Field application of an eDNA assay for the threatened white-clawed crayfish *Austropotamobius pallipes*

Siobhán Atkinson[1,2,3], Jeanette E. L. Carlsson[2,4], Bernard Ball[2,5], Mary Kelly-Quinn[1,6], and Jens Carlsson[2,7]

[1]School of Biology and Environmental Science/Earth Institute, University College Dublin, Dublin, Ireland

[2]Area52 Research Group, School of Biology and Environmental Science/Earth Institute, University College Dublin, Dublin, Ireland

E-mail addresses: [3]siobhan.atkinson@ucdconnect.ie, [4]jeanette.carlsson@ucd.ie, [5]bernie.ball@ucd.ie, [6]mary.kelly-quinn@ucd.ie, [7]jens.carlsson@ucd.ie

Abstract: The white-clawed crayfish *Austropotamobius pallipes* has experienced extensive population declines in its native range in the last century and is now a threatened species. European legislation therefore requires *A. pallipes* populations to be monitored regularly and that Special Areas of Conservation (SACs) be designated in part of their range. These needs cannot be met without knowledge about the distribution of this species. However, common crayfish sampling approaches can be time consuming, expensive, or yield false negatives at low population densities. We therefore present an environmental DNA (eDNA) assay to detect *A. pallipes* in water samples with species-specific primers, a minor groove binding (MGB) probe, and quantitative PCR, based on the mitochondrial cytochrome oxidase I (COI) gene. The results reflected the expected distribution of *A. pallipes*, based on the known locations of this species in the catchment areas sampled. The results of this study indicate that eDNA is an effective tool for detecting *A. pallipes* in a lotic system, and could therefore provide a valuable, non-invasive method for determining its distribution.

Keywords: eDNA, qPCR, conservation, non-invasive, detection, native species, *Austropotamobius pallipes*, Ireland, river

The white-clawed crayfish *Austropotamobius pallipes* is a relatively large, long-lived (>10 y) crustacean that inhabits both rivers and lakes (Reynolds et al. 2010). This species requires alkaline conditions for survival and is commonly found in waterbodies overlying carboniferous limestone bedrock (Lucey and McGarrigle 1987). *Austropotamobius pallipe*s is 1 of the 5 indigenous crayfish species in Europe (Holdich et al. 2009) and was once abundant throughout Europe. However, over the last century, populations of this species have been greatly reduced or gone locally extinct across large parts of its native range (Grandjean et al. 1997). Pollution (Demers and Reynolds 2002, Lyons and Kelly-Quinn 2003), habitat loss, and disease (Matthews and Reynolds 1992) have all contributed to this decline. The crayfish plague, caused by the fungus *Aphanomyces astaci* (Holdich et al. 2009) is of particular concern because *A. pallipes* possesses no resistance to this fungus. Thus, entire populations of *A. pallipes* can be eradicated following an outbreak (Reynolds et al. 2010).

In spite of the crayfish plague, Ireland is considered one of the few remaining strongholds for *A. pallipes* within Europe. One reason for this may be that the invasive signal crayfish (*Pacifastacus leniusculus*), which can cause wide-ranging impacts on freshwater ecosystems and act as a vector for the crayfish plague (Vaeßen and Hollert 2015), has not been reported in Ireland. However, crayfish plague has reached Irish rivers, and large changes in the distribution of *A. pallipes* have been partially attributed to past plague outbreaks (Demers et al. 2005). Additionally, there is currently an outbreak of crayfish plague in Ireland (National Biodiversity Data Center 2018). Thus, there is a pressing need for a non-invasive, rapid, and cost-effective sampling method that can be used to establish the distribution of this species.

Austropotamobius pallipes is protected under the EU Habitats Directive, and is listed under Annexes II and V because of its vital role in freshwater ecosystem functioning and

threatened status (Matthews and Reynolds 1992). Ireland must therefore regularly monitor *A. pallipes* populations and designate Special Areas of Conservation (SACs) for the species under Natura 2000 (Reynolds et al. 2010). Knowledge of the geographic distribution of *A. pallipes* is essential for implementing these conservation measures. Traditional survey methods include night viewing with a strong light, modified quadrat sampling (DiStefano et al. 2003), kick-sampling, baiting traps and enclosures (Byrne and Lynch 1999), snorkeling surveys (Reynolds et al. 2010), and SCUBA diving (Matthews and Reynolds 1992). Many of these methods are effective, but they are also time-consuming, potentially costly, and may be less effective when *A. pallipes* occurs in low abundance. Furthermore, many of these traditional *A. pallipes* surveying methods require a license that allows the holder to catch and disturb *A. pallipes*. Licenses for invasive monitoring procedures are vital for ensuring the protection of *A. pallipes*, however, a non-invasive method that does not require a license would be beneficial.

A promising new tool that can effectively detect species and save significant time in the field (Sigsgaard et al. 2015) is environmental DNA (eDNA) analysis. eDNA is the collective term for DNA that has been shed by organisms and is therefore present freely in the environment. For example, eDNA can occur in the form of mucus, excrement, gametes, or blood (Taberlet et al. 2012, Thomsen and Willerslev 2015). eDNA analysis has effectively detected species that occur in low abundance, such as rare or endangered species (Mächler et al. 2014, Laramie et al. 2015, Sigsgaard et al. 2015, Boothroyd et al. 2016, Carlsson et al. 2017) and invasive alien species (Jerde et al. 2011, Goldberg et al. 2013, Fujiwara et al. 2016). In addition, eDNA may be better able to detect species at low abundance than traditional methods (Dejean et al. 2012, Smart et al. 2015, Dougherty et al. 2016). eDNA assays have been successfully employed for other crayfish species, including *Astacus astacus*, *Astacus leptodactylus*, *P.*

leniusculus (Agersnap et al. 2017, Larson et al. 2017), *Orconectes rusticus* (Dougherty et al. 2016, Larson et al. 2017), *Procambarus clarkii* (Cai et al. 2017), and *Faxonius eupunctus* (Rice et al. 2018).

The aim of this study was to develop a minor groove binding (MGB) probe-based qPCR assay to detect the presence of *A. pallipes* in water samples and to test the reliability of this assay by comparing the results with field distribution data.

METHODS

Study sites and crayfish distribution data

Eight sampling locations within 7 different rivers were selected for field validation of the assay (Table 1). The research presented here was undertaken as part of a larger study assessing the impact of river obstacles on freshwater fauna (e.g. Atlantic Salmon, *Salmo salar*, Atkinson et al. 2018). As part of the broader study, eDNA samples were collected above and below a number of river obstacles (weir, ford crossing, or bridge apron) to determine whether they imposed migratory barriers on *S. salar*, and we used the same samples here. Of the 8 sites sampled, 4 were in rivers that are Special Areas of Conservation (SAC) that include *A. pallipes* as a species of interest. We sampled the Delour and Dinin rivers, which are within the River Barrow and River Nore Special SAC, and the Multeen and Duag rivers, which are within the Lower River Suir SAC. The other 3 rivers that were sampled were the Burren River, Brown's Beck Brook and the Dalligan River. River flow conditions were low at each study site at the time of sampling.

We estimated the current distribution of *A. pallipes* in these rivers with point data provided by the Irish Environmental Protection Agency (EPA). These data were based on

opportunistic field observations (i.e. the EPA was not specifically looking for *A. papilles*) made by EPA staff while undertaking routine biological monitoring between the years 2007 and 2016 (W. Trodd, Irish Environmental Protection Agency, personal communication). Electrofishing surveys were carried out at each site as part of the larger study mentioned previously. At some sites, crayfish were observed during these electrofishing surveys. These additional observations were included in the EPA's *A. pallipes* field observation dataset. We extrapolated each *A. pallipes* observation to the wider subcatchment level. Thus, even if *A. pallipes* was only observed once (between the years 2007 and 2016) in a subcatchment, we assumed that they occurred in the rest of that subcatchment.

eDNA qPCR assay development

We used *A. papilles*-specific primers (forward primer: 5'- GGG TTA GTG GAG AGA GGG GT -3', and reverse primer 5'- AAT CCC CAG ATC CAC AGA CG -3') and a 5'-6-FAM labeled TaqMan® MGB probe (5'- TCA GCT ATT GCC CAC GCA -3') to target a locus within the mitochondrial cytochrome oxidase I (COI) region. We designed these primers in Primer Express 3.0 (Applied Biosystems-Roche, Branchburg, NJ) with Irish *A. pallipes* COI sequences from the National Center for Biotechnology Information (NCBI - http://www.ncbi.nlm.nih.gov/). The total amplicon size was 96 base pairs, including the primers. To verify the in silico species specificity of the *A. pallipes* assay, we combined the probe and primer sequences, and queried them against the NCBI nucleotide database with BLASTn (Basic Local Alignment Search Tool). The BLASTn analyses showed 90% sequence identity for 100% of the assay query only for the target species. The sequence did not align with 100% of the sequence identity in the BLASTn search because there were gaps between the forward and reverse primers and the probe.

However, non-target species showed a maximum of 37% query cover with 100% sequence identity indicating that only the target species *A. pallipes* would bind to all 3 components (forward and reverse primers, probe) of the assay. To verify the in vitro species specificity of the assay, we tested the assay with DNA from the tissue of some freshwater species that co-occur with *A. pallipes*, including Brown Trout (*Salmo trutta*), Sea Lamprey (*Petromyzon marinus*), Atlantic Salmon (*S. salar*), Allis Shad (*Alsoa alosa*), and Twaite Shad (*Alosa fallax*), with conventional PCR techniques followed by visualization of amplicons on agarose. We optimized the qPCR assay with tissue extracted from *A. pallipes*.

eDNA collection, filtration, and extraction

We collected 2 L water samples for eDNA analysis in PET bottles from 2 locations at each study site, above and below the river obstacle. Samples were collected between 45 and 267 m downstream of each obstacle to allow for the accumulation of eDNA from species found potentially only below the obstacle. Upstream eDNA samples were collected between 1 and 193 m upstream of the obstacles (Table 1.). It was not possible to consistently collect samples from the same distance at each location due to health and safety precautions. At each sampling location, 3 replicate water samples were collected, one from each of the left margin, right margin, and the center of the river channel. In addition, we filtered 1 negative field control of double distilled H_2O (ddH_2O) at each location. This procedure resulted in a total of 6 water samples for eDNA analysis and 2 field controls collected per study site. To sterilize the PET bottles before sample collection, we soaked them for 30 min in 50% sodium hypochlorite solution. Prior to collecting the final water samples at each location, the bottles were rinsed at least 5 times with river water to remove any remaining sodium hypochlorite. To further avoid

sample contamination, water samples were taken in an upstream direction before any researchers entered the river. Water samples were filtered through disposable filter funnels on site with a peristaltic pump, through 47-mm glass microfiber filters (1.5 µm). Filters were placed in 2.0 mL Eppendorf tubes and subsequently stored in a cooler box on ice. The filters were frozen at -20˚C in the laboratory ~3-6 h following collection.

All eDNA work was completed in a dedicated Low Copy DNA laboratory to reduce contamination risk. We used a modified CTAB (cetyltrimethylammonium bromide) protocol (Möller et al. 1992) to extract the eDNA. Briefly, ½ of each filter was transferred to a 2.0 mL Eppendorf tube, and the other ½ was archived for future validation or queries. We then added 750 µL CTAB buffer (100 mM Tris-HCL, 20 mM EDTA, 1.4 M NaCl, 2% CTAB) and 7 µL of Proteinase K (20 mg/mL) to the tube with the sample filter. Samples were then vortexed for 10 s, followed by incubation at 56˚C for 2 h. We then added 750 µL of a phenol, chloroform, and isoamyl alcohol (25:25:1 v/v) mixture. We manually mixed the tube contents for 15 s and subsequently centrifuged the tube at 11,000 x g for 20 min. We removed the aqueous solution from the centrifuged tube, and placed it in a new tube that contained 750 µL of a chloroform and isoamyl alcohol (24:1 v/v) mixture. We repeated the manual mixing and centrifugation steps once, and transferred the aqueous solution to a new tube. We precipitated the DNA from the solution by adding 1 volume of isopropanol alcohol, incubating this mixture at -20˚C for 1 h, and centrifuging at 11,000 x g for 20 min. The resulting pellets were washed with 750 µL of 70% ethanol and centrifuged at 11,000 x g for 5 min. We then removed the ethanol, taking care to ensure that the pellet remained in the tube. The tubes containing pellets were dried in a heat block (50˚C, 5 min), and the pellet was then resuspended in 40 µL of molecular-grade ddH_2O.

eDNA concentrations were determined with NanoDrop®-1000, Thermo Scientific, Wilmington, DE.

eDNA assay deployment on field samples

We used an Applied Biosystems ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City, California, USA.) quantitative thermocycler to amplify and quantify eDNA. Briefly, the PCR profile consisted of heating at 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. We generated standard curves from DNA originating from *A. pallipes* tissue extracts (quantified with NanoDrop®-1000, Thermo Scientific, Wilmington, DE). The standard curves served as both a positive control and as a means of calculating the concentration of *A. pallipes* eDNA in each sample. We used seven 10:1 serial dilutions ranging from 2.9 to 2.9 x 10^{-6} ng/µL to make the standard curves. Each qPCR reaction was carried out in a total volume of 30 µL that consisted of 15 µL of TaqMan® Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems, Foster City, CA), 3 µL of each primer (final concentration of 2 µM), 3 µL probe (final concentration of 2 µM), 3 µL eDNA/tissue extracted DNA template, and 3 µL ddH_2O. We generated individual standard curves for each qPCR plate (y = -3.37x + 20.589, efficiency = 98.04 %, R^2 = 0.996 [1], y = -3.442x + 20.392, efficiency = 95.22 %, R^2 = 0.999 [2] and y = -3.441x + 20.723, efficiency = 95.26 %, R^2 = 0.999 [3]). Each plate incorporated 2 no-template controls (NTCs) to identify any contamination that occurred during preparation. Standard curve, field, and control samples were quantified in triplicate (3 technical replicates). For a positive detection to occur at a site, at least 2 of the 3 technical replicates had to contain amplifiable DNA with quantitation cycle (Cq) differences <0.5Cq (to allow for pipetting errors), and be within the detection range of the standard curve. If

1 out of 3 technical replicates showed Cq differences >0.5 Cq, this technical replicate was excluded from the study. In cases when the Cq value of 2 out of 3 technical replicates differed by >0.5 Cq, that specific dilution series or field replicate was removed from further study.

Salmo trutta were present in all rivers, both upstream and downstream of the obstacles, so we used this species as a positive field control to test for the presence of amplifiable eDNA in all samples. To detect the presence of *S. trutta* DNA in our samples, we used the eDNA assay described in Gustavson et al. (2015). Standard curves were not generated, as the purpose of this analysis was to simply establish the presence or absence of *S. trutta* DNA in our samples. Three replicates per location with 1 technical replicate were used.

RESULTS

In this study, we successfully detected *A. pallipes* in silico, in vitro, and in situ. The *A. pallipes* eDNA assay was highly sensitive, the dynamic range of the standard curves was between 19.1 Cq and 39 Cq. The lowest concentration of *A. pallipes* eDNA detected within the range of the standard curve was 0.002 ng/L at Cq 38.2 (average over 3 technical replicates, sd = 0.000059 ng/L). No cross-species amplification occurred. Furthermore, no amplification occurred in the NTCs or field controls. *Salmo trutta* DNA (positive field control) was detected in all the field samples, which confirmed the presence of detectable DNA in them. The eDNA results accurately reflect the *A. pallipes* observation data the EPA collected in the field. *Austropotamobius pallipes* eDNA was positively detected in all subcatchments where EPA staff observed *A. pallipes,* and not detected in any of the subcatchments where *A. pallipes* were not observed (Fig. 1). *Austropotamobius papilles* DNA was detected in all field replicates above and

below the river obstacles within the Burren, Duag, and Multeen rivers. At the Dinin River (Crettyard), we detected *A. pallipes* at 2 out of 3 field replicates above the obstacle, and 3 out of 3 field replicates below the obstacle. At the Dinin River (Castlecomer), we detected *A. pallipes* only in the field replicates above the obstacle (Table 2).

In addition, the eDNA results also reflect the expected distribution of *A. pallipes* based on siliceous dominant bedrock. *Austropotamobius pallipes* usually occurs in waterbodies with calcareous, rather than siliceous, bedrock (Lucey and McGarrigle 1987). The dominant bedrock was either 100% siliceous (Brown's Beck Brook and the Dalligan River) or was 1-25% calcareous (mixed geology, Delour River) at the sites where *A. pallipes* was not detected by either field observation or eDNA.

DISCUSSION

This study showed that eDNA is a reliable tool for detecting *A. pallipes* in river systems. Furthermore, this assay could also be used in lentic systems, such as lakes or reservoirs, where field sampling can be more demanding. eDNA sampling requires only 1 site visit, which involves collecting water samples and filtering them onsite. In contrast, survey methods for monitoring *A. pallipes* can vary in their efficiency depending on the habitat type being surveyed, but they all are usually more involved than what is required for collecting eDNA (Reynolds et al. 2010). Night searches, for example, typically require 2 site visits (for health and safety purposes) and are dependent on water clarity and algal growth (Reynolds et al. 2010). Trapping is time-consuming and requires 2 site visits, whereas snorkeling and hand searches are habitat dependent. Thus, eDNA monitoring may lend itself to surveying more challenging habitats, such

as heavily vegetated lake margins with soft substrates, or locations where health and safety considerations make trapping or night searching impossible or difficult.

In addition to increasing sampling efficiency, eDNA sampling requires substantially less field equipment than traditional sampling methods. Herder et al. (2014) suggested that the eDNA method may help reduce the risk of unintentional pathogen transmission into new areas because of the numerous contamination-prevention precautions that must be taken when sampling. Further, eDNA sampling, and the subsequent disinfection of field equipment is relatively quick. For example, excluding instances where a boat is required, sampling in general only requires the collection of a number of water samples, followed by sterilization of footwear, containers and filter funnels. Snorkeling, SCUBA diving or trapping surveys, in contrast, require time to disinfect and dry equipment (wetsuits, traps etc.) between entering different waterbodies (Reynolds et al. 2010). eDNA sampling could minimize the risk of further spread of crayfish plague by reducing the amount of field equipment required and therefore potential vectors of crayfish plague. eDNA analysis may therefore be particularly suitable for surveys of waterbodies that harbor crayfish plague.

It is surprising that we detected eDNA above but not below the obstacle at the Dinin River (Castlecomer) because eDNA travels downstream in river systems (Deiner and Altermatt 2014, Deiner et al. 2016). This site had the lowest concentration of *A. pallipes* eDNA of all locations (0.002 ng/L), and eDNA amplification occurred in only 1 out of a total of 6 site replicates collected from above and below the obstacle. It is possible that either eDNA concentrations in the other field samples were too low for a positive detection or we obtained false positive results. However, we feel false positives results in this instance are unlikely, as we detected *A. pallipes* in a total of 5 out of 6 field replicates at the Dinin River (Crettyard), which is

located approximately 9 km upstream of the Dinin River (Castlecomer) (Fig. 1). The density or biomass of *A. pallipes*, environmental conditions such as flow, temperature, pH, and sediment, or natural inhibitors such as algae or humic substances, could all have contributed to this lack of amplification in the Dinin River (Castlecomer) (Strickler et al. 2015, Goldberg et al. 2016, Stoeckle et al. 2017) if *A. pallipes* was present there. However, all eDNA samples yielded detectable DNA with the positive field control (e.g. *S. trutta*), so it is unlikely that in this instance the lack of amplification of *A. pallipes* DNA was a result of inhibition of the eDNA samples.

In conclusion, this eDNA assay provides an alternative, rapid method for locating *A. pallipes* in lotic ecosystems that allows for more targeted, efficient surveying strategies. Considering the current crayfish plague outbreak in Ireland (National Biodiversity Data Center 2018) and other countries, there is an urgent need for a non-invasive sampling method for this species. Large scale deployment of this assay would enable managers to rapidly establish the distribution of *A. pallipes* with confidence, which would support the designation of new conservation areas.

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Table 1. Sampling locations from which we collected water to sample *Austropotamobius pallipes* eDNA. Each sampling site had one collection point upstream and one downstream of an obstacle.

River	Obstacle Type	Sampling Location	Distance from Obstacle (m)	Latitude	Longitude	Sampling Date
Burren	Weir	Upstream	1	52.83300	-6.92534	18/07/2017
		Downstream	215	52.83388	-6.92822	18/07/2017
Delour	Weir	Upstream	193	52.97709	-7.54606	19/07/2017
		Downstream	267	52.97957	-7.54100	19/07/2017
Nore (Castlecomer)	Weir	Upstream	11	52.80599	-7.20527	20/07/2017
		Downstream	57	52.80535	-7.20558	20/07/2017
Duag	Weir	Upstream	8	52.27196	-8.01319	21/07/2017
		Downstream	45	52.27174	-8.01248	21/07/2017
Brown's Beck Brook (BBB)	Ford	Upstream	1	53.01627	-6.62311	24/07/2017
		Downstream	60	53.01573	-6.62322	24/07/2017
Dalligan	Weir	Upstream	15	52.11876	-7.51938	25/07/2017

		Downstream	92	52.11862	-7.51803	25/07/2017
Muilteen	Bridge Apron	Upstream	101	52.61304	-8.00872	27/07/2017
		Downstream	34	52.61182	-8.00966	27/07/2017
Nore (Crettyard)	Bridge Apron	Upstream	8	52.84534	-7.12758	28/07/2017
		Downstream	68	52.84584	-7.12891	28/07/2017

Table 2. Mean (± 95% CI) eDNA concentrations (ng/L) from the *A. pallipes* assay at each study river. Concentrations are based on the average eDNA concentration in a minimum of 2 technical replicates and between 1 and 3 field replicates. Confidence intervals (95%) are presented for each location where positive detections occurred in >1 of our field replicates.

River	**Location**	**No. eDNA samples collected**	**No. of positive detections**	**eDNA concentration ng/L (95% CI)**	**Visual observation**
Burren	Upstream	3	3	0.027 (0.01-0.02)	+
	Downstream	3	3	0.02 (0.01-0.04)	
Duag	Upstream	3	3	0.027 (0.005-0.05)	+
	Downstream	3	3	0.157 (0.09-0.23)	
Multeen	Upstream	3	3	0.034 (0.02-0.04)	+
	Downstream	3	3	0.06 (0.04-0.08)	
Dinin (Crettyard)	Upstream	3	2	0.011 (0.004-0.02)	+
	Downstream	3	3	0.006 (0.002-0.01)	
Dinin (Castlecomer)	Upstream	3	1	0.002	+
	Downstream	3	0	0	
Delour	Upstream	3	0	0	-
	Downstream	3	0	0	
Dalligan	Upstream	3	0	0	-
	Downstream	3	0	0	

Brown's Beck Brook (BBB)	Upstream	3	0	0	-
	Downstream	3	0	0	

FIGURE CAPTIONS

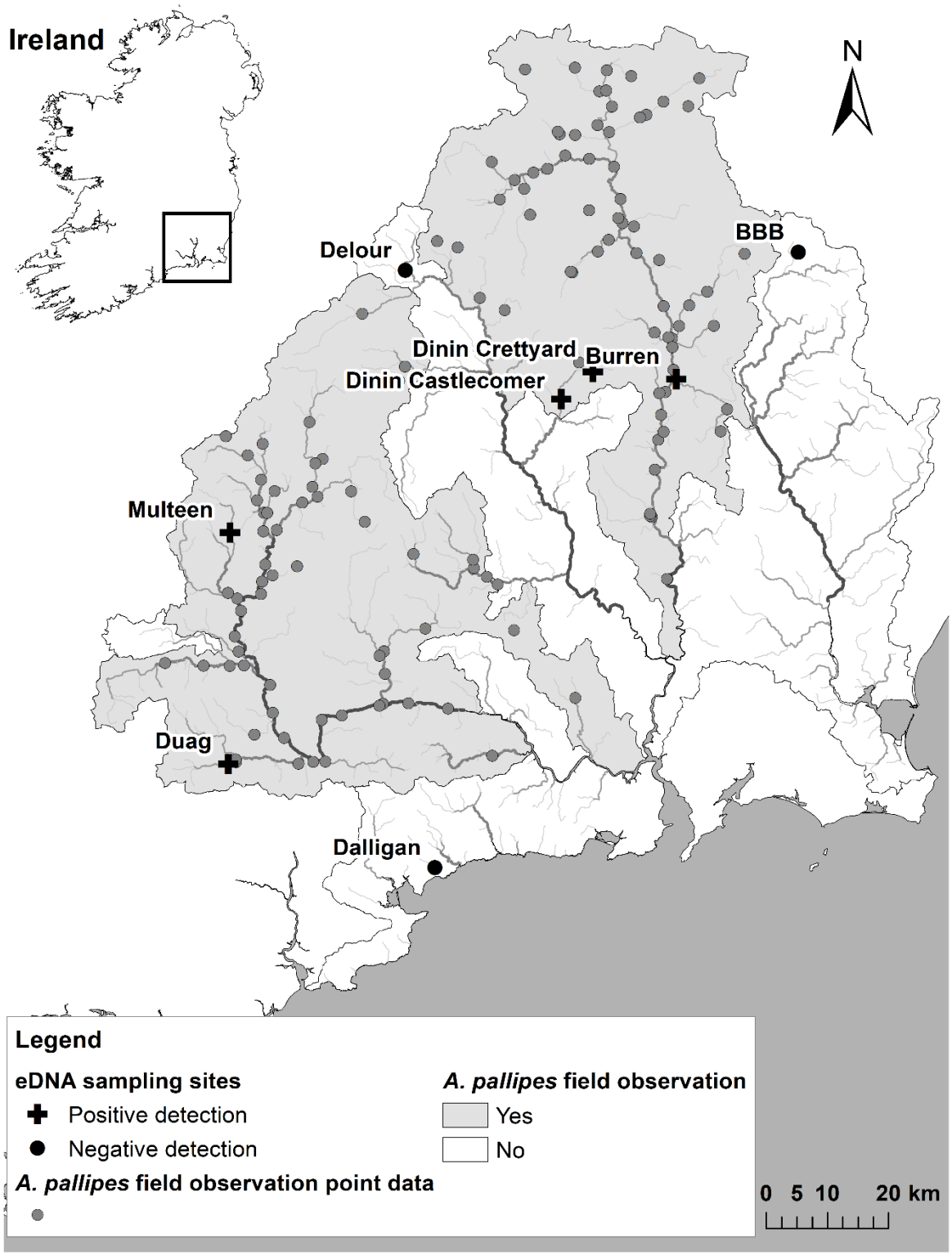

Fig. 1. Map showing the sampling locations for the eDNA analysis. Crosses indicate sites where a positive detection of *A. pallipes* was made in at least 1 field replicate. Circles indicate

sites where *A. pallipes* was not detected in any field replicates. River subcatchments in which field observations of *A. pallipes* were made between the years 2007 and 2016 are highlighted in grey. Grey circles indicate the actual locations where field observations of *A. pallipes* were made.