24 hours after collecting specimens. The flexibility of our mobile laboratory further allowed us to generate sequence information at Universidad Tecnológica Indoamérica in Quito for recently collected rare, endangered, and undescribed specimens. This includes the recently re-discovered Jambato toad, which was thought to be extinct for 28 years. Sequences generated on the MinION required as little as 30 reads to achieve high accuracy relative to Sanger sequencing and with further multiplexing of samples,

nanopore sequencing can become a cost-effective approach for rapid and portable DNA barcoding.

Conclusions

Overall, we establish how mobile laboratories and nanopore sequencing can help to accelerate species identification in remote areas to aid in conservation efforts and be applied to research facilities in developing countries. This opens up possibilities for biodiversity research by promoting local research capacity building, teaching laymen and students about the environment, tackling wildlife crime or by promoting conservation via research focused eco-tourism.

Keywords

Nanopore sequencing, portable, DNA barcoding, biodiversity, field-based, real-time

Data Description

Background

Biodiversity is defined as the variety of life found on Earth, including variation in genes, species, and ecosystems. While about 1.9 million species have been described to date, there are an estimated 5-30 million species in total on the planet, with most of the diversity contained within tropical rainforests [1], [2], [3]. For instance, Ecuador, despite its small size of 283,561 km² (roughly 1.5% of South America), is one of the most biologically diverse countries in the world [4], [5]. Biodiversity is fundamentally important

to natural and agro-ecosystems; it provides humans with an array of foods and materials, contributes to medical discoveries, furnishes the economy, and supports ecological services that make life on our planet possible [6]. Today species are going extinct at an accelerated rate because of environmental changes caused by human activities including habitat loss, spread of non-native species, pollution, and climate change [7], [8]. All of these threats have put a serious strain on the diversity of species on Earth.

In the past decade, an ever-growing body of readily accessible knowledge, coupled with new tools in molecular genetics and bioinformatics, have resulted in species being described with greater accuracy, in greater detail, and with additional information to morphological differences. As a result of this increase in quality and content, desirable as it is, the actual process of species description has become slower, while the rate at which species are being lost to extinction has become faster. For many groups of animals, species delimitation can be challenging using solely morphological characteristics [9], [10], and can be improved by incorporating molecular data [11], [12]. This is relevant for the conservation of threatened animals because programs or laws can be implemented more effectively when the existence of a species or population is formally described. DNA barcoding, which is a diagnostic technique that utilizes short conserved DNA sequences, has become a popular tool for a variety of studies including species identification and molecular phylogenetic inference [13], [14], [15]. Ongoing initiatives, such as ‘Barcode of Life’ (www.barcodeoflife.org), seek to identify species and create large-scale reference databases via diagnostic DNA sequences using a standardized approach to accelerate taxonomic progress.

While projects utilizing standard molecular markers have grown in popularity in the last decade, a fundamental challenge remains in transporting biological material to a site that can carry out the DNA sequencing. Furthermore, complex and overwhelming regulations can impede biological research in biodiverse countries, and can make it challenging to export material out of the country of origin [16], [17]. Additionally, many research institutions in developing parts of the world do not have access to conventional sequencing technologies within the country, further limiting identification options. This is the case for Ecuador, where most laboratories ship their samples internationally to be sequenced, often creating a delay of weeks to months between tissue collection and the availability of the sequence data. Performing genetic analyses on site or at a nearby facility within the country can help to avoid project delays and decrease the risk of sample quality decline associated with extensive transport. Now it has become possible to take portable lab equipment to remote regions, perform *in situ* experiments, and obtain genetic information relevant for biological studies and conservation policies in real-time.

Portable Sequencing

The MinION (Oxford Nanopore Technologies) is a recently developed nanopore-based DNA sequencing platform. This technology has several advantages over traditional sequencing technologies, including long-read output, low initial startup costs relative to other commercial sequencers, portability, and rapid real-time analysis (reviewed by [18], [19]). Due to its small size (10 x 3.2 x 2 cm), light weight (90 grams) and ease of power and data transfer (a single USB connection to a standard laptop computer), the MinION

has emerged as a valuable tool for portable sequencing projects. This device has been applied in remote sites outside of conventional labs including West Africa to monitor the 2014-2015 Ebola outbreak [20] and Brazil for Zika virus outbreak surveillance [21], [22]. It has also been applied in the Arctic to sequence microbial communities [23], [24], in Tanzania to sequence frog DNA [25], and in Snowdonia National Park for shotgun genomic sequencing of closely-related plant species [26]. The MinION has even been run aboard the International Space Station to evaluate performance off-Earth [27], however, the sequencing runs were performed using DNA libraries pre-prepared in a standard laboratory environment, whereas preparing samples outside of a lab with limited infrastructure presents additional challenges. Indeed, nanopore sequencing appears to hold promise for a variety of molecular experiments in the field.

Scientists have mused over the possibility of a portable method for DNA barcoding for over a decade [28], [15] and in this study our goal was to determine if the steps involved in barcoding, including real-time sequencing with the MinION, could be carried out entirely during a field expedition. We specifically targeted DNA barcodes with existing reference databases because they are the standard approach in molecular biodiversity studies, and allowed us to rapidly produce genetic data for the identification of several animal taxa by multiplexing. Our field site was situated in a remote tropical rainforest and did not offer the commodities of a sophisticated laboratory environment, including consistent power sources or internet access. Furthermore, we restricted our laboratory equipment to reasonably affordable technologies. We did this for two reasons, (a) researchers in the field of molecular ecology may have limited funds for biodiversity research projects and (b) to test technologies that are affordable for

research facilities in developing countries. We assessed the feasibility for *in situ* genetic sequencing of reptiles and amphibians for rapid species identification, using a portable laboratory fitting within a single backpack, at one of the world's most imperiled biodiversity hotspots, the Ecuadorian Chocó rainforest (Fig. 1). We demonstrate that portable DNA amplicon sequencing with the MinION allows rapid, accurate, and efficient determination at the species level under remote tropical environmental conditions, as well as quick turnaround time for DNA barcodes of undescribed and threatened species at a research facility within the country.

Analyses

Site, sampling, digital photos, tissue collection

We performed all field-based research in the Canandé Reserve (Fig. 1, 0.52993 N, 79.03541 W, 594 m), a 2000 ha. protected area, owned by Jocotoco Foundation (http://www.fjocotoco.org/canandeacute1.html) in Esmeraldas province in Northwestern Ecuador. The reserve is located in the Chocó ecoregion and is approximately 6 hours by car, depending on road conditions, from the city of Quito. The majority of organisms sampled in this study were located by space-constrained visual examination of ground-level substrates [1]. The remaining individuals were detected by turning over logs, rocks, and other surface objects. All specimens included in the genetic analyses were morphologically identified based on [2] and [3]. The sample (a tadpole, CJ 7191) of *Atelopus ignescens* was provided by the Museum of Centro Jambatu, Ecuador and was preserved in ethanol 95%. We took vouchers for all samples collected and processed in

the field. These were deposited at the Museo de Zoología of the Universidad Tecnológica Indoamérica (MZUTI 5375 *Bothriechis schlegelii*, MZUTI 5383 *Lepidoblepharis* aff. *grandis.* (Gecko 1), MZUTI 5384 *Lepidoblepharis* aff. *buchwaldi.* (Gecko 2)).

Portable laboratory equipment and set-up

The main items for portable laboratory equipment included the following: two MinION devices, a USB 3.0 cable, three SpotON flow cells (R9.5, Oxford Nanopore Technologies (ONT)), one miniPCR thermocycler (miniPCR), and a benchtop centrifuge (USA Scientific), as well as standard laboratory pipettes and sample racks (Fig. 2, Supplementary Figure 3). The MinKNOW offline software (ONT) required for operation of the MinION was installed and ran on a Windows Vaio Sony laptop with an external SSD drive (VisionTek, 240GB). All heat block and temperature cycling steps were performed using the miniPCR machine, which is a portable thermo-cycler weighing 0.45 kg. The miniPCR was programmed via an application on the laptop and powered by an external battery (PowerAdd). The total amount of equipment could fit in one carry-on backpack; a full list of laboratory hardware is provided as Supplementary Table 1. Reagents for sequencing required frozen transport from the US, which was attained by use of packaging with cold packs in a Styrofoam box and was later transferred to a plastic cool box with further cold packs upon arrival to Quito, Ecuador. MinION flow cells require storage at +2-8°C and were therefore transferred in a food storage container with chilled cold packs. At the field site, reagents and supplies were stored inside a local refrigerator and freezer.

Molecular techniques

Genomic DNA was extracted from fresh blood or tissue samples stored in 95% ethanol using either the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol and eluted in 100 µl ddH2O or a modified salt precipitation method based on the Puregene DNA purification kit (Gentra Systems) that involved cellular lysis with SDS and proteinase K, protein precipitation using guanidine isothiocyanate, and DNA precipitation by isopropanol. Tools for manipulating and lysing tissues were sterilized with a flame in between processing samples. We amplified the following mitochondrial DNA fragments: 16S gene using primers 16Sar-L and 16Sbr-H-R from [4], CytB gene using primers L14910 and H16064 developed by [5], and the gene coding for subunit 4 of the NADH dehydrogenase with primers ND4 developed by [6]. All PCR primers contained universal tailed sequences for the Oxford Nanopore Technologies barcoding kit (Supplementary Table 2). We used the ONT PCR Barcoding Kit that allows up to 12 different libraries (barcodes 1-12) to be combined and loaded onto a single flow cell at the same time. PCR reactions contained approximately 1 µl of PCR product, 2.5 µl 10X PCR buffer, 1 µl 25mM $MgCl_2$, 200 µM dNTP mix, 0.2 µM of each primer and 0.25 Platinum Taq DNA Polymerase (Thermo Fisher Scientific) in a 25 µL total volume. All samples for the first PCR run were amplified on the same miniPCR under the following settings: initial denaturation: 94°C for 2 minutes, 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 60 seconds extension for 72°C for 60 seconds, and a final extension of 72°C for 120 seconds. Then a second round of PCR was carried out, including 2 µl of ONT PCR Barcode, 2 µL of first-round

PCR product, 41 µl H20, and 50 µl PCR reaction mix (0.5 µl Taq DNA polymerase, 1 µL dNTP mix, 2 µL MgCl2, 41 µL H2O). The second round of PCR barcode conditions were modified based on ONT protocol for the Platinum Taq polymerase used in this study as follows: initial denaturation at 95°C for 3 minutes, 15 cycles of denaturation at 95°C for 15 seconds, annealing at 62°C for 15 seconds, extension at 72°C for 60 seconds, and final extension at 72°C for 120 seconds. For verification of samples sequenced in the field, PCR products were subsequently cleaned with Exonuclase I and Alkaline Phosphatase (Illustra ExoProStar by GE Healthcare) at the Universidad Tecnológica Indoamérica (UTI) in Quito and sent to Macrogen Inc (Korea) for Sanger sequencing. All PCR products were sequenced on an ABI3730XL sequencer in both forward and reverse directions with the same primers that were used for amplification. The created sequences were deposited in GenBank (and will be available upon publication). All original Sanger and MinION generated consensus sequences can be found in Additional File 1.

MinION sequencing

DNA library preparation was carried out according to the 1D PCR barcoding amplicons SQK-LSK108 protocol (Oxford Nanopore Technologies). Barcode DNA products were pooled with 5 µl of DNA CS control and an end-repair was performed (NEB-Next Ultra II End-prep reaction buffer and enzyme mix, New England Biolabs), then purified using AMPure XP beads. Adapter ligation and tethering was then carried out with 20 µl Adapter Mix (ONT) and 50 µl of NEB Blunt/TA ligation Master Mix (New England Biolabs). The adapter ligated DNA library was then purified with AMPure beads,

followed by the addition of Adapter Bead binding buffer (ONT), and finally eluted in 15 µl of Elution Buffer (ONT). Each R9 flow cell was primed with 1000 µl of a mixture of Fuel Mix (Oxford Nanopore Technologies) and nuclease-free water. Twelve microliters of the amplicon library was diluted in 75 µL of running buffer with 35 µL RBF, 25.5 uL LLB, and 2.5 µL nuclease-free water and then added to the flow cell via the SpotON sample port. The “NC_48Hr_sequencing_FLO-MIN107_SQK-LSK108_plus_Basecaller.py” protocol was initiated using the MinION control software, MinKNOW (offline version provided by ONT).

Bioinformatics

The commands used can be found in the Supplementary Materials and Methods section.

To retrieve the nucleotide sequences from raw signal data generated by the MinKNOW software, we used Albacore 1.2.5 (https://github.com/dvera/albacore) for base calling and de-multiplexing of the ONT barcodes. The FAST5 files were then converted to fastq files using Nanopolish [7]; https://github.com/jts/nanopolish). We then filtered the raw reads for quality (score of >13) and read length (> 200bp) using Nanofilt (https://github.com/wdecoster/nanofilt), and generated consensus sequences using both reference-based mapping and *de novo* assembly. For the reference-based mapping we used BWA 0.7.15 [8]; https://github.com/lh3/bwa/releases) to align the reads to the reference, samtools 1.3 [9] to process the mapping file, and ANGSD [10], to call the consensus sequence. The *de novo* assembly of each amplicon was carried out using Canu [11], https://canu.readthedocs.io), with parameters fitting for our application. Given

that we used short amplicons for the assembly we set the minimum read length to 200bp and the minimum overlap to 50bp. We subsequently extracted the consensus sequences using tgStoreDump. After the consensus calling (for both methods) we mapped the reads back to the consensus sequence (using BWA mem and samtools as described above) and polished the sequencing using Nanopolish [7]. Adapters were removed using Cutadapt [12]. The consensi were then aligned to the Sanger sequences of the same amplicons to investigate the quality of the consensus sequences generated from MinION reads using SeaView [13] and AliView [14]. Sanger sequencing reads were edited and assembled using Geneious R10 software [15] and mapping files inspected by eye using Tablet [16].

We further tested the impact of coverage on the consensus accuracy by randomly subsampling three sets of 30, 100, 300 and 1,000 reads, respectively for the eyelash palm pitviper and gecko 1. Subsampling was performed with famas (https://github.com/andreas-wilm/famas). These sets were assembled *de novo* and processed using the same approach we used for the full data sets (see above).

We then created species alignments for all barcodes (using sequences obtained from Genbank; accession numbers can be found in the phylogenetic tree reconstructions in the Supplementary material). We inferred the best substitution model using jModelTest [17] and reconstructed their phylogenetic trees using the maximum likelihood approach implemented in Mega 5 [18] with 1,000 bootstrap replicates (for bioinformatics workflow see Fig. 3). The output tree files including the Genbank Accession Numbers are provided in the supplementary material.

Discussion

From Snakes to Sequences in 24 Hours

On July 11, 2017, we arrived at the field site at approximately 1500 hours and collected reptile and amphibian samples from 2000 to 2300 hours. Next, back at the field station, we extracted DNA and performed PCR amplification for 16S, CytB, and ND4 genes. On July 12, the PCR barcodes were pooled, the library was prepared, and then sequencing was initiated at approximately 1600 hours on a flow cell using the offline MinKNOW software, generating 16,663 reads after approximately two hours (Fig. 2). The software was then paused in order to assess the reads generated. Within 24 hours of collecting reptiles and amphibians in the Ecuadorian Chocó, we successfully generated consensus sequences for 16S and ND4 genes of an eyelash palm pitviper (*Bothriechis schlegelii*) and 16S for the dwarf gecko (*Lepidoblepharis* sp.; gecko 1). The CytB gene was not successfully sequenced, which was later confirmed at UTI's lab by lack of PCR product on a gel (Supplementary Figure 1). The field-generated sequence data was analyzed that evening on a laptop using a number of open source and custom-developed bioinformatic workflows (see Materials and Methods; Supplementary Figure 2). Blasting of the sequences against the NCBI database, as well as constructing phylogenetic trees using the nanopore sequences and previously generated reference database yielded accurate species identifications and phylogenetic placements (Fig. 3 and Fig. 4).

Upon returning to a local research facility in Quito, the Universidad Tecnológica Indoamérica, which does not have local sequencing capacities, we created one

additional DNA barcode library with new samples. With our remaining flow cell, we were interested in quickly generating genetic information for (a) additional specimens that were collected during our field expedition (gecko 2), (b) undescribed species collected the week before our expedition (Genera: *Dipsas* and *Sibon*), (c) an endangered species that would have been difficult to export out of the country (Jambato toad), (d) a rare species lacking molecular data (Guayaquil blind snake), and (e) combinations of barcoded samples through multiplexing (for the eyelash palm pitviper and gecko 1). Initially, this second sequencing run appeared to perform well. However, after using Albacore to demultiplex the reads, we determined the adapter ligation enzyme likely degraded because the output primarily consisted of adapter sequences (Supplementary Figure 1). Nevertheless, we were able to generate consensus sequences for 16S of the Jambato toad, the two *Dipsas* species, the dwarf gecko, and the Guayaquil blind snake (Fig. 3 and Fig. 4). The reads from both sequencing runs are available at Genbank (pending).

Subsampling

Next, we investigated the read depth needed to call accurate consensus sequences using our approach. We used the eyelash palm pitviper and gecko 1 to test subsampling schemes, since we obtained thousands of reads for these samples. We randomly subsampled to 30, 100, 300 and 1,000 reads (in three replicates; see Supplementary Table 3). For the eyelash palm pitviper we achieved accuracies ranging from 99.4% to 99.8% using only 30 reads, 99.6% to 100% using 100 reads, 99.8% for 300 reads and 99.8% to 100% for 1,000 reads. For gecko 1 we achieved even better

accuracy overall, with 30 reads ranging from 99.4% to 99.8%, 100 reads from 99.8% to 100%, all 300 reads sets achieved an accuracy of 100% and for 1,000 reads all but one set (99.8%) achieved 100% accuracy.

Performance in the field

Our objective was to employ a portable laboratory in a rainforest to identify endemic species with DNA barcoding in real-time (Fig. 2). Our protocols resulted in successful DNA extraction, PCR amplification, nanopore sequencing, and barcode assembly. We observed that the MinION sequencing platform performed well in the field after extended travel, indicating the potential for nanopore-based sequencing on future field expeditions. Although we demonstrate that the successful molecular identification of organisms in a remote tropical environment is possible, challenges with molecular work in the field remain. Our field site was provided with inconsistent electrical power, but still allowed us to use a conventional small centrifuge for several steps of DNA extraction and to power a refrigerator for storage of flow cells and some of the reagents, although temperatures were likely suboptimal. Lack of electrical supply can impede adequate storage of temperature-sensitive reagents for extended periods of time. Our experiments were performed during a relatively short field trial, with 10 days being the longest time period that reagents were kept at inconsistent freezing temperatures. It is uncertain how well nanopore kit reagents or flow cell integrity would endure over longer periods without consistent cooling temperatures, and we suspect the adapter ligation enzyme was compromised during our second nanopore run, as demultiplexing led to a majority of barcode adapters in each folder (Supplementary Figure 1). Furthermore, we

used an external SSD drive with 240 GB space to store raw data generated by MinKNOW. Due to overheating of the external drive, we placed ice packs underneath the USB stick to maintain cooler temperatures, which appeared to maintain the run.

While the MinION sequencer fits in the palm of a hand and needs only a USB outlet to function, bioinformatic analyses can be hampered under remote field conditions, because internet access, large amounts of data storage, and long periods of time are often required for such analytical tasks. In our study, utilizing short DNA fragments with a relatively small number of samples for barcoding allowed us to perform all bioinformatic analyses in the field, but larger data outputs may require additional storage and more computational resources.

Implications for conservation and biodiversity assessments

Tropical rainforests, such as the Ecuadorian Chocó, are often rich in biodiversity, as well as species of conservation concern. The Chocó biogeographical region is one of the world's 25 biodiversity hotspots [29] and several studies have identified the Chocó region of western Colombia and Ecuador as a global conservation priority [29], [30], [31]. We therefore chose this region for proof of principle *in situ* molecular work to highlight the importance of expediting fieldwork in order to produce genetic information of endemic fauna. Our rapidly obtained DNA barcodes allowed us to accurately identify organisms while in the field. When samples are not required to be exported out of the country to carry out molecular experiments, real-time sequencing information can contribute to more efficient production of biodiversity reports that advise conservation policy, especially in areas of high conservation risk.

Of particular note in this study was the critically endangered harlequin Jambato toad, *Atelopus ignescens*. Although not a denizen of the Chocó rainforests, this Andean toad is a good example to demonstrate how nanopore sequencing can aid in the conservation of critically endangered species. *Atelopus ignescens* was previously presumed extinct (it is currently still listed as “extinct” on IUCN; [32]) and was only recently rediscovered [33]. The last confirmed record of *Atelopus ignescens* dates back to 1988, and this species was presumed to be extinct before one population was rediscovered in 2016, 28 years later. *Atelopus* is a species-rich genus of neotropical toads containing 96 species, most of which are possibly extinct or endangered. In Ecuador there are 11 species of *Atelopus* that are Critically Endangered (tagged as Possibly extinct; [34]). Extinctions of *Atelopus* (and other anurans) are beyond control and are increasingly exacerbated by a combination of factors including habitat loss, climate change and pathogens [35], [36], [37]. For the many endangered species that are protected by international laws and treaties, sample transport requires permits that can often be difficult to obtain, even when research is expressly aimed at conservation, resulting in project delays that can further compromise sample quality. By working within the country, under permits issued by Ministerio del Ambiente de Ecuador to local institutions, we were able to generate sequence data for the endangered harlequin Jambato toad *Atelopus ignescens* within 24 hours of receiving the tissue, whereas obtaining permits to ship samples internationally in the same time frame would have not been possible. Rapidly identifying the phylogenetic affinity of populations of *Atelopus* toads could speed up conservation efforts for these animals. Namely, a better understanding of the systematics of the group facilitated by real-time sequencing could

help establish species limits, geographic distributions, in-situ conservation actions and ex-situ breeding programs.

Species identifications

It is important to note that we do not intend for rapidly-obtained portable sequence information to substitute for standard species description processes. Instead, we aim to demonstrate that obtaining real-time genetic information can have beneficial applications for biologists in the field, such as raising the interesting possibility of promptly identifying new candidate species, information which can be used to adjust fieldwork strategies or sampling efforts. As we have shown, the latter could be especially important with organisms and habitats facing pressing threat. Rapidly obtaining genetic sequence information in the field can also be useful for a range of other applications, including identifying cryptic species, hybrid zones, immature stages, and species-complexes.

Furthermore, we acknowledge that in most cases multiple loci are needed to reliably infer species position in a phylogenetic tree. DNA barcoding has been shown to hold promise for identification purposes in taxonomically well-sampled clades, but may have limitations or pitfalls in delineating closely related species or in taxonomically understudied groups [38], [39]. However, our aim in this study was to demonstrate that portable sequencing can be used in the field and that the final sequences have an accuracy needed to achieve reliable identification of a specimen. While a recent study has demonstrated a field-based shotgun genome approach with the MinION to identify closely related plant species [26], DNA barcoding already offers a robust reference

database for many taxa thanks in part to global barcoding initiatives (the current Barcode of Life Data System contains 4,013,927 specimens and 398,087 Barcode Index Numbers http://ibol.org/resources/barcode-library/ as of September 2017).

Finally, while highlighting the value of real-time portable DNA barcoding in this study, we do not wish to downplay the significance of taxonomic experts, who have invaluable specialist knowledge about specific groups of organisms. Even with the advent of molecular diagnostic techniques to describe and discover species, placing organisms within a phylogenetic context based on a solid taxonomic foundation is necessary. An integrative approach utilizing molecular data and morphological taxonomy can lead to greater insight of biological and ecological questions [40]. As noted by Bik, 2017, *"There is much to gain and little to lose by deeply integrating morphological taxonomy with high-throughput sequencing and computational workflows."*

Bioinformatic challenges

While we were able to show that nanopore sequencing results in high quality DNA barcode sequences, some challenges during the read processing remain. To our knowledge, no software solution specifically designed to assemble DNA barcodes from long read technologies is available. Here, we created our own pipeline (Supplementary Figure 2). This required changing the settings for Canu [41], a whole genome *de novo* assembler (see Materials and Methods in the Supplementary Information; and discussion below). However, software geared towards the specifics of assembling DNA

barcodes from long read data would be beneficial to make the bioinformatics analysis easier and more widely applicable.

We were also interested in investigating the minimum coverage needed to create reliable consensus sequences. Therefore, we used different subsampling schemes. Overall, a coverage of 30 reads achieved an accuracy of 99.4 - 99.8%. With 100x read coverage almost all assemblies were 100% accurate, indicating that an excessive number of reads is not needed to produce high quality consensus sequences. Furthermore, we applied Nanopolish to all consensus sequences. This tool has been shown to be very effective at correcting typical nanopore errors, such as homopolymer errors [42], [43]. As can be seen in section “Post-Nanopolish assembly identity” in [43], accuracy of the resulting consensus increases significantly after polishing. While, we did not measure the improvement in accuracy in our study, we did notice a high accuracy after polishing. However, as can be seen in Fig. 4B, nanopolish is not always able to accurately correct homopolymer stretches.

We further tested reference-based mapping versus *de novo* assembly, because a reference-based mapping approach may introduce bias, making it possible to miss indels. Overall, we see that consensus sequences generated using reference-based mapping have slightly lower accuracy. However, in two cases (the eyelash palm pitviper and the Jambato toad) an accuracy of 100% was achieved with reference-based mapping. Interestingly, in the case of *Dipsas* sp. (JMG378), reference-based mapping resulted in a slightly better accuracy than the *de novo* approach (99.4% compared to 99%). However, in general, we recommend the use of a *de novo* assembly approach as this method can be applied even if no reference sequence is available and generally

produced more accurate sequences. An alternative approach would be to generate consensus sequences by aligning the individual reads for each barcode to one another, which would not be affected by a reference bias. This method is implemented in the freely available software tool Allele Wrangler (https://github.com/transplantation-immunology/allele-wrangler/). However, at the time of submission this tool picks the first read as the pseudo reference, which can lead to errors in the consensus if this read is of particularly low quality or an incorrect (contaminant) sequence. Future developments might establish this method as an alternative to *de novo* assembly algorithms, which are typically written for larger genomes (e.g. the minimum genome size in Canu is 1000bp) and can have issues with assemblies where the consensus sequence is roughly the size of the input reads (*personal communications* Adam Phillippy).

Each of our two runs showed a very high number of reads not assigned to any barcode sequence after de-multiplexing with Albacore 1.2.5 (7,780 and 14,272 for the first and second sequencing run, respectively). In order to investigate whether these reads belong to the target DNA barcodes but did not get assigned to sequencing barcodes, or if they constitute other sequences, we generated two references (one for each sequencing run) comprising all consensi found within each individual sequencing run. We then mapped all reads not assigned to barcodes back to the reference. We were able to map 2,874 and 4,997 reads to the reference for the first and the second sequencing run, respectively, which shows that a high number of reads might be usable if more efficient de-multiplexing algorithms become available. Here we used Albacore 1.2.5, an ONT software tool, to de-multiplex the sequencing barcodes. This tool in under constant development and thus might offer more efficient de-multiplexing in later

versions. Alternatively, 3rd party software tools like npBarcode [44] or Porechop (https://github.com/rrwick/Porechop) can be used.

Cost-effectiveness and local resource development

Next-generation sequencing technologies are constantly evolving, along with their associated costs. Most major next-generation sequencing platforms require considerable initial investment in the sequencers themselves, costing hundreds of thousands of dollars, which is why they are often consolidated to sequencing centers at the institutional level [45]. In this study, we used the ONT starter pack, which currently costs $1000, and includes two flow cells and a library preparation kit (6 library preparations), as well as the ONT 12 barcoding kit which is currently $250 for 6 library preparations (for a full list of equipment and additional reagents see Supplementary Table 1). Using this minimal setup, each barcode sequence costs about $45 (this includes cost for the starter pack, etc; a detailed cost account can be found in the Supplementary material). At this cost, further multiplexing of samples on each flow cell is necessary to achieve a cost-effectiveness for DNA sequencing relative to other commercial options. Fortunately, ONT also offers a 96 barcode kit, currently priced at $1,700. While this is a higher upfront cost it can reduce the price for each DNA barcode sequence to about $12. However, it will not be long until much higher multiplexing (>300 samples) becomes achievable. This way per DNA barcode costs can be reduced to less than $1. On the contrary, Sanger sequencing from UTI shipped internationally for processing costs approximately $10 per sample, independent of the through-put. Thus, the Oxford Nanopore MinION has the potential to be a cost-effective sequencing option

for resource-limited labs, especially in developing countries without access to standard sequencing devices.

The small size and low power requirements of the MinION will likely continue to enable its evolution as a field-deployable DNA sequencing device, opening up new avenues for biological research in areas where the typical laboratory infrastructure for genetic sequencing is unavailable. With some training, in the field molecular analyses could also potentially be performed by students (see [46]) or assistants, providing an opportunity for local teaching and research capacity building, and community involvement via research focused ecotourism or citizen-science projects.

Future outlook

Technological developments in lab equipment and reagent chemistry are increasingly enabling the incorporation of genetic analyses into field projects. Several portable technologies have been used to perform molecular experiments in the field, particularly for disease diagnostics [47], [48]. Advances in lyophilized and room-temperature reagents are also promising for field applications, such as EZ PCR Master Mix [49], and loop-mediated isothermal amplification [50], [51]. A hand-powered centrifuge [52] could also act as substitute for a standard benchtop centrifuge during DNA extraction steps. Automatic devices, such as VolTRAX (a compact microfluidic device designed to automate nanopore library preparation, ONT) and improved library construction methods may offer faster and high-throughput methods for preparing nanopore libraries in the future. As the ONT MinION evolves, it could greatly advance field researchers' capacity to obtain genetic data from wild organisms while in the field. These

technologies currently depend on reagents that require freezing, but can be used at field sites with solar or portable freezer options. Faster and more automated sample processing, as well as cost reductions, are needed for adoption in low-income settings.

Beyond short PCR-based amplicons aimed at species identification, other exciting potential applications of nanopore sequencing in the field include sequencing of entire mitochondria from gDNA samples [53] or via long-range PCR, shotgun genome sequencing [26], analysis of environmental DNA [54], [24], sequencing of direct RNA [55], [56] or cDNA to rapidly profile transcriptomes ([57], and pathogen diagnostics and monitoring (such as chytrid fungus; [58]). Rapid portable sequencing can also be applied to wildlife crime to perform species identification of animals affected by illegal trafficking, as well as serve to aid in early detection of invasive species threatening local biodiversity and agriculture, and emerging infectious diseases.

Potential implications

Portable DNA barcoding with the MinION sequencer allows rapid, accurate, and efficient determination at the species level under remote and tropical environmental conditions. We also demonstrate that portable sequencing can allow nimble use of rapidly generating data for endangered, rare, and undescribed species at nearby facilities within the country. In the context of conservation and biodiversity science, portable nanopore sequencing can be beneficial for applications including:

i. When it is exceedingly challenging or not possible to export biological material internationally or to a facility with a conventional sequencing device. Be aware

that the proper permits to collect samples and carry out experiments in the location of the study are still necessary, and collaborating with local researchers is strongly encouraged.

ii. When the material to be sequenced may be compromised during transportation conditions, or during the time in between collection and sequencing. This can be applicable to experiments involving RNA in particular, which is subject to degradation if not adequately preserved or immediately frozen.

iii. For biodiversity reports aimed at quickly generating species data to inform conservation policy decisions, especially in areas of high conservation risk.

iv. To rapidly screen and sequence pathogens, such as chytrid fungus in amphibians or infectious agents in fecal samples. Studies using the MinION in the field have been applied during epidemics, including recent outbreaks of Ebola and Zika, and can be applied to non-human pathogens as well.

v. To perform on-site identification of organisms, immature life stages, or sexes that are difficult to distinguish morphologically, such as larvae or pupae of insects, plants when they are not actively flowering such as orchids, or cryptic species. This can help guide specimen collection in the field.

vi. To assist with rapid species identification in the fight against illegal wildlife trade.

vii. To identify organisms in the field that are difficult to locate or capture by sampling environmental DNA (eDNA).

viii. To build up local sequencing capacity of laboratories and field stations in developing countries, and to promote training of local students or citizen-science (e.g. via research focused eco-tourism).

While we live in a period of amazing technological change, biodiversity and ecosystem health are decreasing worldwide. Portable sequencing will not be a silver bullet for conservation biology, but it can be a powerful tool to more efficiently obtain information about the diversity of life on our planet. This is particularly important for many biodiversity hotpots, such as tropical rainforests like the Ecuadorian Chocó, which are often under high risk of habitat loss. We anticipate that as portable technologies develop further, this method will broaden the utility of biological field analyses including real-time species identification, cryptic species discovery, biodiversity conservation reports, pathogen detection, and environmental studies.

Competing interests

The authors report no competing interests.

Author contributions

AP and SP designed the project. AP, NP, AA, LB, FP, CB, DV and SP carried out specimen collection; AP and NP laboratory work; AA, LB, FP, LC, CB and DV morphological species identification and SP computational analyses. AP, NP, AA, LB, FP, LC, CB, DV and SP wrote the paper.

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Figures

Figure 1. Site where field-based nanopore research was conducted within the Chocó biogeographical region in Ecuador, which is one of the world’s 25 biodiversity hotspots.

This area has experienced one of the highest rates of deforestation in the country and is considered a global conservation priority.

Figure 2. Process of nanopore sequencing in the Ecuadorian Chocó rainforest. A) Sampling endemic fauna; eyelash viper next to MinION. B) Extraction of blood or tissue samples. C) DNA extraction using the DNeasy kit and benchtop centrifuge, and PCR amplification with the MiniPCR. D) Oxford nanopore library preparation of DNA barcodes. E) Bioinformatic processing of nanopore data in the field. F) Primary equipment used in portable sequencing, left to right: MiniPCR sitting atop Poweradd external battery, MinION plugged into a Windows laptop displaying Geneious Pro software of raw nanopore data.

Figure 3. Species investigated, nucleotide alignments of nanopore and Sanger sequences comparing consensus accuracy, and Maximum Likelihood trees of 16S sequences for: A) Eyelash pitviper, *Bothriechis schlegelii*, B) two species of dwarf gecko, *Lepidoblepharis* sp, and C) the Jambato toad, *Atelopus ignescens.* Red labels in the phylogenetic trees indicate the sequences generated by the MinION.

Figure 4. Species investigated, nucleotide alignments of nanopore and Sanger sequences comparing consensus accuracy, and Maximum Likelihood trees of 16S sequences for: A) Guayaquil blind snake, *Trilepida guayaquilensis* and B) two species *Dipsas* snakes. Red labels in the phylogenetic trees indicate the sequences generated by the MinION.

References

1. Erwin TL. Tropical Forests Their Richness in Coleoptera and Other Arthropod Species. The Coleop Bull. 1982;36:74-75.

2. Stork NE. How many species are there? Biodiv Conserv. 1993;2:215-232.

3. Scheffers BR, Joppa LN, Pimm SL, Laurance WF. What we know and don't know about Earth's missing biodiversity. Trends Ecol Evol. 2012;27:501-510.

4. Sierra R, Campos F, Chamberlin J. Assessing biodiversity conservation priorities: ecosystem risk and representativeness in continental Ecuador. Landscape and Urban Planning. 2002;59:95-110.

5. Cuesta F, Peralvo M, Merino-Viteri A, Bustamante M, Baquero F, et al. Priority areas for biodiversity conservation in mainland Ecuador. Neotrop Biodiv. 2017;3:93-106.

6. Gascon C, Brooks TM, Contreras-MacBeath T, Heard N, Konstant W, et al. The importance and benefits of species. Curr Biol. 2015;25:R431-438.

7. Pimm SL, Jenkins CN, Abell R, Brooks TM, Gittleman JL, et al. The biodiversity of species and their rates of extinction, distribution, and protection. Science. 2014;344:1246752.

8. Ceballos G, Ehrlich PR, Dirzo R. Biological annihilation via the ongoing sixth mass extinction signaled by vertebrate population losses and declines. Proc Natl Acad Sci USA. 2017;114:E6089-E6096.

9. Colonma LA, Duellman WE, Almendariz A, Ron, SR. Five new (extinct?) species of Atelopus (Anura: Bufonidae) from Andean Colom-bia, Ecuador, and Peru. Zootaxa. 2010;2574:1-54.

10. Loetters S, Van der Meijden A, Coloma LA, Boistel R, Cloetens P, et al. Assessing the molecular phylogeny of a near extinct group of vertebrates: the Neotropical harlequin frogs (Bufonidae; Atelopus). System Biodiv. 2011;9:45-57.

11. Barley AJ, White J, Diesmos AC, Brown RM. The challenge of species delimitation at the extremes: diversification without morphological change in philippine sun skinks. Evolution. 2013;67:3556-3572.

12. Carstens BC, Pelletier TA, Reid NM, Satler JD. How to fail at species delimitation. Mol Ecol. 2013;22:4369-4383.

13. Hebert PD, Cywinska A, Ball SL, deWaard JR. Biological identifications through DNA barcodes. Proc Biol Sci. 2003;270:313-321.

14. Hebert PD, Gregory TR. The promise of DNA barcoding for taxonomy. Syst Biol. 2005;54:852-859.

15. Savolainen V, Cowan RS, Vogler AP, Roderick GK, Lane R. Towards writing the encyclopedia of life: an introduction to DNA barcoding. Philos Trans R Soc Lond B Biol Sci. 2005;360:1805-1811.

16. Gilbert N. Biodiversity law could stymie research. Nature Publishing Group. 2010; http://www.nature.com/news/2010/100203/full/463598a.html

17. Fernández F. The greatest impediment to the study of biodiversity in Colombia. Caldasia. 2011;33:3-5.

18. Laver T, Harrison J, O'Neill PA, Moore K, Farbos A, et al. Assessing the performance of the Oxford Nanopore Technologies MinION. Biomol Detect Quantif. 2015;3:1-8.

19. Mikheyev AS, Tin MM. A first look at the Oxford Nanopore MinION sequencer. Mol Ecol Resour. 2014;14:1097-1102.

20. Quick J, Loman NJ, Duraffour S, Simpson JT, Severi E, et al. Real-time, portable genome sequencing for Ebola surveillance. Nature. 2016;530:228-232.

21. Faria NR, Sabino EC, Nunes MR, Alcantara LC, Loman NJ, et al. Mobile real-time surveillance of Zika virus in Brazil. Genome Med. 2016;8:97.

22. Faria NR, Quick J, Claro IM, Theze J, de Jesus JG, et al. Establishment and cryptic transmission of Zika virus in Brazil and the Americas. Nature. 2017;546:406-410.

23. Edwards A, Debbonaire AR, Sattler B, Mur LAJ, Hodson AJ. Extreme metagenomics using nanopore DNA sequencing: a field report from Svalbard, 78 °N. bioRxiv. 2016; https://doi.org/10.1101/073965

24. Johnson SS, Zaikova E, Goerlitz DS, Bai Y, Tighe SW. Real-Time DNA Sequencing in the Antarctic Dry Valleys Using the Oxford Nanopore Sequencer. J Biomol Tech. 2017;28:2-7.

25. Menegon M, Cantaloni C, Rodriguez-Prieto A, Centomo C, Abdelfattah A, Rossato M, Bernardi M, Xumerle L, Loader S, Delledonne M. On site DNA barcoding by nanopore sequencing. PLoS One. 2017;https://doi.org/10.1371/journal.pone.0184741

26. Parker J, Helmstetter AJ, Devey D, Wilkinson T, Papadopulos AST. Field-based species identification of closely-related plants using real-time nanopore sequencing. Sci Rep. 2017;7:8345.

27. Castro-Wallace SL, Chiu CY, John KK, Stahl SE, Rubins KH, et al. Nanopore DNA Sequencing and Genome Assembly on the International Space Station uploaded to BioRxiv. 2016; https://doi.org/10.1101/077651

28. Janzen DH, Hajibabaei M, Burns JM, Hallwachs W, Remigio E, et al. Wedding biodiversity inventory of a large and complex Lepidoptera fauna with DNA barcoding. Philos Trans R Soc Lond B Biol Sci. 2005;360:1835-1845.

29. Myers N, Mittermeier RA, Mittermeier CG, da Fonseca GA, Kent J. Biodiversity hotspots for conservation priorities. Nature. 2000;403:853-858.

30. Dinerstein E. A Conservation assessment of the terrestrial ecoregions of Latin America and the Caribbean. Washington, D.C.: World Bank. 1995;xvii, 129 p. p.

31. Olson DM, Dinerstein E. The Global 200: a representation approach to conserving the Earth's most biologically valuable ecoregions. Conservation Biology. 1998;12:502-515.

32. Santiago Ron LAC, Stefan Lötters, William Duellman, Martín R. Bustamante, Wilmar Bolívar, Enrique La Marca. 2004 IUCN Red List Atelopus ignescens

33. Coloma L. El Jambato negro del páramo, Atelopus ignescens, resucitó. 2016 IMCiencia.

34. Tapia EE, Coloma LA, Pazmiño-Otamendi G, Peñafiel N. Rediscovery of the nearly extinct longnose harlequin frog Atelopus longirostris (Bufonidae) in Junín, Imbabura, Ecuador. Neotropical Biodiversity. 2017;3:157-167.

35. Kiesecker JM. Global stressors and the global decline of amphibians: tipping the stress immunocompetency axis. Ecological research. 2011;26:897-908.

36. Pounds JA, Coloma LA. Beware the lone killer. Nature Reports Climate Change. 2008;57-59.

37. Lips KR, Diffendorfer J, Mendelson III JR, Sears MW. Riding the wave: reconciling the roles of disease and climate change in amphibian declines. PLoS biology. 2008;6: e72.

38. Elias M, Hill RI, Willmott KR, Dasmahapatra KK, Brower AV, et al. Limited performance of DNA barcoding in a diverse community of tropical butterflies. Proc Biol Sci. 2007;274:2881-2889.

39. Meyer CP, Paulay G. DNA barcoding: error rates based on comprehensive sampling. PLoS Biol. 2005;3: e422.

40. Bik HM. Let's rise up to unite taxonomy and technology. PLoS Biology. 2017;15: e2002231.

41. Koren S, Walenz BP, Berlin K, Miller JR, Bergman NH, et al. Canu: scalable and accurate long-read assembly via adaptive k-mer weighting and repeat separation. Genome Res. 2017;27:722-736.

42. Loman NJ, Quick J, Simpson JT. A complete bacterial genome assembled de novo using only nanopore sequencing data. Nat Methods. 2015;12:733-735.

43. Wick RR, Judd LM, Gorrie CL, Holt KE (2017) Completing bacterial genome assemblies with multiplex MinION sequencing. bioRxiv. 2017;160614.

44. Nguyen SH, Duarte TDS, Coin LJ, Cao MD. Real-Time Demultiplexing Nanopore Barcoded Sequencing Data With npBarcode. bioRxiv. 2017;134155.

45. Glenn TC. Field guide to next-generation DNA sequencers. Molecular ecology resources. 2011;11: 759-769.

46. Zeng, Y., & Martin, CH. Oxford Nanopore sequencing in a research-based undergraduate course. bioRxiv *2017;*227439.

47. Canier L, Khim N, Kim S, Sluydts V, Heng S, et al. An innovative tool for moving malaria PCR detection of parasite reservoir into the field. Malar J. 2013;12:405.

48. Marx V. PCR heads into the field. Nature methods. 2015;12:393-397.

49. Guevara EE, Frankel DC, Ranaivonasy J, Richard AF, Ratsirarson J, et al. A simple, economical protocol for DNA extraction and amplification where there is no lab. Conservation Genetics Resources. 2017;1-7.

50. Centeno-Cuadros A, Abbasi I, Nathan R. Sex determination in the wild: a field application of loop-mediated isothermal amplification successfully determines sex across three raptor species. Mol Ecol Resour. 2017;17:153-160.

51. Howson ELA, Armson B, Madi M, Kasanga CJ, Kandusi S, et al. Evaluation of Two Lyophilized Molecular Assays to Rapidly Detect Foot-and-Mouth Disease Virus Directly from Clinical Samples in Field Settings. Transbound Emerg Dis. 2017;64:861-871.

52. Bhamla MS, Benson B, Chai C, Katsikis G, Johri A, et al. Hand-powered ultralow-cost paper centrifuge. Nature Biomedical Engineering. 2017;1: 0009.

53. Chandler J, Camberis M, Bouchery T, Blaxter M, Le Gros G, et al. Annotated mitochondrial genome with Nanopore R9 signal for Nippostrongylus brasiliensis. F1000Res. 2017;6: 56.

54. Rees HC, Maddison BC, Middleditch DJ, Patmore JR, Gough KC. The detection of aquatic animal species using environmental DNA–a review of eDNA as a survey tool in ecology. Journal of Applied Ecology. 2014;51:1450-1459.

55. Ayub M, Hardwick SW, Luisi BF, Bayley H. Nanopore-based identification of individual nucleotides for direct RNA sequencing. Nano Lett. 2014;13: 6144-6150.

56. Garalde DR, Snell EA, Jachimowicz D, Heron AJ, Bruce M, et al. Highly parallel direct RNA sequenc ing on an array of nanopores bioRxiv. 2016;https://doi.org/10.1101/068809

57. Hargreaves AD, Mulley JF. Assessing the utility of the Oxford Nanopore MinION for snake venom gland cDNA sequencing. PeerJ. 2015;3:e1441.

58. Weldon C, du Preez LH, Hyatt AD, Muller R, Spears R. Origin of the amphibian chytrid fungus. Emerg Infect Dis. 2004;10:2100-2105.

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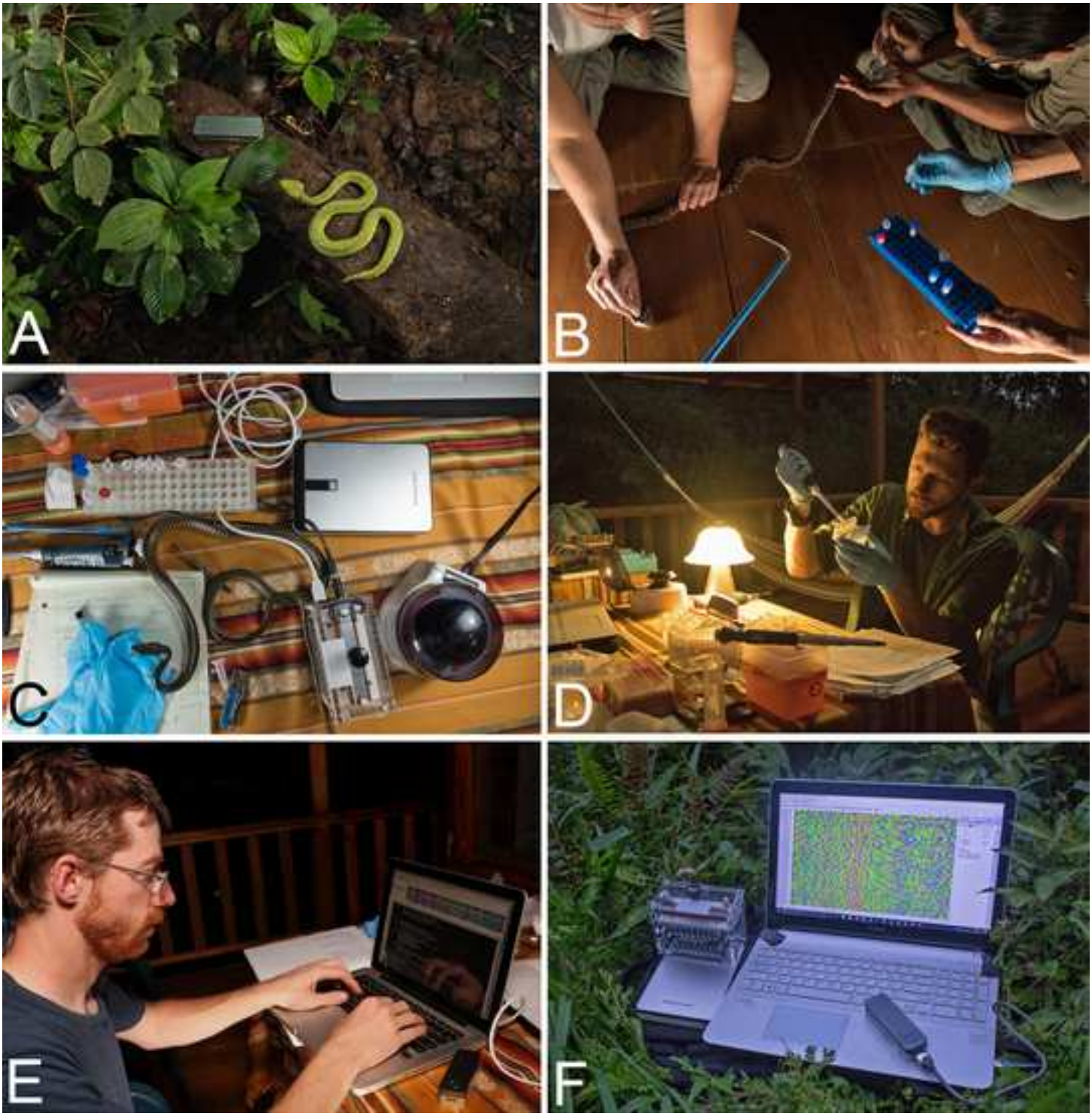

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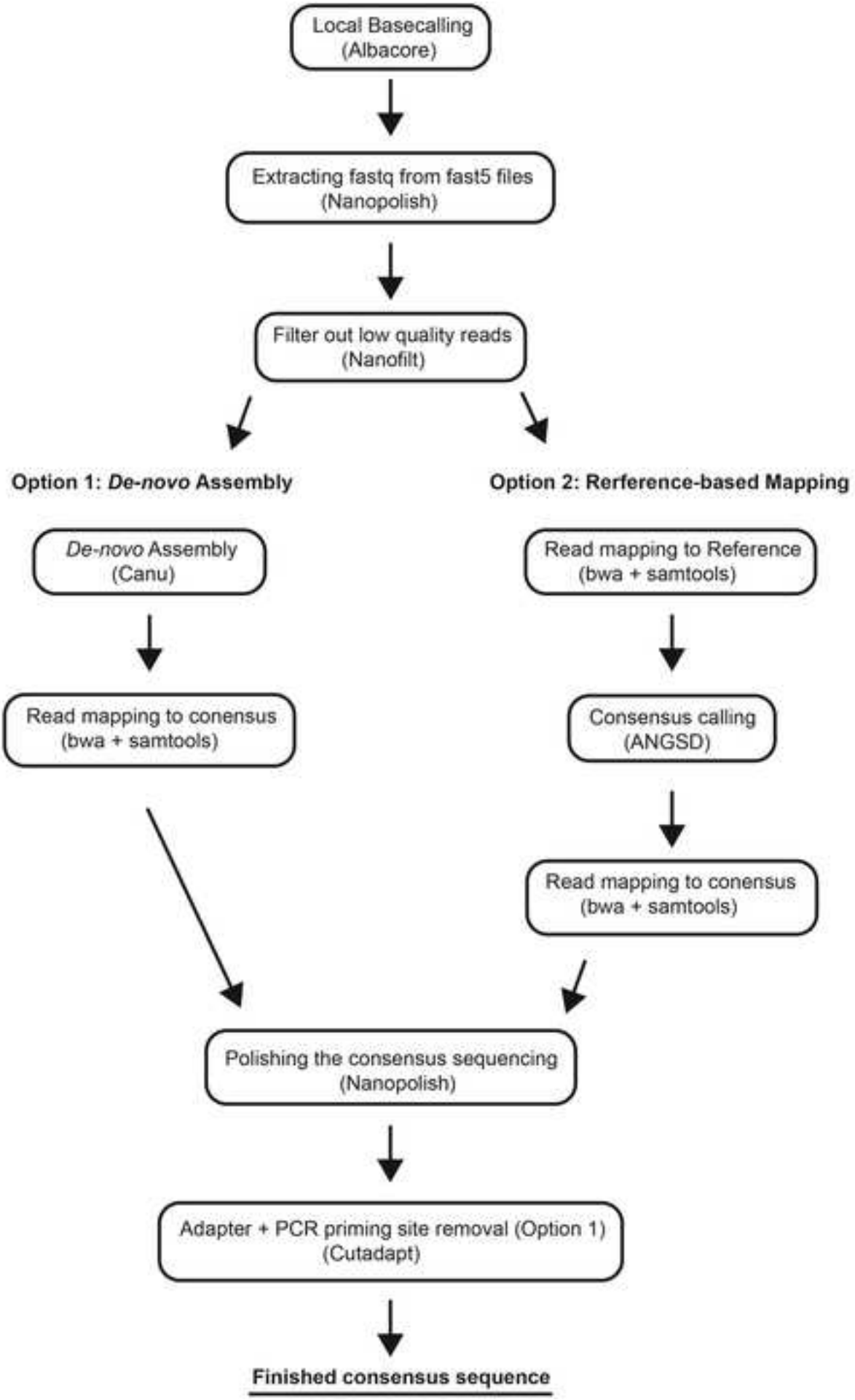

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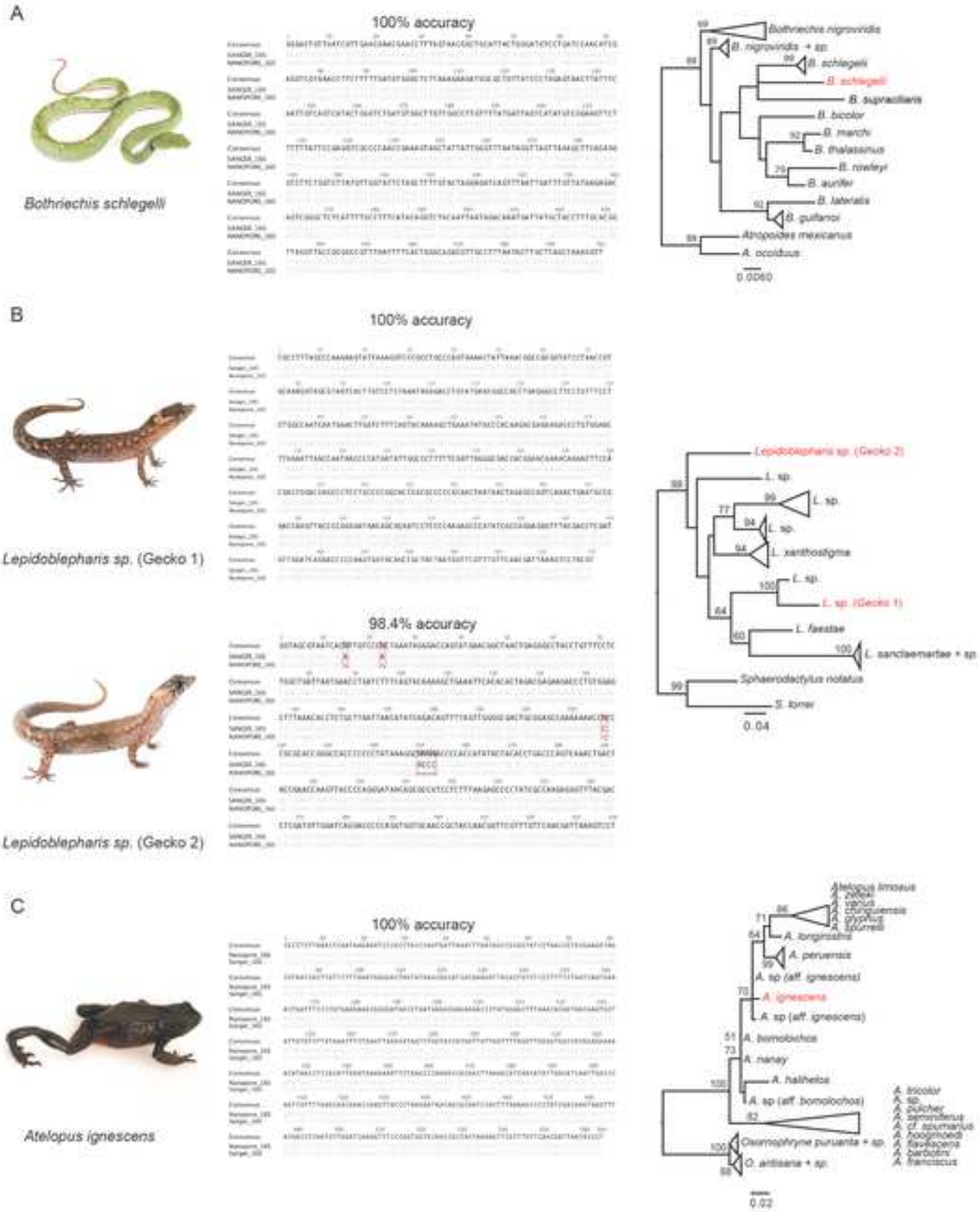

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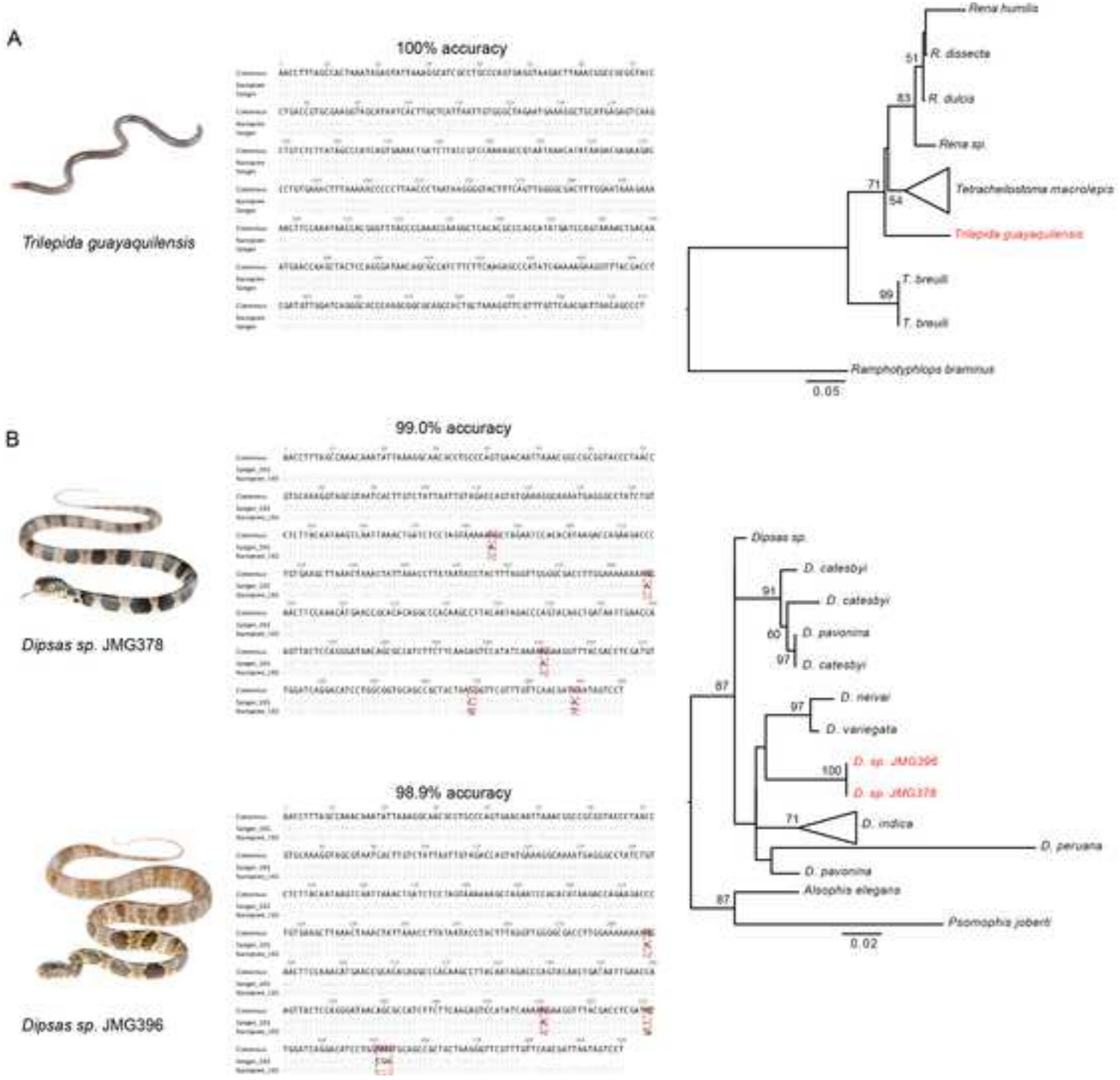

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