

BRIEF COMMUNICATIONS**Sampling large geographic areas for rare species using environmental DNA: a study of bull trout *Salvelinus confluentus* occupancy in western Montana**

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This study tested the efficacy of environmental DNA (eDNA) sampling to delineate the distribution of bull trout *Salvelinus confluentus* in headwater streams in western Montana, U.S.A. Surveys proved fast, reliable and sensitive: 124 samples were collected across five basins by a single crew in c. 8 days. Results were largely consistent with past electrofishing, but, in a basin where *S. confluentus* were known to be scarce, eDNA samples indicated that *S. confluentus* were more broadly distributed than previously thought.

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The inventory and monitoring of rare species are problematic. Even the most basic data on these species, such as presence or absence, are costly to obtain. If, as is often the case, rare species have special legal protections, monitoring activities and methods may be constrained due to conservation concerns. Environmental DNA (eDNA) sampling infers a species' presence by detecting its genetic material in environmental samples. This technique can yield high detection rates (Pilliod *et al.*, 2013; Rees *et al.*, 2014; Jane *et al.*, 2015), is simple and fast to conduct (Biggs *et al.*, 2015), and is non-invasive, particularly important for monitoring species with legal protections (Janosik & Johnston, 2015). In lotic systems, samples are commonly collected by filtering several litres of water (Goldberg *et al.*, 2011; Jane *et al.*, 2015; Laramie *et al.*, 2015) permitting the rapid collection of large numbers of samples each with a high probability of species detection. Hence, this tool shows great promise for the broad-scale

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monitoring of rare species (Laramie *et al.*, 2015). This study examines eDNA-based detections to locate and delineate bull trout *Salvelinus confluentus* (Suckley 1859) populations in western Montana, U.S.A. *Salvelinus confluentus* are currently listed as threatened under the U.S. Endangered Species Act (USFWS, 1999), are rare but widely distributed and are more difficult to detect through traditional electrofishing than other salmonids in streams (Peterson *et al.*, 2004).

The eDNA sampling for *S. confluentus* was performed in multiple streams in each of the five small first to fourth-order drainages (Fig. 1) which had previously been extensively sampled. In two streams (Little Joe and Albert), *S. confluentus* were known to exist throughout most of the basins. In the headwaters of Lolo Creek, past detections of *S. confluentus* were intermittent, scarce and confined to a single headwater tributary. The other two basins (Big and Twelvemile Creeks) contained *S. confluentus* habitat (Fig. 1; Isaak *et al.*, 2015), but *S. confluentus* had not been reported since 1999 (Montana Fish, Wildlife and Parks, unpubl. data). All sampling was carried out during base flows from August to October 2014, an interval when autumn-spawning adults are expected to home to their natal habitats. Each creek was sampled at roughly 1.5 km intervals within reaches where habitat modelling (Isaak *et al.*, 2015) indicated $\geq 50\%$ probability of juvenile *S. confluentus* presence (Fig. 1). This sampling interval was used because populations of *S. confluentus* were unlikely to persist at smaller spatial scales (Isaak *et al.*, 2015). To evaluate temporal patterns, each site in the headwaters of Lolo Creek was sampled three times at *c.* 1 month intervals [Fig. 1(c)]. At each site, samples were collected by drawing 5 l of stream water through a 47 mm diameter, 1.5 μm pore glass filter (GE Healthcare; www.gehealthcare.com) following the protocol outlined in Carim *et al.* (2015). Samples were preserved by folding the filter in half with forceps and storing it in silica desiccant. Filters were transferred to a -20°C freezer within 1 day of sample collection.

All extractions were performed in a room reserved for extracting non-invasive genetic samples where no PCR products or other sources of high concentration DNA were handled. One half of each filter was extracted using the Qiagen Blood and Tissue DNeasy Kit with QIASHredder columns (QIAGEN; www.qiagen.com; protocol adapted from Goldberg *et al.*, 2011), eluting into 100 μl of sterile Tris -EDTA buffer (Integrated DNA Technologies; www.ididna.com). All extractions were then stored in a -20 or -80°C freezer until quantitative PCR (qPCR) analysis. The other half of each filter was stored at -20°C for future analyses.

A species-specific qPCR assay (BUT1; Wilcox *et al.*, 2013) was used. This assay was obtained from Life Technologies (www.thermofisher.com) and contained a primer set and a FAM-labelled, minor groove-binding, non-fluorescent quencher (MGB-NFQ) probe. Experiments were run in 15 μl volumes with 4 μl of template, 7.5 μl of 2X TaqMan Environmental Mastermix 2.0 (Life Technologies), 0.75 μl of 20X assay mix (primers each at 18 μM , probe at 5 μM), a VIC-labelled exogenous internal amplification control assay and template (Life Technologies TaqMan Exogenous Control Kit) and 0.95 μl deionized H_2O following standard cycling conditions [95°C for 10 min (95°C for 15 s, 60°C for 60 s) \times 45 cycles] on a StepOne Plus Real-time PCR Instrument (Life Technologies). All reactions were run in triplicate, along with triplicate no-template control wells and positive controls on each PCR plate. Because detection rather than quantification was the goal, samples were not paired with standard curves. Amplification in any wells during the process was considered to be a positive detection. Reactions were set up inside of a hood which was irradiated with UV for

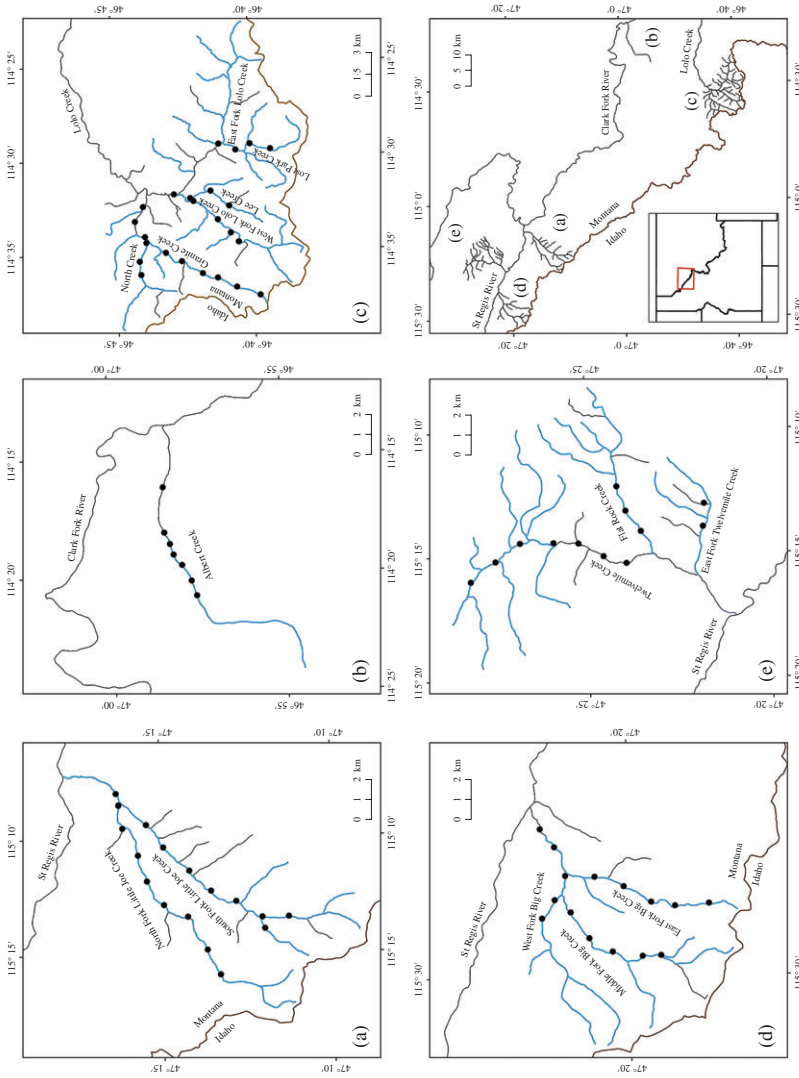


FIG. 1. Maps of the five western Montana drainages surveyed: (a) Little Joe, (b) Albert, (c) Lolo, (d) Big and (e) Twelvemile. Nodes represent environmental DNA (eDNA) sample locations. Based on Isaak *et al.* (2015), reaches are represented as — if they have a predicted probability of $\geq 50\%$ of containing juvenile *Salvelinus confluentus* given the absence of *Salvelinus fontinalis*. —, state boundary.

at least 30 min prior to PCR set-up. If any samples appeared inhibited (as evidence by a >1 cycle-threshold (C_T) shift in the internal amplification control), DNA was extracted from the second half of the filter and DNA from both filter halves was combined and cleaned by running through an inhibitor removal column (Zymo Research; www.zymoresearch.com) and then re-analysed.

Detections of *S. confluentus* by eDNA sampling were compared with those by electrofishing in the interval between 1999 and 2014 (Montana Fish, Wildlife and Parks, unpubl. data). Electrofishing data were limited to the last 15 years because *S. confluentus* were more likely to have undergone range contractions over longer intervals (Eby *et al.*, 2014). The locations of eDNA sampling and electrofishing were not always the same. Because of the downstream distance at which fishes may be detected by using eDNA sampling (Jane *et al.*, 2015), electrofishing and eDNA sampling sites within 750 m were considered comparable. For method comparison, if sites were sampled more than once using either method, the site was considered positive for *S. confluentus* for that method if any sample was positive. Because the eDNA assay was based on a mitochondrial DNA sequence, the assay cannot distinguish between pure *S. confluentus* and *S. confluentus* \times brook trout *Salvelinus fontinalis* (Mitchill 1814) hybrids. Therefore, when comparing methods, detection of hybrids during electrofishing was considered equivalent to detecting *S. confluentus*.

All five drainages had roads that paralleled the valley floor, allowing for rapid eDNA sampling (*c.* 20 min per sample for most sites, including travel between sites). Overall, 124 samples were collected from 76 sites and permitted assessment of *S. confluentus* occupancy across 98 km of streams (Fig. 1) by a single crew in *c.* 8 days. A field crew of one to two people sampled Little Joe (18 sites) and Big Creek (15 sites) in 1 day each, Twelvemile Creek (12 sites) in 2 days (because a closed road required hiking to sites), Albert Creek (seven sites) in 0.5 days and the headwaters of Lolo Creek (24 sites) in 1–1.5 days per sample pass.

Salvelinus confluentus DNA was not detected in any qPCR negative control samples; nor was there evidence of DNA contamination leading to false positive detections of *S. confluentus* in this study. Patterns of detection between eDNA sampling and past electrofishing were generally consistent. Of the 76 eDNA sampling sites, 47 were within 750 m of electrofishing sites. At all paired sites where *S. confluentus* or *S. confluentus* \times *S. fontinalis* hybrids had been captured in electrofishing surveys ($n = 16$ of 47), *S. confluentus* eDNA was also detected. Neither method detected *S. confluentus* at 24 of the 47 paired sites, including all sites in Big and Twelvemile Creeks. *Salvelinus confluentus* eDNA was detected at seven sites where past electrofishing had failed to detect them [one each in Little Joe and Albert Creeks, and five in Lolo Creek; Fig. 2 and Table SI (Supporting Information)]. In Lolo Creek, each site was sampled three times [Fig. 2(e)]. Past electrofishing indicated that *S. confluentus* or *S. confluentus* \times *S. fontinalis* hybrids were consistently located in the middle portion of Granite Creek and occasionally in North Creek, but nowhere else in the drainage [Fig. 2(f)]. *Salvelinus confluentus* eDNA was not only detected in Granite and North Creeks where anticipated by past electrofishing but also in other areas within the basin [Fig. 2(e)].

In the three basins where *S. confluentus* had been detected, the extent of occupied habitat estimated from 3 days of eDNA sampling in 2014 was virtually identical to that based on previous electrofishing. At the lowest site in Albert Creek, *S. confluentus* DNA was detected although this species had not been captured there [Fig. 2(c), (d)]. Whether this is due to the drift of eDNA from an upstream source or from nearby fish is uncertain

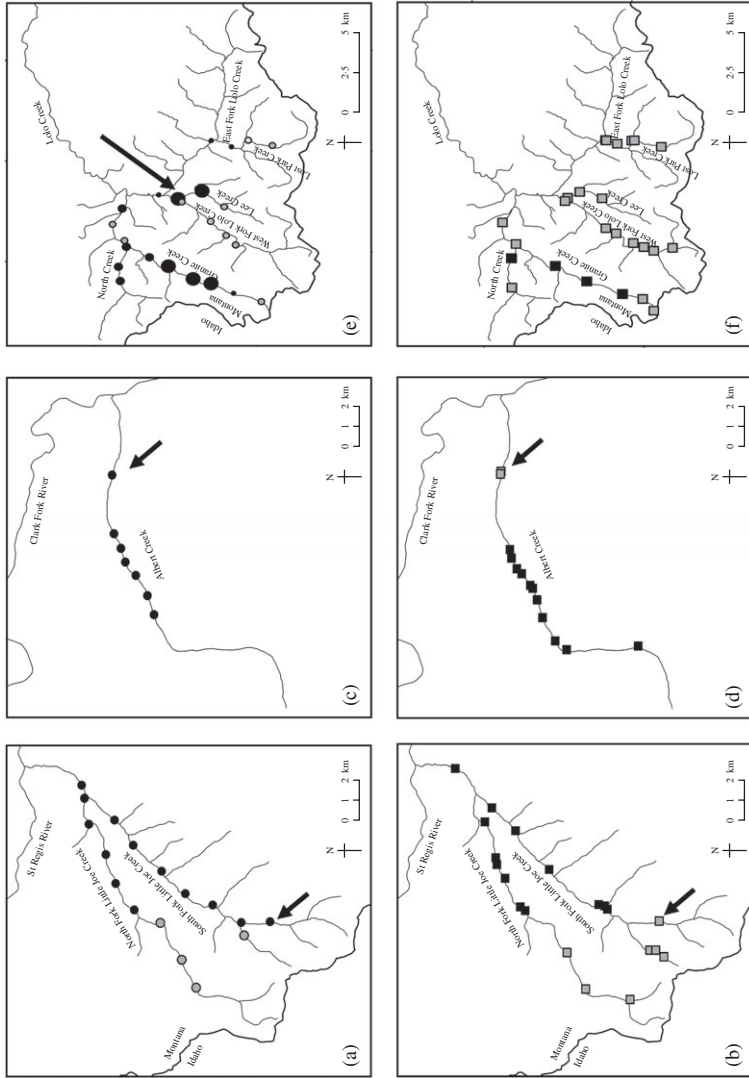


FIG. 2. Environmental DNA (eDNA; circles) and electrofishing (squares) sample results for (a, b) Little Joe, (c, d) Albert and (e, f) Lolo Creeks. ● and ■, positive detections; ○ and □, no detections. eDNA sampling in Lolo Creek was repeated three times. The size of the filled circles in (e) varies with the number of positive detections; the largest symbols indicate that all three samples detected *Salvelinus confluentus*. → in (a–d) indicate sampling locations where eDNA and electrofishing detections do not match. The → in (e) points to a putative small population identified through eDNA sampling.

because detections of eDNA some distance from their source are likely even when fish are rare (Jane *et al.*, 2015). Jane *et al.* (2015), however, only examined eDNA transport over distances up to 240 m below a source. Here, sampling was at 1500 m intervals; in Granite Creek, no eDNA was detected downstream from the population in areas where electrofishing also failed to detect *S. confluentus* [Fig. 2(e)]. At the population margins, detection is expected to vary temporally because range boundaries will fluctuate as fish move over the course of days to weeks and as population size changes over years to generations (Cole *et al.*, 2006). In Big and Twelvemile Creeks, *S. confluentus* were not detected by eDNA sampling or in 28 previous electrofishing samples.

In the upper Lolo Creek, where each site was sampled three times, the wider distribution of *S. confluentus* associated with eDNA sampling when compared with electrofishing [Fig. 2(e), (f)] may be partially explained by the increased sampling intensity; additional electrofishing might have located *S. confluentus* in more stream reaches. Electrofishing effort in this basin was not trivial [Fig. 2(f)] and fish were detected outside of the area indicated by past electrofishing in each of the eDNA sample passes. In the lower Lee Creek, eDNA samples were positive for *S. confluentus* in two adjacent samples across all sample passes [Fig. 2(e)] and may indicate the presence of a small, previously undetected, population. In contrast, the one-time detection of *S. confluentus* at two sites in Lost Park Creek is consistent with ephemeral occupation of this basin. Both sub-adult and adult *S. confluentus* are known to move widely and temporarily occupy various habitats (Swanberg, 1997). Environmental DNA collection is fast, easy and highly sensitive. This unique combination of properties encourages the collection of spatially and temporally dense samples that will detect both temporary use and small, spatially discrete, populations of fish missed by other methods, albeit with greater uncertainty about the downstream bounds of a population.

In summary, detections of *S. confluentus* based on eDNA sampling were comparable to those obtained through electrofishing, but appeared to offer greater reliability. The eDNA inventories were not susceptible to false positive detections and were performed rapidly, often by a single individual (Carim *et al.*, 2015), whereas electrofishing generally requires a greater investment in labour, equipment and time (Dunham *et al.*, 2009). These findings corroborate previous observations that eDNA sampling provides a viable alternative to electrofishing for determining species occupancy (Thomsen *et al.*, 2012a; Pilliod *et al.*, 2013; Janosik & Johnston, 2015). Electrofishing and other sampling methods such as snorkelling and redd counts remain essential for determining population characteristics such as age structure or abundance. At present, eDNA sampling provides no information on fish age, population genetic structure or introgressive hybridization, and DNA copy number is a relatively weak index of abundance (Thomsen *et al.*, 2012b; Takahara *et al.*, 2013; Kelly *et al.*, 2014). Additionally, direct observations to validate eDNA detections may be necessary if false positive detections due to eDNA transport into the system are a concern (*e.g.* by birds and boats; Darling & Mahon, 2011), or to identify the precise downstream extent of the population given uncertainties associated with downstream transport. Much remains to be learned about the factors influencing spatial and temporal variation in eDNA detection rates (Laramie *et al.*, 2015), but the same is true for electrofishing, where knowing detection probability remains an issue (Price & Peterson, 2010). Nonetheless, the successful application of eDNA sampling to detect an array of species in aquatic environments indicates that this method has matured sufficiently to constitute a useful addition to a biologist's toolbox.

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Supporting Information

Supporting Information may be found in the online version of this paper:

Table SI. Results from eDNA sampling for all sites within the three basins where bull trout (*Salvelinus confluentus*) were located (Granite, North, West Fork (WF) Lolo, Lee and Lost Park are all tributaries of the Upper Lolo Creek Basin). Locations on a stream are ordered upstream to downstream with 1 representing the uppermost reach sampled. Each eDNA sample was run in triplicate; 'Wells Amplified' indicates the number of wells in which *S. confluentus* DNA was detected in each analysed sample. In Lolo Creek, three samples were taken at each site. Electrofishing sites were considered to match the location of eDNA sample locations if the coordinates were within 750 m. Electrofishing data consist of the total number of visits to a site, fish sampled and the proportion of sampled fish that were either *S. confluentus* or *S. confluentus* × brook trout (*Salvelinus fontinalis*) hybrids. For comparison, a site was considered positive for *S. confluentus* if *S. confluentus* or hybrids were collected in any of the electrofishing samples or if any of the eDNA samples tested positive. Under the 'Match' column, an '=' indicates that eDNA and electrofishing bull trout detection results matched (e.g. both positive or both negative), a '+' indicates that eDNA located bull trout where electrofishing did not and 'NA' indicates that the eDNA sample location was not within 750 m of a historical electrofishing site

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