Field validation of an eDNA assay for the endangered white-clawed crayfish *Austropotamobius pallipes*

Siobhán Atkinson[1,2*], Jeanette E.L. Carlsson[2], Bernard Ball[2], Mary Kelly-Quinn[1], Jens Carlsson[2]

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

*Corresponding author: Siobhán Atkinson, siobhan.atkinson@ucdconnect.ie

Abstract

The white-clawed crayfish *Austropotamobius pallipes* has undergone extensive declines within its native range in the last century. Because of its threatened status, European legislation requires the species to be regularly monitored and that Special Areas of Conservation (SACs) be designated for it. Knowledge on the distribution of this species is vital for addressing these needs. This study presents an environmental (e)DNA assay to detect *A. pallipes* in water samples, based on the mitochondrial cytochrome oxidase I (COI) gene, utilizing species-specific primers, a minor groove binding (MGB) probe and quantitative PCR. The results of this study indicate that eDNA is an effective tool for detecting *A. pallipes* in a lotic system, and could provide a valuable, non-invasive method for determining the distribution of this species.

Keywords: eDNA, qPCR, conservation, non-invasive, detection, native species, river.

Introduction

The white-clawed crayfish *Austropotamobius pallipes* is a relatively large, long-lived (>10 years) crustacean that inhabits both rivers and lakes (Reynolds et al. 2010). They require alkaline conditions for survival and are commonly found in waterbodies overlying carboniferous limestone bedrock (Lucey and McGarrigle 1987). *A. pallipe*s is one of the five indigenous crayfish species in Europe (Holdich et al. 2009). This once abundant species has, however, become greatly reduced or locally extinct across large parts of its native range during the last century (Grandjean et al. 1997). Pollution (Demers and Reynolds 2002, Lyons and Kelly-Quinn 2003), habitat loss and disease (Matthews and Reynolds 1992) have contribution to this decline. Of particular concern is the crayfish plague, caused by the fungus *Aphanomyces astaci* (Holdich et al. 2009). *A. pallipes* possess no resistance to this

fungus, so eradication of entire populations of *A. pallipes* is possible following an outbreak (Reynolds et al. 2010). Despite this, Ireland is considered one of the few remaining strongholds for *A. pallipes* within Europe. One of the reasons for this is that the invasive signal crayfish *Pacifastacus leniusculus,* which can impose wide-ranging impacts on ecosystems and act as a vector for the crayfish plague (Vaeßen and Hollert 2015), has not been reported in Ireland to date. However, the crayfish plague has reached Ireland's rivers, and large changes in the distribution of *A. pallipes* have been attributed, at least in part, to past plague outbreaks (Demers et al. 2005). Additionally, there is at present an outbreak of the crayfish plague in Ireland (National Biodiversity Data Centre 2018), so more than ever there is a need for non-invasive sampling methods that do not have the potential to further spread the crayfish plague.

Because of its vital role in freshwater ecosystem functioning and its threatened status (Matthews and Reynolds 1992), *A. pallipes* is afforded protection under the EU Habitats Directive and is listed under Annexes II and V. This means Ireland is required to regularly monitor *A. pallipes*, and to designate Special Areas of Conservation (SACs) for the species under Natura 2000 (Reynolds et al. 2010). Knowledge on the geographic distribution of *A. pallipes* is essential for implementing these conservation measures. Traditional survey methods include night viewing with a strong torch, modified quadrat samplers (DiStefano et al. 2003), kick-sampling, baited traps and enclosures (Byrne and Lynch 1999) and SCUBA diving (Matthews and Reynolds 1992). While many of these methods are effective, they are time-consuming, potentially costly, and may not be effective when *A. pallipes* occur in low abundance. Furthermore, as white-clawed crayfish are a protected species in Ireland, several surveying methodologies require a licence from the National Parks and Wildlife Service. Certainly, a low-cost, non-invasive and effective methodology for assessing the distribution

57 of *A. pallipes* is warranted. A promising new tool that has been shown to be both effective at
58 detecting species and to save significant time in the field (Sigsgaard et al. 2015) is
59 environmental DNA (eDNA) analysis. Environmental DNA is the collective term for DNA
60 present freely in the environment (Taberlet et al. 2012, Thomsen and Willerslev 2015). The
61 analysis has been successful in detecting species that occur in low abundance such as rare or
62 endangered species (Mächler et al. 2014, Laramie et al. 2015, Sigsgaard et al. 2015,
63 Boothroyd et al. 2016, Carlsson et al. 2017), or invasive alien species (Jerde et al. 2011,
64 Goldberg et al. 2013, Fujiwara et al. 2016). Futhermore, eDNA assays have been successfully
65 deployed for other crayfish species including *Astacus astacus*, *Astacus leptodactylus*, *P.*
66 *leniusculus* (Agersnap et al. 2017, Larson et al. 2017), *Orconectes rusticus* (Dougherty et al.
67 2016, Larson et al. 2017), *Procambarus clarkii* (Cai et al. 2017) and *Faxonius eupunctus* (Rice
68 et al. 2018).

69 The aim of this study was to develop an MGB based qPCR assay to detect the presence of
70 the white-clawed crayfish, *A. pallipes* in environmental samples, and to test the reliability of
71 the assay by comparing the results with field observation data.

72 **Methods**

73 *Study sites and crayfish distribution data*

74 Eight sampling locations within seven different rivers were selected for field validation of the
75 assay. This research was carried out as part of a wider study assessing the impact of river
76 obstacles on Atlantic salmon *Salmo salar*. An obstacle, (weir, ford crossing or bridge apron),
77 was therefore present in the river at each sampling location and eDNA samples were
78 collected both above and below the structure. Out of the eight sites sampled, four were in
79 rivers with SAC status in which *A. pallipes* featured as a species of interest. Study rivers

80 within the River Barrow and River Nore SAC included the Delour and Dinin rivers and study
81 rivers within the Lower River Suir SAC included the Multeen and Duag rivers. Although the
82 Burren River is a tributary of the River Barrow, it does not have SAC status.

83 The presence of *A. pallipes* in these rivers was confirmed using data provided by the Irish
84 Environmental Protection Agency (EPA). These data are point data based on casual field
85 observations made of *A. pallipes* by EPA staff whilst undertaking routine biological
86 monitoring between the years 2007 and 2016 (W. Trodd, Irish Environmental Protection
87 Agency, personal communication). In addition, it was possible at some sites to confirm the
88 presence of crayfish by field observations made while electrofishing for *S. salar* and *S. trutta*.
89 For the purposes of this study, *A. pallipes* observations were extrapolated to the wider
90 subcatchment level. This meant that even if only one field observation of *A. pallipes*
91 occurred in a subcatchment, it was assumed that there were likely to be *A. pallipes* in the
92 rest of that subcatchment. This was considered the most meaningful way to present the
93 data, because the EPA were not specifically looking for *A. pallipes* when their observations
94 were made, so there is no certainty that *A. pallipes* did not occur in other parts of the
95 subcatchment.

96 *eDNA qPCR assay development*

97 Species-specific primers (forward primer: 5'- GGG TTA GTG GAG AGA GGG GT -3', and
98 reverse primer 5'- AAT CCC CAG ATC CAC AGA CG -3') and a 5'-6-FAM labelled TaqMan®
99 minor groove binding probe (5'- TCA GCT ATT GCC CAC GCA -3') for *A. pallipes*, which
100 targeted a locus within the mitochondrial cytochrome oxidase I (COI) region, were designed
101 in Primer Express 3.0 (Applied Biosystems-Roche, Branchburg, NJ). Including primers, the
102 total amplicon size was 96 base pairs. To verify the species specificity for the *A. pallipes*

103 assay in silico, probe and primer sequences were queried against the National Centre for
104 Biotechnology Information (NCBI - http://www.ncbi.nlm.nih.gov/) nucleotide database with
105 BLASTn (Basic Local Alignment Search Tool). The assay was tested in vitro with freshwater
106 species including brown trout *S. trutta*, sea lamprey *Petromyzon marinus*, Atlantic salmon *S.*
107 *salar*, allis shad *Alsoa alosa* and twaite shad *Alosa fallax*. The qPCR assay was optimized
108 using tissue extracted from *A. pallipes*.

109 *eDNA collection, filtering and extraction*

110 Water samples for eDNA analysis were collected in sterilized 2 L PET bottles from two
111 locations at each study site, above and below the river obstacle, and filtered on site using a
112 peristaltic pump. To avoid contamination, the water samples were taken in an upstream
113 direction, prior to any individuals entering the river. At each sampling location, three
114 replicate water samples were collected, one from each of the left margin, right margin and
115 the center of the river channel. In addition, one negative field control consisting of ddH_20
116 was also filtered. This resulted in a total number of six water samples for eDNA analysis and
117 two field controls collected per study site. All water samples were filtered through 47 mm
118 glass microfiber filters (1.5 µm). Filters were stored in 2.0 mL Eppendorf tubes and
119 subsequently frozen at -20˚C. To reduce contamination risk, all eDNA work was performed
120 in a dedicated Low Copy DNA laboratory. A modified CTAB (cetyltrimethylammonium
121 bromide) protocol (Möller et al. 1992) was employed for eDNA extractions. Briefly, one-half
122 of the filter was transferred to a 2.0 mL Eppendorf tube. A total volume of 750 µL CTAB
123 buffer (100 mM Tris-HCL, 20 mM EDTA, 1.4 M NaCl, 2% CTAB), and 7 µL of Proteinase K (20
124 mg mL^{-1}) were added to the tube. Samples were then vortexed for 10 seconds followed by
125 incubation at 56˚C for 2 hours, after which 750 µL of phenol/chloroform/isoamyl alcohol

126 (25:25:1 v/v) was added. The contents of the tube were manually mixed for 15 seconds and
127 subsequently centrifuged (11,000 x g, 20 min). A new tube contained 750 µL of
128 chloroform/isoamyl alcohol (24:1 v/v) was prepared, and the aqueous phase was transferred
129 to it. The manual mixing followed by centrifugation steps were repeated, and the aqueous
130 phase was again transferred to a new tube. The DNA was precipitated using one volume of
131 isopropanol alcohol that was added to the aqueous phase and incubated at -20˚C for 1 hour
132 followed by centrifugation (11,000 x g, 20 min). The resulting pellets were washed with 750
133 µL of 70% ethanol and centrifuged (11,000 x g, 5 min). The ethanol was removed, and care
134 was taken to ensure that the pellet remained in the tube. The tubes containing pellets were
135 dried in a heat block (50˚C, 5 min) followed by resuspension in molecular-grade ddH_2O.

136 *eDNA assay deployment*

137 An Applied Biosystems ViiA™ 7 (Life Technologies, Inc., Applied Biosystems, Foster City,
138 California, U.S.A.) quantitative thermocycler was used for eDNA amplification and
139 quantification. Briefly, the PCR profile consisted of 50°C for 2 min and 95°C for 10 min,
140 followed by 40 cycles between 95°C for 15 s and 60°C for 1 min. Standard curves were
141 generated from DNA originating from *A. pallipes* tissue extracts (quantified with NanoDrop®-
142 1000, Thermo Scientific, Wilmington, DE). This served as both a positive control and as a
143 means of calculating the concentration of *A. pallipes* eDNA in each sample. Seven 10:1 serial
144 dilutions ranging from 2.9 ng μL^{-1} to 2.9 x 10^{-6} ng μL^{-1} were used for the standard curve. Each
145 qPCR reaction was carried out in a total volume of 30 µL, consisting of 15 µL of TaqMan®
146 Environmental Master Mix 2.0 (Life Technologies, Applied Biosystems, Foster City, CA), 3 µL
147 of each primer (final concentration of 2 µM), probe (final concentration of 2 µM), 3 µL
148 eDNA/tissue extracted DNA template and 3 µL ddH_2O. Individual standard curves were

149 generated for each qPCR plate (y = -3.37x + 20.589, efficiency = 98.04 %, R^2 = 0.996 (1), y = -
150 3.442x + 20.392, efficiency = 95.22 %, R^2 = 0.999 (2) and y = -3.441x + 20.723, efficiency =
151 95.26 %, R^2 = 0.999 (3)). Each plate had 2 no-template controls (NTCs) to identify any
152 contamination that may have occurred during preparation. Standard curve, field and control
153 samples were quantified in triplicate (3 technical replicates). A positive detection was
154 defined as being when at least 2 out of the 3 technical replicates contained amplifiable DNA
155 with Cq differences not exceeding 0.5, and when the samples were within the detection
156 range of the standard curve. If 1 out of 3 technical replicates showed Cq differences
157 exceeding 0.5, the replicate was excluded from the study. In cases when the Cq value of 2
158 out of 3 technical replicates differed by more than 0.5 Cq, that specific dilution series or field
159 replicate was removed from further study. As *S. trutta* was present in all rivers, both
160 upstream and downstream of the obstacles, this species was used as a positive field control
161 to test for the presence of amplifiable DNA in all samples. The *S. trutta* assay from previously
162 published work (Gustavson et al. 2015) was used for this analysis. Three replicates per
163 location with 1 technical replicate were used.

164 **Results and Discussion**

165 The present study was successful in detecting *A. pallipes* in silico, in vitro and in situ. The
166 assay was highly sensitive and could detect *A. pallipes* eDNA concentrations as low as 0.002
167 ng L^{-1} at Cq 38.2 (average over 3 technical replicates, standard deviation 0.000059 ng L^{-1}). No
168 cross-species amplification occurred when the assay was tested using conventional PCR
169 visualized on agarose. Furthermore, zero amplification occurred in the NTCs and field
170 controls. While all field samples analysed yielded detectable DNA with the positive field
171 control (*S. trutta* assay), this was not the case for the *A. pallipes* assay. *A. pallipes* was only

172 detected in 5 out of 8 sites (Table 1, Fig. 1). Within the Burren, Duag and Multeen rivers,
173 positive detections were observed in all field replicates above and below the river obstacles.
174 At the Dinin (Cretty Yard) site, positive detection was observed in 2 out of 3 field replicates
175 above the obstacle, and 3 out of 3 field replicates below the obstacle. At the Dinin
176 (Castlecomer) site, positive detection of *A. pallipes* occurred in only 1 field replicate, which
177 was located above the obstacle (Table 1). The detection of *A. pallipes* eDNA above the
178 obstacle but not below it was a surprising result. Intuitively, one would expect that *A.*
179 *pallipes* would have been detected below the obstacle also, as eDNA travels downstream in
180 a river system. It is worth noting that it was at this site that the lowest concentration of *A.*
181 *pallipes* eDNA was detected (0.002 ng L^{-1}), and amplification of eDNA occurred in only 1 out
182 of 6 site replicates. It is possible that eDNA concentrations in the other field samples were
183 too low for a positive detection.

184 When compared with the EPA's *A. pallipes* observation data, the eDNA results reflected
185 what was observed in the field. In all subcatchments where *A. pallipes* was observed by EPA
186 staff, the eDNA results revealed a positive detection, and in all subcatchments where *A.*
187 *pallipes* was not observed by EPA staff, the eDNA results revealed a negative detection (Fig.
188 1). Furthermore, it is worth noting that the eDNA results also reflected the expected
189 distribution of *A. pallipes* based on environmental conditions. In all sites where *A. pallipes*
190 was not detected by either field observation or eDNA (Delour, Brown's Beck Brook and the
191 Dalligan), the dominant bedrock was siliceous. *A. pallipes* are known to occur in waterbodies
192 with underlying calcareous bedrock rather than siliceous bedrock (Lucey and McGarrigle
193 1987).

194 It was not possible in this study to ascertain the impact of the river obstacles on *A. pallipes*
195 using eDNA. This was because in most sites, *A. pallipes* were either detected both above and
196 below the obstacle, or as observed at the Dinin (Castlecomer) site, above the obstacle only.
197 However, studies where crayfish have been physically marked or tagged have shown that
198 river obstacles can hinder their upstream migration (Kerby et al. 2005, Bubb et al. 2008,
199 Rosewarne et al. 2013). Environmental DNA assays for diadromous species such as sea
200 lamprey *P. marinus* (Gustavson et al. 2015) or Chinook salmon *Oncorhynchus tshawytscha*
201 (Laramie et al. 2015) are likely to be better indicators for assessing the migratory impact of
202 river obstacles. Alternatively, eDNA could be used for monitoring whether invasive species
203 have moved above the barriers designed to block them (Cowart et al. 2018). Nonetheless,
204 the present research shows that eDNA is a reliable tool for detecting *A. pallipes* in river
205 systems. Furthermore, while the focus of this study was in lotic systems, the assay could be
206 readily deployed in lentic systems such as lakes or reservoirs where field sampling may be
207 more challenging.

208 To conclude, this assay provides an alternative, rapid method for locating *A. pallipes* in both
209 lentic and lotic ecosystems, allowing for more targeted, efficient surveying strategies.
210 Considering the current crayfish plague outbreak in Ireland (National Biodiversity Data
211 Centre 2018), and indeed other countries where it is present, there is an urgent need for a
212 non-invasive sampling methodology that will minimise further spread of the pathogen. Large
213 scale deployment of this assay would enable managers to rapidly establish, with confidence,
214 the distribution of *A. pallipes*, which would support the designation of new conservation
215 areas for the species.

216 **Acknowledgements**

This research was funded by the Irish Environmental Protection Agency (Reconnect project - 2015-W-LS-8). The authors would also like to thank the Irish Environmental Protection Agency for providing the data on *A. pallipes* distributions.

Literature Cited

Agersnap, S., W. B. Larsen, S. W. Knudsen, D. Strand, P. F. Thomsen, M. Hesselsøe, P. B. Mortensen, T. Vrålstad, and P. R. Møller. 2017. Monitoring of noble, signal and narrow-clawed crayfish using environmental DNA from freshwater samples. PLoS ONE 12:1–22.

Boothroyd, M., N. E. Mandrak, M. Fox, and C. C. Wilson. 2016. Environmental DNA (eDNA) detection and habitat occupancy of threatened spotted gar (*Lepisosteus oculatus*). Aquatic Conservation: Marine and Freshwater Ecosystems 26:1107–1119.

Bubb, D. H., T. J. Thom, and M. C. Lucas. 2008. Spatial ecology of the white-clawed crayfish in an upland stream and implications for the conservation of this endangered species. Aquatic Conservation: Marine and Freshwater Ecosystems 18:647–657.

Byrne, C., and J. Lynch. 1999. A sampling strategy for stream populations of white-clawed crayfish, *Austropotamobius pallipes* (Lereboullet)(Crustacea, Astacidae). Biology and Environment: Proceedings of the Royal Irish Academy 99b:89–94.

Cai, W., Z. Ma, C. Yang, L. Wang, W. Wang, G. Zhao, Y. Geng, and D. W. Yu. 2017. Using eDNA to detect the distribution and density of invasive crayfish in the HongheHani rice terrace World Heritage site. PLoS ONE 12:1–13.

Carlsson, J. E. L., D. Egan, P. C. Collins, E. D. Farrell, F. Igoe, and J. Carlsson. 2017. A qPCR MGB probe based eDNA assay for European freshwater pearl mussel (*Margaritifera margaritifera* L.). Aquatic Conservation: Marine and Freshwater Ecosystems 27:1341-1344.

239 Cowart, D. A., K. G. H. Breedveld, M. J. Ellis, J. M. Hull, and E. R. Larson. 2018. Environmental
240 DNA (eDNA) applications for the conservation of imperiled crayfish (Decapoda: Astacidea)
241 through monitoring of invasive species barriers and relocated populations. Journal of
242 Crustacean Biology doi:10.1093/jcbiol/ruy007.

243 Demers, A., J. Lucey, M. L. McGarrigle, and J. D. Reynolds. 2005. The distribution of the
244 white-clawed crayfish, *Austropotamobius pallipes*, in Ireland. Biology & Environment:
245 Proceedings of the Royal Irish Academy 105b:65–69.

246 Demers, A., and J. D. Reynolds. 2002. A survey of the white-clawed crayfish,
247 *Austropotamobius pallipes* (Lereboullet), and of water quality in two catchments of eastern
248 Ireland. Bulletin Francais de Pêche et de Pisciculture 367:729–740.

249 DiStefano, R. J., C. M. Gale, B. A. Wagner, and R. D. Zweifel. 2003. A sampling method to
250 assess lotic crayfish communities. Journal of Crustacean Biology 23:678–690.

251 Dougherty, M. M., E. R. Larson, M. A. Renshaw, C. A. Gantz, S. P. Egan, D. M. Erickson, and D.
252 M. Lodge. 2016. Environmental DNA (eDNA) detects the invasive rusty crayfish *Orconectes*
253 *rusticus* at low abundances. Journal of Applied Ecology 53:722–732.

254 Fujiwara, A., S. Matsuhashi, H. Doi, S. Yamamoto, and T. Minamoto. 2016. Use of
255 environmental DNA to survey the distribution of an invasive submerged plant in ponds.
256 Freshwater Science 35:748–754.

257 Goldberg, C. S., A. Sepulveda, A. Ray, J. Baumgardt, and L. P. Waits. 2013. Environmental
258 DNA as a new method for early detection of New Zealand mudsnails (*Potamopyrgus*
259 *antipodarum*). Freshwater Science 32:792–800.

260 Grandjean, F., C. Souty-Grosset, R. Raimond, and D. M. Holdich. 1997. Geographical variation

261 of mitochondrial DNA between populations of the white-clawed crayfish *Austropotamobius*
262 *pallipes*. Freshwater Biology 37:493–501.

263 Gustavson, M. S., P. C. Collins, J. A. Finarelli, D. Egan, R. Ó. Conchúir, G. D. Wightman, J. J.
264 King, D. T. Gauthier, K. Whelan, J. E. L. Carlsson, and J. Carlsson. 2015. An eDNA assay for
265 Irish *Petromyzon marinus* and *Salmo trutta* and field validation in running water. Journal of
266 Fish Biology 87:1254–1262.

267 Holdich, D. M., J. D. Reynolds, C. Souty-Grosset, and P. J. Sibley. 2009. A review of the ever
268 increasing threat to European crayfish from non-indigenous crayfish species. Knowledge and
269 Management of Aquatic Ecosystems 394-395:11.

270 Jerde, C. L., A. R. Mahon, W. L. Chadderton, and D. M. Lodge. 2011. "Sight-unseen" detection
271 of rare aquatic species using environmental DNA. Conservation Letters 4:150–157.

272 Kerby, J. L., S. P. D. Riley, L. B. Kats, and P. Wilson. 2005. Barriers and flow as limiting factors
273 in the spread of an invasive crayfish (*Procambarus clarkii*) in southern California streams.
274 Biological Conservation 126:402–409.

275 Laramie, M. B., D. S. Pilliod, and C. S. Goldberg. 2015. Characterizing the distribution of an
276 endangered salmonid using environmental DNA analysis. Biological Conservation 183:29–37.

277 Larson, E. R., M. A. Renshaw, C. A. Gantz, J. Umek, S. Chandra, D. M. Lodge, and S. P. Egan.
278 2017. Environmental DNA (eDNA) detects the invasive crayfishes *Orconectes rusticus* and
279 *Pacifastacus leniusculus* in large lakes of North America. Hydrobiologia 800:173–185.

280 Lucey, J., and M. L. McGarrigle. 1987. The distribution of the crayfish *Austropotamobius*
281 *pallipes* (Lereboullet) in Ireland. Irish Fisheries Investigations A29:1–13.

282 Lyons, R., and M. Kelly-Quinn. 2003. An investigation into the disappearance of

Austropotamobius pallipes (Lereboullet) populations in the headwaters of the Nore River, Ireland and the correlation to water quality. Bulletin Français de la Pêche et de la Pisciculture 370-371:139–150.

Mächler, E., K. Deiner, P. Steinmann, and F. Altermatt. 2014. Utility of environmental DNA for monitoring rare and indicator macroinvertebrate species. Freshwater Science 33:1174–1183.

Matthews, M., and J. D. Reynolds. 1992. Ecological impact of crayfish plague in Ireland. Hydrobiologia 234:1–6.

Möller, E. M., G. Bahnweg, H. Sandermann, and H. H. Geiger. 1992. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Research 20:6115–6116.

National Biodiversity Data Centre. 2018. Crayfish plague. (Available from: http://www.biodiversityireland.ie/projects/invasive-species/crayfish-plague/)

Reynolds, J. D., W. O'Connor, C. O'Keeffe, and D. Lynn. 2010. A technical manual for monitoring white-clawed crayfish *Austropotamobius pallipes* in Irish lakes. (Available at: https://www.npws.ie/sites/default/files/publications/pdf/IWM45.pdf)

Rice, C. J., E. R. Larson, and C. A. Taylor. 2018. Environmental DNA detects a rare large river crayfish but with little relation to local abundance. Freshwater Biology 63:443–455.

Rosewarne, P. J., A. T. Piper, R. M. Wright, and A. M. Dunn. 2013. Do low-head riverine structures hinder the spread of invasive crayfish? Case study of signal crayfish (*Pacifastacus leniusculus*) movements at a flow gauging weir. Management of Biological Invasions 4:273–282.

Sigsgaard, E. E., H. Carl, P. R. Møller, and P. F. Thomsen. 2015. Monitoring the near-extinct European weather loach in Denmark based on environmental DNA from water samples. Biological Conservation 183:46–52.

Taberlet, P., E. Coissac, M. Hajibabaei, and L. H. Rieseberg. 2012. Environmental DNA. Molecular Ecology 21:1789–1793.

Thomsen, P. F., and E. Willerslev. 2015. Environmental DNA - An emerging tool in conservation for monitoring past and present biodiversity. Biological Conservation 183:4–18.

Vaeßen, S., and H. Hollert. 2015. Impacts of the North American signal crayfish (*Pacifastacus leniusculus*) on European ecosystems. Environmental Sciences Europe 27:1–6.

316 Table 1. Environmental DNA concentrations (ng L^{-1}) from the *A. pallipes* assay at each study
317 river. Concentrations are based the average eDNA concentration in a minimum of 2 technical
318 replicates and between 1 and 3 field replicates. Confidence intervals (95%) are given, where
319 possible, for each location (upstream or downstream of the river obstacle).

River	Location	No. eDNA samples collected	No. of positive detections	eDNA concentration ng L^{-1} (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 (Cretty Yard)	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 [a]	+
	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

[a] It was not possible to calculate a 95% confidence interval for this site as a positive detection was only detected in one out of three site replicates

Figure Heading

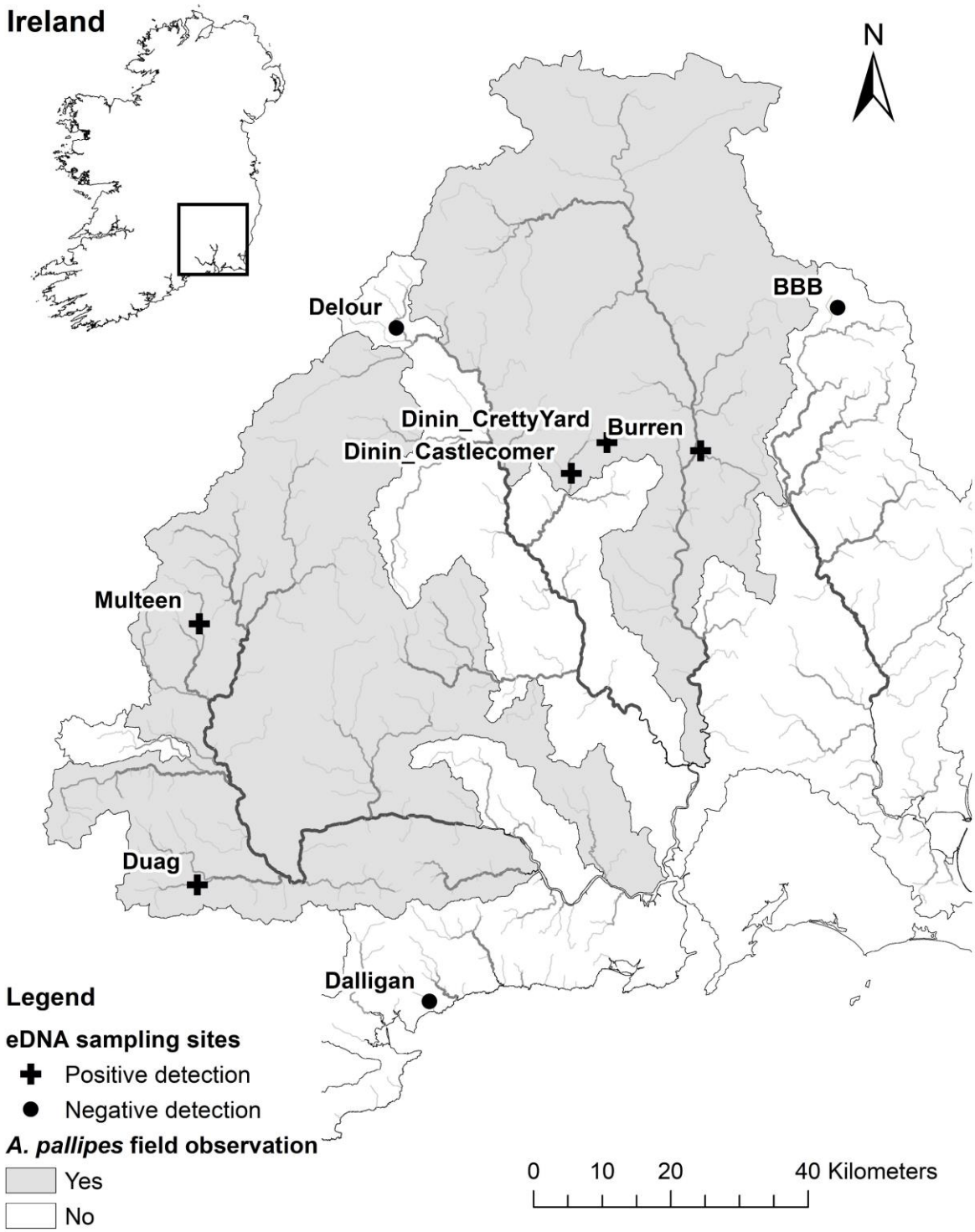

Figure 1. Map showing the sampling locations for the eDNA analysis. Crosses indicate sites where a positive detection of *A. pallipes* was made in at least one field replicate. Circles

328 indicate sites where no positive detection of *A. pallipes* was made in all field replicates. River
329 subcatchments in which field observations of *A. pallipes* were made between the years 2007
330 and 2016 are highlighted in grey.