ARTICLE

INVESTIGATING THE DISTRIBUTION OF THE SMOOTH-COATED OTTER *(Lutrogale perspicillata)* USING ENVIRONMENTAL DNA: PRELIMINARY RESULTS

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Abstract: The analysis of environmental DNA (eDNA) can be an effective tool for detecting the presence of elusive or low-density organisms. While this technique has been utilized in many ecosystems, it has yet to be applied in mangrove ecosystems to detect aquatic mammals. Extreme environmental conditions (heat, salinity, turbidity) pose challenges for detection of rare species using eDNA in mangrove systems. We conducted a pilot study to test the sensitivity of eDNA methods for detecting the smooth-coated otter (*Lutrogale perspicillata*; IUCN classification: vulnerable) in mangrove ecosystems in India. This species can be difficult to monitor due to their elusiveness and the challenges of working in these complex systems. Over 11 weeks, we collected 30 water samples where signs of *L. perspicillata* were noted around Chorão Island in Goa, India, filtering on-site immediately after collection. We designed and validated a species-specific probe-based quantitative PCR assay for this species and used it to detect DNA of *L. perspicillata* in the filtered samples. We found our assay to be effective in detecting *L. perspicillata* within the mangrove ecosystem of Goa. Our results show that the detection probability likely decreases with time and that detection is possible at both high and low tide. This method could provide a sensitive, efficient way to detect elusive semi aquatic or aquatic species in mangrove systems.

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INTRODUCTION

Monitoring rare or vulnerable species can be incredibly difficult. Because of the elusiveness of many species and the difficulty of working in systems with dense vegetation or rough terrain, traditional field techniques (e.g., manual tracking, camera trapping, telemetry) might not be feasible or effective for some species. The analysis of environmental DNA (eDNA) shed by species in aquatic systems can be a sensitive and effective tool for species monitoring, ecology, and conservation (Rees et al., 2015). The use of eDNA techniques has been demonstrated to be more effective at detecting small populations of wildlife than traditional monitoring methods in many systems (Pilliod et al., 2013; Biggs et al., 2015; Ushio et al., 2017; Goldberg et al., 2018; Franklin et al., 2019; Sutter and Kinziger, 2019). Additionally, eDNA surveys can help mitigate issues of limited field resources, funding constraints, restricted access to habitats, reliable

species identification, and negative impacts on organisms through invasive field survey techniques (Bohmann et al., 2014).

Otters can be challenging to survey for because they are rare and elusive, can be nocturnal, sometimes live in difficult to access habitats, and have large territories and home ranges (Gorman et al., 2006; Crimmins et al., 2009; Kruuk, 2011). Because of these factors, collecting data can be expensive and labor-intensive (Kruuk, 2011). Though surveying otters is difficult, it can be of vital importance to monitor otter populations because otters are considered ecological indicators, with their presence or absence reflecting the health of a wetland (Khan et al., 2010). *Lutrogale perspicillata* (smooth-coated otter) is distributed throughout South and South-east Asia and is found in a wide variety of habitat types that include lowland riverine systems, mangrove forests along coast and estuaries, and freshwater systems which include large rivers, lakes, streams, reservoirs, canals, and flooded agricultural fields (Houghton, 1987; Hussain and Choudhury, 1997; Anoop and Hussain, 2004; Shenoy et al., 2006; de Silva et al., 2015; Gomez et al., 2017). *Lutrogale perspicillata* is protected in India under Schedule I & II of the Indian Wildlife (Protection) Act of 1972 and is listed in Appendix II of CITES which prohibits its trade, resulting in it being a protected species in almost all countries within its range (Gomez et al., 2017). The IUCN Red List categorizes *L. perspicillata* as vulnerable due to an inferred population decline caused by habitat loss and exploitation (de Silva et al., 2015). Loss of habitat caused by anthropogenic factors such as large-scale hydroelectric projects, reclamation of wetlands for settlements, agriculture and infrastructure development, reduction of prey biomass, and poaching are some of the factors that are responsible for the decline in population sizes across most of their range (Houghton, 1987; Hussain and Choudhury, 1997; Khan et al., 2010; Acharya and Lamsal, 2011).

Though *L. perspicillata* are found in a variety of habitat types, one of the most ecologically important ecosystems they reside in are mangroves. Mangroves are highly specialized ecosystems characterized by salt-resistant plants growing in intertidal areas along sheltered seacoasts and estuaries in tropical and subtropical regions. They provide a variety of goods and services to flora and fauna, including humans, both directly and indirectly. Mangroves protect and stabilize coastlines, enrich coastal waters, yield commercial forest products, and support coastal fisheries, making them a tremendous benefit to humans (Kathiresan and Bingham, 2001). Despite these ecological and economic services, mangroves globally have decreased between 0.16 and 0.39% per year from 2000 to 2012 (Hamilton and Casey, 2016). Mangroves in India are spread over an area of 4,921 km², which represents 3.3% of the global mangrove vegetation. In contrast to the global trend, the extent of mangroves in India increased up to 875 km² during 1987-2017 (Ragavan and Mandal, 2018). However, despite this overall expansion of mangroves in India, the biodiversity of mangroves has declined in many regions. On the east coast of India, the extent of the mangrove species *Heritiera fomes*, is estimated to have declined by 76% since 1959 and approximately 70% of the remaining *Heritiera* trees surveyed were affected by the 'top dying' disease (Ragavan and Mandal, 2018).

The loss of habitat and vulnerable status highlight the importance of accurate detection for an ecological indicating species such as *L. perspicillata*. We piloted an eDNA detection method to help fill in the gaps about this elusive species. Previous studies have used eDNA detection methods for otter species in freshwater ecosystems (Thomsen et al., 2012; Padgett-Stewart et al., 2015) and to evaluate microbial organisms in mangrove sediments (Andreote et al., 2012). However, this is the first

study we are aware of evaluating mammal species detection using eDNA within a mangrove ecosystem.

METHODS

Study Area

Our study was conducted in an area of mangrove habitats along the Mandovi-Zuari estuarine complex on Chorão Island in Goa, India (Fig. 1). Goa, located on the west coast of India, is the smallest state in the country (3,702 sq. km) and has a wide range of ecosystems and habitats including forests, alluvial plains, coasts, rivers, estuaries, mangroves, and wetlands. This area is under the influence of both the marine biome of the Arabian sea and the terrestrial forest biome of the Western Ghats, leading to a high level of biodiversity (Singh and Chaturvedi, 2017). Of Goa's 37,000ha land area, 500 ha is comprised of mangrove forest; a sizable portion of this (178 ha) is found on the island of Chorão. Today, the mangroves have grown to all parts of the island and *L. perspicillata* are found throughout the island's human-dominated landscapes (K. Fernandez, personal communication, 5 September 2017).

Figure 1. Chorão Island, Goa, India where aquatic environmental DNA samples were collected for *Lutrogale perspicillata* from October 2018 to January 2019. Imagery provided by Esri, DeLorme, HERE, MapmyIndia.

Sample Collection

We collected 30 water samples from 6 sites around Chorão Island from areas of known *L. perspicillata* presence. Camera traps (Bushnell 6MP Trophy Cam Essential Trail Camera; Bushnell, Overland Park, KS) were placed opportunistically and fixed to a tree or sturdy surface angled towards location of known otter presence (e.g., latrine, den) within the boundary of Chorão Island to determine the time between otter presence and sample collection. Camera traps were triggered by a passive infrared sensor with an LED (Light-Emitting Diode) illuminator. In addition to camera trap data (video documentation of the exact time of otter presence at three sampling locations), positive otter presence was determined from signs (footprints, scat (spraint), tail drag marks, grooming sites, and den sightings). Samples were taken as close to otter presence time and location as possible. If not directly observed, otter presence was determined by the

amount of degradation to the sign with consideration of the weather conditions (temperature, humidity, wind, precipitation, and hours of direct sunlight). The age of the sign was defined into three age categories: 1 - 24 hrs, 24 hrs - 7 days, and > 7 days. A subset of samples was collected at high and low tides to determine if tidal movement within the mangroves affected detection.

We collected 1L of surface water using clean gloves and a single-use Whirl-pak bag (Nasco, Madison, WI, USA). We manually filtered the sample immediately on-site using a hand-powered vacuum pump through 0.45-µm cellulose nitrate filter membrane in a single-use filter funnel (Sterlitech, Inc., Auburn, WA, USA) as outlined by Laramie et al. (2015). Filters were removed with a freshly gloved hand or forceps that had been decontaminated in 50% bleach solution prior to use and stored with silica beads until DNA extraction. A field negative was collected at each sampling location by filtering 1L of distilled water to test for contamination. Collection materials not in contact with the sample (vacuum flask, rubber stopper, silicone tubing, vacuum hand pump, and boots) were cleaned in a 10% bleach solution to prevent DNA contamination between sampling events. We attempted to collect environmental parameters such as temperature, pH, and salinity using the Eutech Instruments multi-parameter tester 35 series (Oakton Instruments, Vernon Hills, IL, USA). The tester had multiple malfunctions and was replaced with the Aquasol multi-parameter handheld meter (Rakiro Biotech Systems Private Limited, Navi Mumbai, India), which also immediately developed malfunctions and was unable to provide accurate readings.

Assay Development

We developed and validated a species-specific quantitative PCR (qPCR) assay (primers and probe) for *L. perspicillata* (Table 1) based on previously published cytochrome b sequences (Koepfli et al., 2008; Omer et al., 2012; Moretti et al., 2017) obtained through GenBank (NCBI) and processed using Primer Express 3.0.1 (Applied Biosystems, Foster City, CA, USA). To validate the assay for specificity *in silico*, we used PrimerBlast (Ye et al., 2012). We then validated the assay against filtered water from fecal samples from non-target species (small-clawed otter (*Aonyx cinereus)* and brown palm civet (*Paradoxurus jerdoni)*) to confirm there was no cross-amplification.

Table 1. Primer and probe sequences developed and validated for *Lutrogale perspicillata* assay.

Primer/Probe	Sequence
LUPEF	CCTACTTCTGGYCCTAGTACTAATAACC
LUPER	GGCGRGGGTGTAGTTGTC
LUPEProbe	6FAM-AGGTCTGGGGAGAATAGTACT-MGBNFQ

DNA Extraction and Quantitative PCR Analysis

We extracted DNA from filter membranes using the QIAshredder/ Qiagen DNeasy Blood and Tissue DNA extraction method (Qiagen, Germantown, MD, USA; Goldberg et al. 2011) in a limited access clean room where no high-quality DNA extraction or PCR product has been handled. We analyzed each sample in triplicate and recorded a zero detection when all reactions showed no amplification and a positive detection if all replicates tested positive. If sample results were mixed, the sample was retested in triplicate and considered a positive if one or more replicates amplified on the second round. Reactions consisted of 1X TaqMan Environmental PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and 0.4 µM of each primer and probe and were analyzed using a Bio-Rad CFX96 Real-Time System (Bio-Rad, Hercules,

California, USA). The volume of each reaction was 15 µl, including 3 µl of sample. PCR cycling began with 10 minutes at 95 °C followed by 45 cycles of 95 °C for 15 s and 60 °C for 60s. All reactions included an internal positive control (Thermo Fisher Scientific, Waltham, MA, USA) to test for inhibition, all extraction sets included an extraction negative, and all reaction plates included a negative and positive control (IPC, Thermo Fisher Scientific, Waltham, MA, USA). All positive controls tested positive, and all negative controls tested negative for *L. perspicillata.*

RESULTS

Environmental DNA detection of *Lutrogale perspicillata*

The assay we developed detected eDNA of smooth-coated otters in 14 of the 30 total samples collected. Non-target samples and all negative control samples tested negative. One of our five field negative samples, however, tested positive for the presence of *L. perspicillata* DNA. The field negative sample that tested positive was taken approximately 5 hours after a sample that did detect *L. perspicillata* and taken immediately before a sample that did not detect *L. perspicillata*. However, the glove that was used to remove the filter was not fresh, contrary to field protocols. Supplies had run out and care was taken to try and avoid touching the filter with parts of the glove that had been previously used but this is the likely source of contamination. Positive detections occurred within seven days of otter presence, with a higher proportion of positive detections in samples collected within 24 hours of otter presence (Figure 2). All sites where otters were observed within an hour of sample collection tested positive for eDNA detection, except for one where the otter was >100 m away, with detection rate decreasing to 0.50 between 1 and 24 hours.

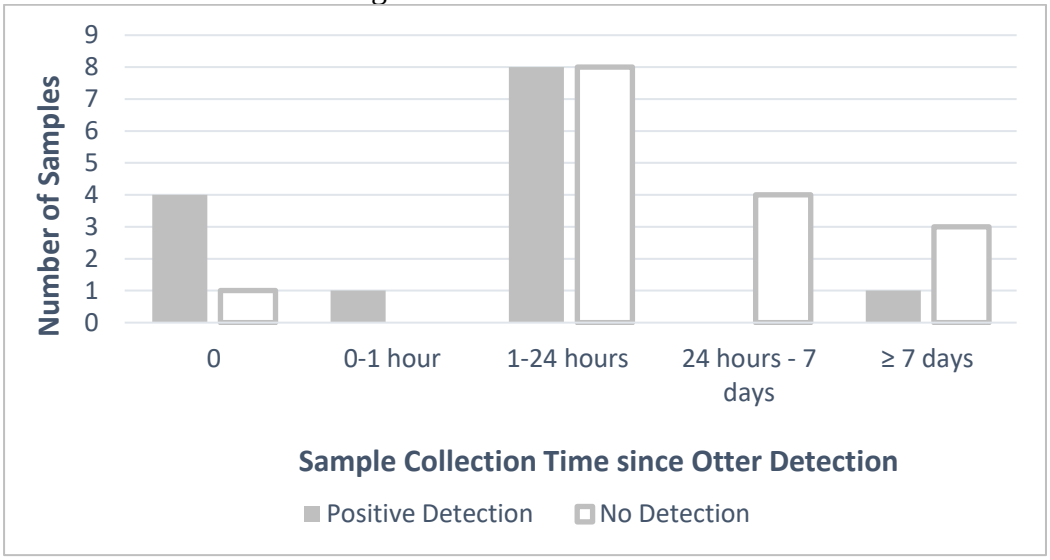

Figure 2. Number of eDNA samples for Lutrogale perspicillata presence, collected around Chorão Island, Goa from various times between October 2018 and January 2019, showing detection or no detection.
* At time 0, otter(s) were actively swimming in water during sampling

The one positive detection of *L. perspicillata* occurrence in a sample collected more than 7 days after otter observations was at an active otter den site. The last recorded footage of otters at the site occurred 11 days prior to sampling and signs such as scat were noted to be 7 days or older in age; however, the camera malfunctioned after that. At the time of sampling, the den had collapsed due to construction in the area and appeared to be abandoned by the otters. When eDNA sampling first occurred at this site, a fishing dam just upstream was under construction, allowing for a more

dramatic tidal movement within the area. When the sample was collected, the dam had been completed and restricted the flow of water to the site, leaving a small amount of water near the den.

Environmental DNA Detection of Smooth-coated Otters in Relation to Tidal Movement

We collected 16 samples at high tide and 14 samples at low tide; we did not find evidence for a difference in detection between tides, (χ^2_1 = 0.12, P=0.73; Figure 3). However, a sample taken at high tide within 24 hours of otter presence tested positive; when the same location was sampled roughly 5 hours later at low tide, the sample showed no detection.

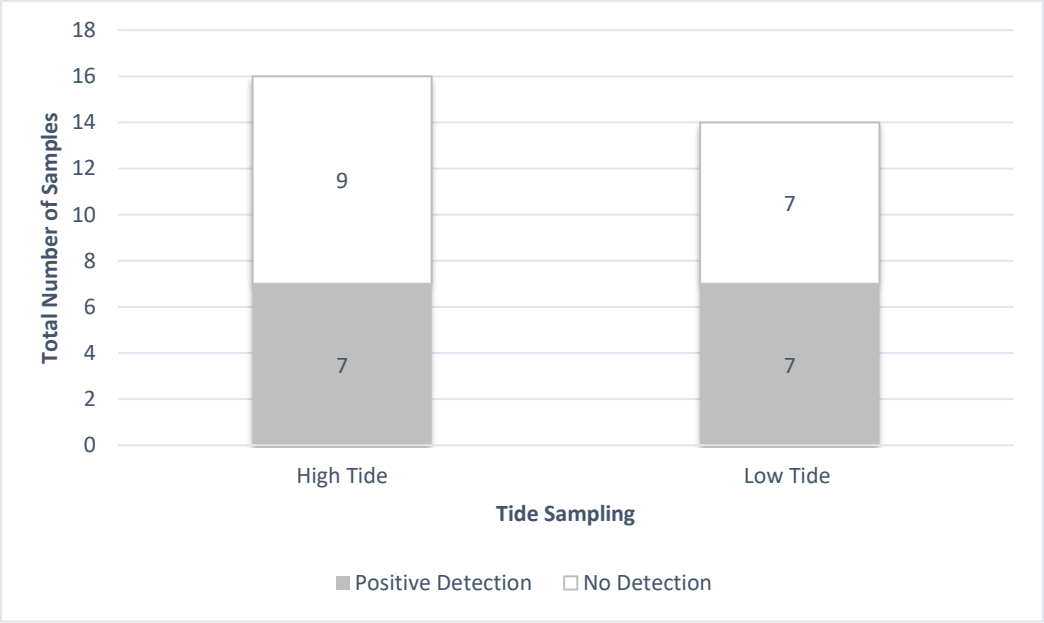

Figure 3. Number of eDNA samples collected for the detection of *L. perspicillata* between October 2018 and January 2019 around Chorão Island, Goa, India at low and high tide showing detection or no detection.

DISCUSSION

We successfully detected *L. perspicillata* in mangrove systems in India using aquatic eDNA methods. The assay we developed was effective and species-specific. Our results indicate that the probability of detecting *L. perspicillata* in mangrove ecosystems was high in areas they had recently visited (<24 hours) and that this detection rate decreased with time. We also found that *L. perspicillata* can be detected at both low and high tides.

Determining the location or presence of a species in a system from aquatic environmental DNA must consider the transport of DNA within the system. Environmental DNA has been shown to travel long distances (up to 12km) in large rivers and much shorter distances in small streams (Deiner et al., 2014; Jane et al., 2015; Deiner et al., 2016; Shogren et al., 2017). The travel and detectability of eDNA are influenced by the physical structure and velocity of a stream (Jane et al., 2015; Fremier et al., 2019). For example, eDNA can be retained in substrate through adsorption to biofilms or other streambed surfaces, resulting in a decrease in eDNA concentration downstream from its source (Jerde et al., 2016; Fremier et al., 2019). Although the travel of eDNA in streams has received some research attention, the movement of eDNA in coastal environments is not as well understood. Studies have shown that

nearshore eDNA detection from benthic and planktonic organismal communities (Kelly et al., 2018) and marine fishes (Lafferty et al., 2021) was not influenced by tidal flow. Not all coastal environments are equally affected by tidal flow, however, and these studies focused only on fjord and atoll ecosystems.

Because coastal ecosystems are subject to considerable effects from water movement, tropical mangrove ecosystems typically experience considerable water movement from tidal flow, conditions to which mangroves are well adapted (Mazda and Wolanski, 2009). The effects of this water flow on eDNA in mangrove ecosystems, however, is not well known, and has not yet been adequately studied. Understanding the movement of DNA in mangroves is particularly essential to determining the utility of eDNA to detect species that inhabit this ecologically important ecosystem. Some organisms of research interest in mangroves are semi-aquatic by necessity (Ong et al., 2007; Ansari et al., 2014) because mangrove ecosystems are often dry during low tide and inundated at high tide. Since semi-aquatic organisms do not spend their entire life cycle in water, successfully detecting them with eDNA techniques in mangrove ecosystems necessitates understanding ecosystem-specific impacts on DNA movement to maximize detectability. Further research is thus needed to establish this baseline understanding of DNA movement in mangrove ecosystems.

In addition to water movement, DNA can be broken down by biotic and abiotic factors such as extracellular enzymes, high temperatures, and UV radiation, all of which could affect detectability. To determine if non-detection results could be caused by abiotic factors, environmental measurements such as temperature, pH, salinity, and sun exposure should be collected at the time of sampling (Strickler et al., 2015). We attempted to collect water quality variables but were not successful due to equipment malfunction. Thus, we could not determine if our non-detection results were due to differences in environmental variables between sampling times. To eliminate this potential problem, we recommend that biologists use new equipment that is tested in the field prior to sampling.

Environmental DNA is a sensitive tool that can produce false positives if measures are not taken to decrease contamination. Although our reusable equipment that was not in contact with the sample (vacuum flask, silicone tubing, rubber stopper, boots, forceps, and vacuum pump), was cleaned in a 10% bleach solution, and single use disposable equipment (Whirl-Pak (Nasco, Madison, WI, USA), nitrile gloves, and filter funnel) was used at each sampling, our contaminated negative control sample highlights how difficult it can be to eliminate contamination in the field. As equipment was cleaned between samplings the sample taken after the field negative resulted in a non-detection, this positive result is likely due to contamination in the reused glove. Early in the study, we had difficulties with our square tipped forceps tearing filters upon removal. To avoid tearing, we resorted to removing the filters with a gloved hand but ran out of supplies and were not able to obtain more gloves by the time sampling needed to be completed. To avoid this, we recommend that consideration is taken in planning to have more supplies than needed, especially when working where supplies are difficult to replenish.

CONCLUSION

This study demonstrates that *L. perspicillata* can be detected within the mangrove ecosystems of Goa using environmental DNA. With this information and the developed assay, biologists have a clear path to monitor the distribution of *L. perspicillata* using eDNA throughout Goa and in other mangrove ecosystems.

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RESUME

ÉTUDE DE LA RÉPARTITION DE LA LOUTRE À PELAGE LISSE *(Lutrogale perspicillata*) À L'AIDE DE L'ADN ENVIRONNEMENTAL : RÉSULTATS PRÉLIMINAIRES

L'analyse de l'ADN environnemental (ADNe) peut être un outil efficace pour détecter la présence d'organismes indécelables ou en faible densité. Bien que cette technique ait été utilisée dans de nombreux écosystèmes, elle n'a pas encore été appliquée dans les écosystèmes de mangrove pour détecter les mammifères aquatiques. Les conditions environnementales extrêmes (chaleur, salinité, turbidité) constituent des défis pour la détection d'espèces rares à l'aide de l'ADNe dans les écosystèmes de type mangrove. Nous avons mené une étude pilote pour tester la sensibilité des méthodes d'ADNe afin de détecter la loutre à pelage lisse (*Lutrogale perspicillata* ; classée vulnérable par l'IUCN) dans les écosystèmes de mangrove en Inde. Cette espèce peut être difficile à suivre en raison de son caractère insaisissable et des défis liés au travail dans ces écosystèmes complexes. Pendant 11 semaines, nous avons prélevé 30 échantillons d'eau filtrés sur place, là où des indices de présence de *L. perspicillata* ont été observés autour de l'île de Chorão à Goa, en Inde. Nous avons conçu et validé un test PCR quantitatif basé sur une sonde adaptée à l'espèce et l'avons utilisée pour détecter l'ADN de *L. perspicillata* dans les échantillons filtrés. Nous avons constaté que notre test était efficace pour détecter *L. perspicillata* dans l'écosystème mangrove de Goa. Nos résultats montrent que la probabilité de détection diminue avec le temps et que la détection est possible à la fois à marée haute et à marée basse. Cette méthode pourrait donc fournir un moyen sensible et efficace de détection des espèces semi-aquatiques ou aquatiques indécelables dans les écosystèmes des mangroves.

RESUMEN

INVESTIGANDO LA DISTRIBUCIÓN DE LA NUTRIA LISA *(Lutrogale perspicillata)* UTILIZANDO ADN AMBIENTAL: RESULTADOS PRELIMINARES

El análisis de ADN ambiental (eDNA) puede ser una herramienta útil para detectar la presencia de organismos elusivos o que viven en bajas densidades. Aunque ésta técnica ha sido utilizada en muchos ecosistemas, todavía no ha sido aplicada en ecosistemas de manglar para detectar mamíferos acuáticos. Las condiciones ambientales extremas (calor, salinidad, turbidez) plantean desafíos para la detección de especies raras mediante eDNA en sistemas de manglar. Condujimos un estudio piloto para testear la sensitividad de los métodos con eDNA para detectar a la nutria lisa (*Lutrogale perspicillata*; clasificación de UICN: vulnerable) en ecosistemas de manglar en India.

Esta especie puede ser difícil de monitorear debido a su elusividad y a los desafíos de trabajar en estos sistemas complejos. A lo largo de 11 semanas, colectamos 30 muestras de agua en sitios donde se habían observado signos de *L. perspicillata*, alrededor de la Isla Chorão en Goa, India, filtrando in-situ inmediatamente después de la recolección. Diseñamos y validamos un ensayo cuantitativo de PCR, especie-específico y basado en sondas, para esta especie, y lo usamos para detectar ADN de *L. perspicillata* en las muestras filtradas. Encontramos que nuestro ensayo fue efectivo para detectar *L. perspicillata* en el ecosistema de manglar de Goa. Nuestros resultados muestran que la probabilidad de detección parece disminuir con el tiempo, y que la detección es posible tanto con alta como con baja marea. Este método podría proporcionar una manera sensitiva y eficiente de detectar especies semi-acuáticas ó acuáticas elusivas en los ecosistemas de manglar.