Using environmental DNA to monitor the spatial distribution of the California Tiger Salamander

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Abstract

Global efforts to conserve declining amphibian populations have necessitated the development of rapid, reliable, and targeted survey methods. Environmental DNA (eDNA) surveys offer alternative or complementary methods to traditional amphibian survey techniques. The California Tiger Salamander *Ambystoma californiense* (CTS) is endemic to California where it breeds in vernal pools. In the past 25 years, CTS has faced a 21 percent loss of known occurrences, largely through habitat loss, and is threatened by hybridization with an introduced congener. Protecting and managing remaining CTS populations relies on accurately monitoring changes in their spatial distribution. Current monitoring practices typically employ dip-net surveys, which are time-consuming and prone to false negative errors. To provide a new resource for monitoring and surveying larval CTS, we designed an assay and tested it on water samples collected from 29 vernal pools at two locations in California. We compared eDNA results to contemporaneous dip-net surveying results and found the assay agreed with positive dip-net results in 100% of cases. In several instances we also detected the presence of CTS genetic material in the early spring months before larvae hatched, potentially offering a new, earlier detection option for this imperiled species. This assay provides a valuable, non-invasive molecular tool for monitoring the spatial distribution of CTS in vernal pools.

Keywords: California and Southwest U.S., *Ambystoma californiense*, environmental dna

Received: May 24, 2019; Accepted: March 31, 2020; Published Online Early: April 2020; Published: xxx

Citation: Kieran SR, Hull JM, Finger AJ. 2020. Using environmental DNA to monitor the spatial distribution of the California Tiger Salamander *Ambystoma californiense*. Journal of Fish and Wildlife Management 11(2):xx-xx; e1944-687X. https://doi.org/10.3996/052019-JFWM-041

Introduction

Amphibian declines in California's Great Central Valley mirror global trends (Fisher and Shaffer 1996), leading to local and regional efforts to protect and recover vulnerable species. The California Tiger Salamander *Ambystoma californiense*, (CTS) is endemic to California and faces ongoing threats throughout its range that have led to a 21 percent reduction in known CTS occurrences since 2002 (USFWS 2017). There are three distinct population segments Federally recognized by the U.S. Fish and Wildlife Service: the Central Valley distinct population segment, which is listed as threatened under the US Endangered Species Act (ESA 1973, as amended), and the Sonoma County and Santa Barbara distinct population segments, which are listed as endangered under the same Act (ESA 1973). In the state of California, the species is listed as threatened throughout its range under the California Endangered Species Act (CESA 1973).

A number of specific threats have spurred the protection and recovery of CTS, including habitat destruction and fragmentation, invasive predators, and hybridization with the introduced Barred Tiger Salamander *A. tigrinum mavortium* (USFWS 2017). Larval and breeding adult CTS are threatened by the loss of the vernal pools that serve as their breeding and spawning habitat from November to April; 80-90% of vernal pool habitat has been lost since Spanish settlement (King 1998). These losses are largely due to urbanization, agricultural land conversion and other anthropogenic factors. During the spawning season CTS adults and larvae congregate in vernal pools and ponds and can be readily monitored by visual inspection or dip-netting. Outside of this season, CTS live underground up to 1.86 km from the breeding site (Searcy and Shaffer 2011) making population-level monitoring infeasible. Consequently, CTS monitoring is carried out during wet season using dip-net monitoring or trapping.

Conservation and restoration of amphibian populations rely on ongoing monitoring to provide consistent, accurate data in order to facilitate status reviews and consequent management decisions. Such spatial distribution monitoring tracks the presence/absence of a species in its habitat and changes in its distribution over time (Greenberg et al. 2018). To date, dip-net surveys

are the most common survey method for monitoring the aquatic stage of terrestrial salamanders, though they are not without limitations (Skelly and Richardson 2009). For example, dip-netting causes substantial disturbance to pools, because it is carried out by entering the pool and moving a large D-frame mesh and aluminum net through the water column. This process can disturb CTS habitat through destruction of substrate and can result in direct injury to CTS if larvae become severely entangled in nets or are crushed by surveyors walking through pools (Anderson and Davis 2013). The accuracy of dip-netting has also been called into question. Curtis and Patton (2010) modeled the detection rate of dip-net surveys on *Ambystomatidae* larvae in isolated ponds on the East Coast and found that detection rates varied across species and throughout the field season, but peaked at only 77%. Furthermore, surveyor movement among pools may increase the risk of spreading diseases such as ranavirus and chytridiomycosis between amphibian populations, which is implicated in the decline of more than 500 amphibian species (Greenberg and Palen 2019).

Environmental DNA (eDNA) monitoring is an emerging tool that can complement dip-net monitoring of amphibians and alleviate some of the associated concerns. Environmental DNA monitoring is a survey method in which environmental samples (here, water) are processed for genetic traces of a target species using specially-designed quantitative Polymerase Chain Reaction (qPCR) assays. Throughout the world, eDNA has been successfully used to track and monitor salamander species including the Great Crested Newt *Triturus cristatus* in the UK (Biggs et al. 2015), the endangered Hellbender Salamander *Cryptobranchus alleganiensis* in Pennsylvania (Pitt et al. 2017), and the Idaho Giant Salamander *Dicamptodon aterrimus* (Pilliod et al. 2014), among others (Goldberg et al. 2018; Preißler et al. 2019; Vörös et al. 2017). The method may be particularly appropriate for CTS monitoring in part because dip-net surveys for CTS must be carried out by trained surveyors carrying federal permits. Collecting water from vernal pools for eDNA sampling requires no permits and minimal on-site training, can frequently be carried out without humans entering pools, and can provide highly accurate results. Here, we developed and field tested a qPCR-based eDNA assay for the detection CTS from water samples and examined its potential as a survey method to monitor CTS presence/absence in California's Central Valley vernal pools.

Methods

Assay Development and Optimization

To develop an eDNA assay for monitoring larval CTS, we first obtained representative DNA sequences from CTS and other *Ambystomatidae* covering the entire mitochondrial genome from Genbank (Table S1) and aligned them using the software program MEGA7 (Kumar et al. 2016). To design the qPCR assay, we identified candidate assays on the Cytochrome Oxidase I mitochondrial gene with Primer3Plus (Untergasser et al. 2007) and PrimerQuest (IDT). We then developed several candidate assays with a forward primer, a reverse primer and a fluorophore-labeled DNA probe. Next we checked each set of assays against our aligned sequences for the presence of species-specific single-nucleotide polymorphisms (SNPs). We selected the candidate assay that had the highest number of inter-specific SNPs without compromising optimal reaction kinetics (Table 1). To validate and optimize assays, we ran the assay using tissue-derived CTS DNA taken from adult individuals in the Santa Rosa Plains in Sonoma County. The resulting optimized reaction recipe and thermocycling protocol for each sample was 1X Taqman Environmental DNA Mastermix (Thermo Fisher Scientific), 0.9 µM each primer, 0.15 µM probe, 1X bovine serum albumin (Life Science) and 6 µL eDNA template in a reaction volume of 20 µL with the following thermocycling conditions: an initial denaturing step at 95 ºC for 10 minutes, followed by 45 cycles of 95 ºC for ten seconds, then 56 ºC for one minute. To estimate assay specificity, we performed *in silico* PCR using ecoPCR (Ficetola et al. 2010) with approximately 70,000 available mitochondrial sequences from all *Ambystomatidae* species in Genbank, including the invasive *A. tigrinum mavortium.* There are no natively-occurring *Ambystomatidae* whose ranges are thought to overlap with CTS (USFWS 2017).

Measurement of Limit of Detection and Limit of Quantitation

The Limit of Detection (LOD) is a parameter used to evaluate the sensitivity of qPCR assays; LOD is a measure of the lowest concentration of analyte (in this case, target genetic material) detectable in a qPCR assay and distinguishable from the concentration plateau (Hunter et al. 2016). We used the calculation of the LOD and Limit of Quantitation (LOQ) described by the United States Geological Survey's Ohio Water Microbiology Laboratory (Francy et al. 2017) based on work by Armbruster and Pry (2008). To determine the LOD and LOQ of the assay, we used synthetic double-stranded DNA fragments (gBlock Gene Fragments; IDT) matching the target amplicon. gBlock gene fragments allow for precise measurement of the number of input

DNA copies in each reaction. We produced a standard curve ranging from 900 copies/reaction to 0.6 copies/reaction with the gBlock gene fragments. Each concentration of gBlock standards was replicated eight times and the standard curve included eight no-template controls.

Field Sampling

For the purpose of this study, we define "eDNA sample" as a volume of water collected from a vernal pool, passed through a filter and processed for DNA extraction. A "sampling event" is the process of collecting three replicate water samples, a negative field control sample, and performing a dip-net survey of a single pool at a single visit. We collected our eDNA samples during three wet seasons (January-March in 2016, 2017 and 2018) at two vernal pool complexes regularly monitored for CTS: The Jepson Prairie Preserve in Solano County and the Dutchman Creek Conservation Bank in Merced County (Figure 1).

We sampled pools that ranged in size from 3 m^2 to nearly 6.8 km^2. We collected replicate eDNA samples in each pool by submerging a sterile 1L Nalgene container into the pool by hand with a single-use nitrile glove. Between uses, Nalgene containers were submerged in 10% bleach for 30 minutes, rinsed in DI water and placed under a UV hood for 15 minutes. For pools larger than approximately 500 m^2, the collector wore sterile, single-use boot covers and waded a short distance into the pool to collect one or more of the replicate samples. For pools larger than approximately 10,000 m^2, a single transect of approximately 10,000m^2 was sampled. In total, 29 vernal pools were sampled between one and six times each for 51 total sampling events. To evaluate the rate of false positive detections and ensure the assay did not amplify CTS when not present in a pool, 16 of our sampling events were from pools with no current or historical presence of CTS (Table S2). Because CTS has been carefully tracked and monitored for multiple years at these sites, testing historically CTS-negative pools is a reliable way to monitor for false positives. To ensure that any positive amplifications we found were not the result of contamination, we included sterilized water as negative controls alongside each sample.

Sample Filtration

Filtration occurred concurrently with sample processing whether in the field or in the laboratory. To filter our eDNA samples, water samples were filtered through a 47mm diameter

filter (glass fiber, 0.45µm or 1.2 µm, cellulose nitrate, 0.45 µm) using a peristaltic pump attached to the vacuum flask with silicon tubing. Water was filtered until 500 mL was passed through the filter or the filter clogged. All three replicates were filtered sequentially. Immediately following field sample filtration, a 500 mL negative control was filtered using sterilized Nanopure water. Between samples, all reusable filtration materials (tubing, filter manifolds, etc.) were replaced with clean units. Contaminated gear was stored in sealed zip-top bags until sterilization in the laboratory with 30 minutes in 20% bleach, a triple-rinse with deionized water, and UV-sterilization in a UV hood or crosslinker for 15 minutes.

Dip-net surveys were conducted immediately after eDNA sampling following USFWS survey guidelines (USFWS 2015). The presence or absence of any CTS larvae in dip-nets was recorded. The dip-net surveys did not record the presence of CTS eggs in the pools. When pools were larger than 10,000m^2, a transect was sampled identically to eDNA sample collection.

Sample processing optimization

To developing the most efficacious field protocol, we refined a number of steps across the three years of sampling (see Figure S1 for details). We made all changes incrementally to limit how sampling protocols may confound results. One change included varying filter materials. We used glass fiber filters in 2016 and 2018 and cellulose nitrate filters in 2017. We also varied the how the filters were stored. In 2016, filters were stored dry in silica gel while in 2017 and 2018 we stored filters in the proprietary Qiagen reagent Buffer ATL at room temperature for up to five days before DNA extraction. Both dry and buffer storage for filters are effective methods for storing eDNA filters at room temperature (Renshaw et al. 2015; Spens et al. 2017; Majaneva et al. 2018), and cellulose nitrate and glass fiber are both proven filter materials for eDNA (Goldberg et al. 2016).

Additional refinements of our field protocol included varying filtration to test the efficacy of laboratory filtration methods. To do this, we varied the filtering protocols between field and laboratory filtration during repeat visits to the same pools. When samples were filtered in the lab, unfiltered water samples were transported in a cooler on ice from the field to the lab and stored at 4 ºC until filtration within 6 hours. The costs and benefits of filtering in the lab are still being studied, and the ideal method may be ecosystem- and assay-specific but the temporary storage of

unfiltered water samples up to 24 hours is common practice (Handley et al. 2016; Bastos Gomes et al. 2017; Gingera et al. 2017; Hinlo et al. 2017; Fernández et al. 2018; Takahara et al. 2019).

DNA Purification and Analysis

After filtration, we extracted DNA from filters in a clean laboratory to minimize the potential for contamination. The clean laboratory contained no tissue or high-concentration (tissue-derived or PCR-amplified) DNA from any amphibian or vernal pool species, following the recommendations of Goldberg et al. (2016). Filter-bound DNA was extracted with the DNeasy Blood and Tissue kit (Qiagen) with the following modifications: 20 µL proteinase K was added to the microcentrifuge tubes containing filters stored in Qiagen Buffer ATL. Dry filters were cut into quarters, placed in separate microcentrifuge tubes and inundated with 20 µL proteinase K and 180 µL buffer ATL. Samples were incubated on a rotary incubator overnight (or for at least 12 hours). Any filter material remaining after incubation was discarded and not transferred to the spin column. Finally, two final elutions of 60 µL nanopore water were used with a 15-minute incubation at 56 °C. After extraction, all samples were treated proactively for inhibition using the Zymo One-Step PCR Inhibitor Removal Kit.

We tested extracted DNA for the presence of our target species using the optimized protocols on a qPCR machine (Bio-Rad CFX). We initially tested seven sampling events, with each of the three field replicates run separately. Of these seven sets of three, all field replicate results from a sample were either positive or negative, indicating perfect agreement between field replicates. As a result, in order to preserve samples and reduce reagent cost we tested pooling the DNA extracts from the three field replicates from each sampling event. For this test, our PCR replicates per pooled sample were run and assessed with the following criteria: if one PCR replicate out of four amplified, the sample was re-run. If two or more amplified, it was considered a positive detection. If zero of four amplified, it was considered a non-detection. Each plate also included four no-template controls (water blanks) and eight standardized positive gBlock controls.

Results

Limit of detection and quantitation.

We determined the LOD and LOQ for our eDNA assay from the standard curve we produced using our gBlock gene fragments. We calculated the equation of our standard curve to be $Ct = -2.121[log10(concentration)] + 40.632$. We used this equation to determine LOD and LOQ. Following the USGS definition, we calculated the LOD to be 23 copies/reaction and the LOQ for this assay to be 75 copies/reaction (Table 2).

Field sampling results

Our assay and the dip-net surveys both detected CTS larvae in the same 14 of 51 sampling events, for a 100% agreement rate between positive dip-net results and positive eDNA surveys. In an additional 14 sampling events, our eDNA assay detected CTS when the dip-net surveys did not, suggesting recent presence. In 23 sampling events, neither the dip-net nor the eDNA assay detected CTS, including the 16 sampling events from sites where CTS was historically absent. There were no instances where the dip-nets detected CTS but the eDNA assay did not (Table 3).

Protocol optimization results

We compared our detection results between filtration location protocols and filter materials. In this study the results suggests our assay was robust to sampling methodology, with no evidence that variation in protocol methods affected detection rates (Table S3). Because we had no instances where dip-net surveys detected CTS but our eDNA assay did not, any analysis of the impact of protocol variation on the assay must necessarily be speculative, and we cannot conclude that any combination of filter or filtration location outperformed any other.

Discussion

We developed a new qPCR-based eDNA assay that offers improved detection of the presence of CTS larvae in vernal pools compared to dip net survey methods. This assay gives highly concordant results with traditional dip-net surveys, with all positive dip-net detections also detected by our eDNA assay. The assay was found to perform well even with some variations in field sampling protocols. Our early-season positive results point to further uses for our assay, including detection of reproductive material (gametes) left behind by adults. The CTS eDNA assay presented here offers an effective new tool to monitor Central Valley CTS larvae that could, in the future, be expanded and used in other geographic areas with additional development.

One advantage of eDNA monitoring for CTS is that it minimizes human disturbance of vernal pools while determining presence/absence. For vernal pool sampling, eDNA samples can be collected without entering pools by using commercially-available long-pole samplers or sampling backpacks (Thomas et al. 2018). Environmental DNA sampling can reduce habitat disturbance and may limit disease transmission between sites via human contact with water during dip net sampling. Furthermore, eDNA sample collection can be performed without the need for federal permits or species-specific training.

Our early-season eDNA detections were found in samples collected up to a month before dip-net surveys detected the presence of larval CTS. It is possible that the DNA detected in these was sourced from gametes or embryonic CTS before larval hatch, or it is possibly detecting reproductive material or other DNA shed from breeding adults. Larval hatch occurs 10-28 days after breeding (USFWS 2017), after adult salamanders have left the breeding pool. We had no positive detections for CTS in pools considered historically negative for CTS. Therefore we have no reason to believe that our early season detections are false positives. In addition, the 14 sampling events that were positive with our eDNA assay but negative in dip-net surveys were all in pools that supported populations of CTS larvae later the same year. While reproductive material is abundant in the pools during breeding season, it would likely degrade rapidly in warm weather; elevated temperatures change a number of biotic and abiotic factors known to influence the decay of genetic material (e.g., water pH, microbial community abundance; Eichmiller et al.

2016). Elevated early season temperatures may reduce the abundance (and thus detection) of gametes and produce eDNA false negatives between egg laying and larval emergence, although during this period dip-nets would also detect no CTS larvae. Managers will have to decide when to employ eDNA surveys based on their needs and questions.

Our assay is designed to detect a locus on the maternally-inherited mitochondrial DNA of a CTS salamander. We used mitochondrial DNA because of its high copy number relative to nuclear DNA, which increases the likelihood of a detection in an eDNA sample. However due to its maternal inheritance, mitochondrial DNA cannot distinguish between a pure CTS and a hybrid CTS x *Ambystoma tigrinum mavortium* that is maternally CTS. Our assay will not detect hybrids that are paternally CTS.

Management recommendations

For managers wanting an efficient way to monitor the presence/absence CTS without dip-netting, we recommend use of this assay with eDNA sampling in Spring after larval emergence. However it is important to remember that our assay cannot provide data about CTS abundance or health, so dip-netting is still necessary when more than presence/absence data is required, such as count data or larval maturity information. Additional field testing of our assay could introduce potential other uses for this survey methodology, such as its use as an early-season predictive tool for pools that are expected to support CTS larvae later in the year. This could allow managers to take early action to protect these pools. As global amphibian decline has intensified the monitoring and management of salamander populations, environmental DNA has proven to be a successful method for monitoring freshwater amphibians in general and salamanders in particular (Pilliod et al. 2014; Spear et al. 2015; Katano et al. 2017; Preißler et al. 2019). Our assay expands the utility of eDNA to monitoring the threatened CTS, providing managers with an additional highly-accurate method of tracking the spatial distribution of larval CTS in the Central Valley.

Supplemental Materials

Table S1: This table is a record of the mitochondrial genetic sequences used to develop and validate our *Ambystoma californiense* quantitative PCR assay *in silico* using MEGA7 (Kumar et al. 2016) and ecoPCR (Ficetola et al. 2010). The scientific name and common name for each species is given, along with the National Center for Biotechnology Information (NCBI) accession number that can be used to reference the original sequence at https://www.ncbi.nlm.nih.gov. When the original sequence is published, the citation is included in the table. These accession numbers represent whole mitochondrial genome sequences for our target salamander species and a variety of other *Ambystomatidae*. Not all *Ambystomatidae* species were available for comparison. No other *Ambystomatidae* species is known to natively co-occur with *Ambystoma californiense* (USFWS 2017).

Table S2: Record of California Tiger Salamander (CTS) *Ambystoma californiense* environmental DNA (eDNA) and dip-net sampling events undertaken during January-March 2016, 2017 and 2018. This record includes: a unique identifier for each pool (pools that begin with D are located at the Dutchman Creek Conservation Bank in Merced County, CA; pools that begin with J are located at the Jepson Prairie Preserve in Solano County, CA); the latitude and longitude of the vernal pool (decimal degrees) the sampling date; the dip-net sampling results (0 for a non-detection and 1 for a detection); the eDNA assay results (0 for a non-detection and 1 for a detection); the filter type (GF for glass fiber or CN for cellulose nitrate); the filtration protocol (in the field or in the laboratory); the historical status of CTS in that pool; the water volume filtered (per biological replicate and averaged across replicates, in milliliters); and pool area (in square meters).

Table S3: Results of *Ambystoma californiense* eDNA and dip-net surveys broken down by (a) filter type and (b) filtration protocol. Because our assay has perfect agreement with positive dip-net survey results, all considerations about the effect of various protocols on eDNA assay detection rate must be speculative. There are seven cases where there is historical or known presence of CTS in the pool, but neither the dip-net nor the eDNA assay detected CTS. These cases may speculatively represent failures of our assay to detect CTS. Of these, three used a

cellulose nitrate filter while four used glass fiber. Three were filtered in the field and four in the laboratory. We conclude that there is no evidence that protocol variation impacted the detection rate of our assay.

Figure S1: Flowchart of protocol variations for our 51 sampling events ("samples"), each comprising three replicate filters and a negative control filter. In 2016, we used a 0.45µm glass fiber filter with a reusable 47mm filter manifold (Advantec), filtered immediately in the field and stored filters dry in silica gel. In 2017, we used a 0.45µm cellulose nitrate filter (Sterlitech) housed in a single-use filter manifold. We varied our filtration between the field and the lab, and stored filters in Qiagen Buffer ATL. In 2018 we used a 1.2µm glass fiber filter (Whatman), did all filtration in the lab and stored filters in Qiagen Buffer ATL. All reusable equipment (tubing, filter manifolds, flasks) were bleach sterilized in 20% bleach for 30 minutes, triple rinsed and then UV sterilized for 15 minutes between uses.

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Acknowledgements

The findings and conclusions in this article are those of the author(s) and do not necessarily represent the views of the U.S. Fish and Wildlife Service. This project was funded through cooperative agreement R15AC00040 between University of California, Davis, and the United States Fish and Wildlife Service, with additional funding from the University of California Natural Reserve System. The authors would like to thank the land managers who provided access to study sites: the University of California Natural Reserve System and Virginia Boucher at Jepson Prairie Preserve, and Matthew Gausse and Tara Collins with Westervelt Ecological Services at Dutchman Creek Conservation Bank. We would like to thank and acknowledge the two anonymous reviewers and Associate Editor for their thoughtful commentary which greatly improved an earlier version of this manuscript. We would also like to thank Alisha Goodbla, Alyssa Benjamin, Amanda Coen, Kaitlyn McGee, Henry Hwang, Pauline Tran, Jason Bachiero and Daniel Prince for their help collecting samples in the field. We would also like to acknowledge the help of Ravi Nagarajan, Kristy Deiner, Andrea Schreier, Ann Holmes and Daphne Gille, who provided feedback and guidance.

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Figure and Table Captions

Figure Captions

Figure 1: Map of locations of environmental DNA and dip-net surveys carried out to detect the California Tiger Salamander *Ambystoma californiense*. Environmental DNA sample collection at vernal pools on each reserve were immediately followed by dip-net surveys. Sampling was carried out January-March of 2016, 2017 and 2018 at (a) The Jepson Prairie Preserve in Solano County, CA, and (b) Dutchman Creek Conservation Bank in Merced County, CA.

Table Captions

Table 1: Sequences of DNA primers and Taqman quantitative Polymerase Chain Reaction probe used to assay environmental DNA samples for the presence of the California Tiger Salamander *Ambystoma californiense*. Primers and probes were designed using mitochondrial genome sequences obtained from Genbank and selected using Primer3Plus and Primerquest (IDT). The fluorescent Taqman probe employs the commercially-available FAM fluorophore and proprietary Black Hold Quencher (BHQ-1) quencher molecule (IDT).

Table 2: We used synthetic gBlock gene fragments (IDT) and our novel qPCR primers and probe to produce a standard curve and dilution series for *Ambystoma californiense.* We report the target DNA concentration (DNA copies/reaction), number of positive amplifications (of eight replicates), average cycle quantification (Cq) value and the standard deviation of Cq values, which we used to calculate the Limit of Detection (LOD) and Limit of Quantitation (LOQ) scores for the assay. The Cq value is the PCR cycle number at which the signal amplifies beyond the threshold and is considered a real amplification by the qPCR measurement equipment. We additionally plotted the $\log_{10}$(DNA concentration) (copies/reaction) against the Cq value of each replicate to produce a line of regression. We used the slope and intercept of this line to produce an equation used to calculate the LOD and LOQ of the assay.

Table 3: The results of *Ambystoma californiense* (CTS) environmental DNA (eDNA) and dip-net surveys. Sites were defined as historically present (35) or historically absent (16) of CTS**.**

Results compare eDNA samples taken immediately before dip-net sampling at a single vernal pool during a single visit. Dip-net surveys were performed according to USFWS guidelines to identify larval CTS.

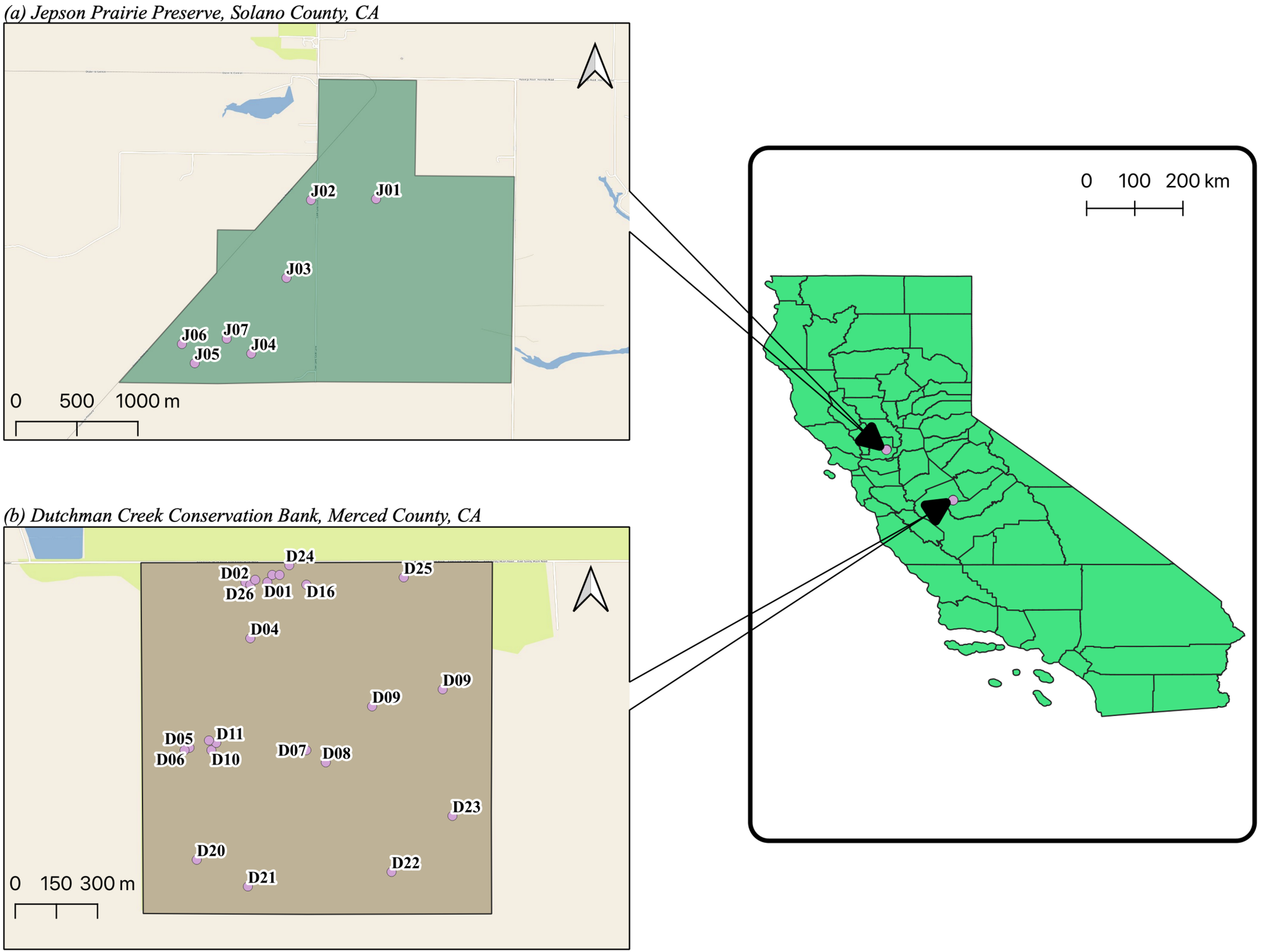
(a) Jepson Prairie Preserve, Solano County, CA
J02
J01
J03
J06
J07
J04
J05
0 500 1000 m
0 100 200 km
(b) Dutchman Creek Conservation Bank, Merced County, CA
D24
D25
D02
D26
D01
D16
D04
D09
D09
D05
D11
D06
D10
D07
D08
D23
D20
D22
D21
0 150 300 m

Table 1

Name	Sequence
Forward Primer (CTSCOIF)	GATCAGTATTAATTACAGCAGTCCTTC
Reverse Primer (CTSCOIR)	GTTTCGATCCGTCAGCAGTAT
Probe (CTSCOI)	/FAM/TCTCTTCCGGTTTTAGCAGCG/BHQ1/

Table 2

Concentration (DNA copies/reaction)	Positive Reactions (of 8)	Mean Cq
900	8	33.79
180.73	8	35.84
90	8	36.84
30	3	40.44
24	8	35.65
18.1	8	41.4
12	8	37.16
6	8	37.75
3	8	39.89
1.8	0	0
0.6	1	40.24
0 (Blank)	0	0

Cq SD
0.145
0.23
0.54
0.766
0.457
4.904
0.409
0.548
1.084
0
0
0

Table 3

CTS Detection Type	Dip-net and eDNA	eDNA Only	Dip-net Only	Neither eDNA nor Dip-net
Historically Present	14	14	0	7
Historically Absent	0	0	0	16