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**THE GENETIC DIFFERENTIATION OF COMMON TOADS ON UK FARMLAND:  
THE EFFECT OF STRAIGHT-LINE (EUCLIDEAN) DISTANCE AND ISOLATION  
BY BARRIERS IN A HETEROGENOUS ENVIRONMENT**

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LRH: D. Macdonald et al.  
RRH: Common toad genetic distance

30 Abstract. - Genetic differentiation plays an integral role in species persistence.  
31 However, it remains challenging to quantify the ways in which the degree of isolation affects  
32 animal populations. The common toad (*Bufo bufo*) is a species of conservation concern,  
33 particularly in the United Kingdom where populations have undergone large-scale declines.  
34 There are two types of isolation that are relevant to toad population persistence. These are: *i*)  
35 isolation by distance (IBD), where populations that are further apart become more isolated  
36 with time, and *ii*) isolation by barrier (IBB), where the presence of barriers to movement can  
37 isolate populations. Both IBB and IBD are relevant to common toad survival, and thus  
38 conservation, in fragmented landscapes typical of farmlands. We collected common toad  
39 genetic material from eight different breeding ponds amongst the rural farmland landscapes  
40 of Oxfordshire, England to test the effect of IBD and IBB on toad genetic differentiation. We  
41 detected no significant effect of IBD (range 2-22 km between breeding ponds) on common  
42 toad genetic differentiation at this scale. We did however, identify a significant and positive  
43 relationship between IBB and common toad genetic differentiation. Breeding populations  
44 were more genetically different with increasing barrier distance. The lack of a relationship  
45 between IBD and toad genetic differentiation could suggest that common toads are not as  
46 philopatric as previously thought, with reduced availability of suitable breeding ponds  
47 possibly driving more migrants to disperse greater distances and thereby possibly improving  
48 genetic mixing of the metapopulation.

49 *Keywords:* Bufo bufo; Common toad; Genetic distance; IBB; IBD; Isolation by barrier;  
50 Isolation by distance.

51

52 Genetic differentiation is recognized as a vital component of long-term species  
53 persistence as evidenced by the incorporation of genetic research techniques to conservation  
54 initiatives (Lynch, 1995; Sunnucks, 2000; Hedrick, 2001). Genetic research now commonly  
55 assesses how habitats and landscape configurations may restrict migration, and therefore gene  
56 flow (O'Grady et al., 2006; Reed et al., 2007; Sarre & Georges, 2009). Such approaches are  
57 particularly relevant for amphibian species that tend to use disparate habitats over large areas,  
58 potentially magnifying the effects of genetic isolation (Hanski et al., 2004; Roe & Georges,  
59 2007; Ficetola, Padoa-Schioppa & De Bernardi, 2009). Two common mechanisms of  
60 isolation for amphibian species include isolation by distance (IBD) and isolation by barrier  
61 (IBB). Isolation by distance suggests that populations that are farther apart should be more  
62 genetically dissimilar than populations closer together (Beaumont & Nichols, 1996; Palumbi,  
63 2003). Isolation by barrier implies that populations separated by barriers are more genetically  
64 dissimilar because of the increased travel distance necessary to circumvent these obstacles to  
65 movement (Keyghobadi et al., 1999; Bush et al., 2011). Identification of the influence of IBD  
66 or IBB can be used to better allocate conservation attention toward isolated populations and  
67 determine the appropriate course of mitigation action (e.g. translocation or habitat  
68 improvement; Reinert, 1991; Cabezas & Moreno, 2007; Stamps & Swaisgood, 2007).

69 Common toads (*Bufo bufo* L.) are a highly philopatric (tending to return to or remain  
70 near a particular site or area) species (e.g. Reading, Loman & Madsen, 1991) with limited  
71 mobility (e.g. Moore, 1954; Sinsch, 1988), and so are likely to be susceptible to isolation of  
72 their breeding populations by distance. Further, where the species has undergone population  
73 declines (e.g. Beebee, 2012), common toads tend to be rare and particularly vulnerable to the  
74 effects of habitat change and land fragmentation which can increase isolation (Ficetola & De  
75 Bernardi, 2004). Large urban areas and roads can be particularly costly for migrating  
76 common toads, presenting barriers to movement and leading to direct mortality from vehicle

77 traffic (Gibbs & Shriver, 2005; Puky, 2006; Elzanowski *et al.*, 2009). Populations that are  
78 isolated exchange fewer individuals, and thereby less genetic material, reducing their  
79 genetically similarity over time.

80 Common toads are now a species of conservation concern in the United Kingdom  
81 following the large and unexplained local population declines occurring over the last 30 years  
82 (Carrier & Beebee, 2003; Petrovan & Schmidt, 2016). Common toads tend to have lower  
83 genetic diversity in small urban ponds compared with larger, more rural ponds (Hitchings &  
84 Beebee, 1998). Further, common toads inhabiting more fragmented landscapes have higher  
85 levels of stress hormones and poorer body condition than toads residing in more contiguous  
86 habitats (Janin, Léna & Joly, 2011). Thus, these dynamics may affect fecundity of individual  
87 animals with scaling consequences to the population level. Despite these conservation issues,  
88 the principles of IBD and IBB, relevant to many anuran species, have not yet been applied to  
89 the study of common toads. Natterjack toads (*Bufo calamita*), for example, in Denmark were  
90 found to have no detectable pattern of IBD, supporting the notion that the remaining distinct  
91 populations are genetically isolated by a different mechanism (Allentoft *et al.* 2009; but see  
92 Rowe & Beebee, 2007). Studies of the common frog (*Rana temporaria*) showed reduced  
93 population size, genetic differentiation and fitness of populations in fragmented habitats when  
94 compared with those from continuous habitats (Johansson, Primmer & Merilä, 2007). Finally,  
95 moor frogs (*Rana arvalis*) are especially sensitive to road and railway barriers, the presence  
96 of which better explained genetic distance than isolation by distance alone (Vos *et al.*, 2001).

97 To apply conservation action in timely and efficient ways that can prevent future  
98 population declines, it is crucial that we determine the current threats to species persistence.  
99 This should include threats to the genetic differentiation of species of conservation concern.  
100 Here we examine the mechanistic connections between common toad genetic differentiation

101 and IBB and IBD among eight breeding populations of common toads in Oxfordshire,  
102 England, hypothesising that both IBB and IBD significantly influence common toad genetic  
103 differentiation.

104

105

## Materials and Methods

106 *Study Area and Sampling.* – We positioned our study among eight common toad  
107 breeding ponds in West Oxfordshire, England (Fig. 1). To identify our study ponds we  
108 collaborated with the Thames Valley Environmental Records Centre ([www.tverc.org](http://www.tverc.org)), which  
109 holds all available information about the plants, animals, wildlife habitats and important  
110 wildlife and geological sites in Berkshire and Oxfordshire. In 2011, we visited ponds to  
111 detect evidence of common toad breeding, to select our study ponds and to collect samples  
112 for screening and optimisation of the genetic extraction and amplification protocol.

113 We collected genetic samples, in the form of common toad tadpoles, from a sample of  
114 ponds at each of these eight toad breeding ponds (Fig. 1) between 18<sup>th</sup> June and 4<sup>th</sup> July 2012.  
115 All animal capture and handling protocols were reviewed and approved by the Oxford Local  
116 Ethical Review Committee. Access to private land for collection of samples was granted by  
117 each individual landowner. The breeding ponds in our sample averaged an area of 4,484 m<sup>2</sup>  
118 (range 795 m<sup>2</sup> – 11,436 m<sup>2</sup>) and were between 3.8 km and 21.5 km (Euclidean distance)  
119 apart.

120 Ponds were selected where toad tadpoles were considered to be present in sufficient  
121 numbers so that collection of a sample (26-48 tadpoles, Table 1) would not detrimentally  
122 impact the breeding success of that pond in the year of collection (i.e. sample size <5%  
123 population size).

124 Sibling tadpoles from *Bufo spp.* tend to shoal together (Blaustein *et al.*, 1990), so we  
125 stratified our sampling by shoals so as to avoid sampling closely-related individuals. Where  
126 shoals were not easily visible because of dense vegetation or poor water clarity, we spaced  
127 collections a minimum of two metres apart. Via these sampling efforts we minimized the  
128 potential negative impacts of our collection efforts on the breeding success of any one  
129 common toad breeding pair and maximized the potential genetic differentiation of the  
130 samples collected from each pond. We controlled sampling effort between ponds by  
131 completing each collection during a single visit to each pond. Within this context we had two  
132 surveyors complete a circuit of the pond and collect tadpoles from each shoal or net sweep at  
133 2 m intervals as appropriate.

134 To facilitate genetic analysis, we euthanized tadpoles using an approved *Schedule 1*  
135 technique (therefore not a regulated procedure under the Animals Scientific Procedures Act  
136 1986). We administered a lethal dose of anaesthetic using Benzocaine (Orajel®; Cecala,  
137 Price & Dorcas, 2007), followed by pithing before storing in the freezer in 90% ethanol.  
138

139 *DNA extraction and genotyping.* – We extracted DNA from tadpole tails (remainder  
140 of the tissue retained for future use) using QIAGEN DNeasy blood and tissues extraction kit.  
141 We amplified the samples by multiplex polymerase chain reaction (PCR) by following the  
142 QIAGEN Type-it kit protocol (details in Table 2). We randomly selected three individuals for  
143 use in the PCR protocol optimisation: Ga21, Hi23 and St4. We used primers for seven  
144 microsatellite loci previously developed for this species (Brede *et al.*, 2001) (Table 2). Due to  
145 the overlapping size of the loci analysed we created two separate multiplex associations and  
146 one singleplex (Table 2). The labels used for multiplex were 6FAM: blue, PET: red, NED:  
147 yellow and VIC: green. The details of the PCR cycles are provided in Table 2.

148 Using GENEPOP 4.2 (Raymond & Rousset, 1995; Rousset, 2008) and FSTAT 2.9.3  
149 (Goudet, 1995) software we measured the number of alleles, observed and unbiased expected  
150 heterozygosity  $H_e$  (Nei, 1978), genetic differentiation, allelic richness and inbreeding  
151 coefficient ( $F_{IS}$ ) for each breeding pond and each locus. We used program FreeNA (Chapuis  
152 & Estoup, 2007) to perform checking for genotyping errors such as allelic dropout, stuttering  
153 and null alleles so as to infer their possible impact on the population analysis.

154 Using the exact test in GENEPOP we tested for departure from Hardy-Weinberg  
155 equilibrium for each breeding pond at each locus and tested for linkage disequilibrium with  
156 probability tests across pairs of loci. When necessary, we adjusted significance levels using  
157 the sequential Bonferroni correction (Rice, 1989) in the R statistical software built in function  
158 p.adjust (R Core Team, 2008).

159

160 *Genetic differentiation.* – We measured genetic differentiation, in the form of  $F_{ST}$   
161 values, among all possible combinations (28 pairs) of the toad breeding pond pairs from the  
162 eight ponds using seven microsatellite loci. As microsatellites are neutral markers (not under  
163 selection) we assume that genetic differentiation (change in these traits) between populations  
164 has occurred through genetic drift (LD was tested using GENEPOP and none of the tests was  
165 significant). Calculation and significance of genetic differentiation, using permutation test,  
166 between pairs of breeding ponds was estimated using FSTAT 2.9.3 (Goudet, 1995).

167

168 *IBD and IBB.* – To measure IBD, we quantified Euclidean distance in ArcMap v10.0  
169 (ESRI, 2011) using the Point Distance tool, with each pond paired with all others to produce  
170 28 distances. Next, we mapped IBB using cost distance estimation. Cost distance (in metres)  
171 was calculated around natural barriers to toad movement. We described these barriers by  
172 mapping the predicted relative probability of toad occurrence in the Oxfordshire pond cluster

173 based on a resource selection function (RSF) developed in a previous study (Salazar *et al.*,  
174 2016). We fit the RSF as a function of environmental features including proximity to; *i*)  
175 wooded habitat, *ii*) urban areas, and *iii*) water bodies. Barriers in the cost distance prediction  
176 (Fig. 2) were areas of 0% relative probability of toad occurrence, further from woodland and  
177 water bodies and closer to urban areas (Salazar *et al.*, 2016). The spatial prediction, based on  
178 the environmental parameter estimates of the RSF, displayed the habitat units within the  
179 study area that were suitable for toads. We used the cost distance tool in ArcMap to calculate  
180 the least cost distance between all possible pairs of ponds within the configuration of habitat  
181 within this RSF prediction (Fig. 2).

182

183 *Statistical analyses.* – We created matrices describing the pairwise physical separation  
184 between ponds using two metrics. We used both the simple Euclidian distance between them  
185 and the metric intended to incorporate the cost of movement across the landscape between  
186 them (i.e., cost distance described above). These matrices were then compared with the  
187 matrix describing the pairwise genetic similarity between toad populations inhabiting the  
188 sampled ponds. We measured the association between the genetic and separation matrices  
189 using Spearman’s correlation coefficient. The significance of this statistic was estimated by  
190 randomly reallocating the order of the elements in one of the matrices. We did this with the *zt*  
191 software (Bonnet & Van de Peer, 2002). We also performed the same procedure to derive a  
192 partial correlation between genetic distance and cost distance controlling for Euclidean  
193 distance with partial Mantel test using the same software. This tests a null hypothesis that the  
194 association between genetic similarity and cost distance is not accounted for by Euclidean  
195 distance.

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197

## RESULTS

198           We extracted biological material from a total of 259 tadpoles collected from the eight  
199 studied populations. All seven microsatellite loci were successfully amplified and  
200 polymorphic. The number of alleles detected varied from nine (*Bbufjμ14*) to 31 (*Bbufjμ65*),  
201 indicating new alleles in all loci compared to Brede *et al.* (2001). Expected heterozygosity  
202 ranged from 0.57 to 0.89 while observed heterozygosity varied from 0.55 to 0.83. The allelic  
203 richness values per breeding pond varied from 7.9 (Cumnor) to 9.5 (Bagley Wood). After  
204 correction using the ENA tool provided by FreeNa (Chapuis & Estoup, 2007), null alleles  
205 were present on average 2.4% over all breeding ponds and loci and between 1.1 % over all  
206 ponds for the locus *Bbufjμ62* and 5.3 % over all ponds for the locus *Bbufjμ15*.

207           Genetic differentiation across ponds was not significantly different from zero for three  
208 pairs of ponds: Fawler-Bagley Wood, Cothill-Garford, Ducklington-Standlake (Table 3) and  
209 therefore these pairs were considered genetically homogenous. Ducklington-Standlake (~ 6  
210 km apart) were the most genetically similar breeding ponds pair and were notable for the high  
211 density of ponds between the two locations.

212           We detected no significant relationship between Euclidean distance and genetic  
213 differentiation (Mantel test:  $r = -0.17$ ,  $p = 0.237$ ). The relationship between cost distance and  
214 genetic differentiation was positive and significant both with and without control of  
215 Euclidean distance (simple Mantel test:  $r = 0.48$ ,  $p < 0.05$ , partial Mantel test (Euclidean  
216 controlled):  $r = 0.48$ ,  $p < 0.05$ ).

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218

## DISCUSSION

219 We assessed the impacts of IBB and IBD on common toad genetic differentiation  
220 among eight breeding ponds in Oxfordshire, England. The number of alleles, their size  
221 ranges, as well as expected and observed heterozygosity were mostly higher than in Brede *et*  
222 *al.* (2001), possibly as a result of the larger number of alleles identified in our study. The  
223 genetic differentiation that we detected between toad breeding ponds was possibly best  
224 explained by IBB. We suspect that the size and strength of these barriers determined how  
225 much the cost distance would need to increase to circumvent the barrier. This result  
226 demonstrates the effect of urban areas with reduced density of more hospitable habitats  
227 (woodland, water bodies) as barriers to migration, affecting the ability of toads to move  
228 around the landscape to breed away from their natal pond (Hitchings & Beebee, 1997; Hamer  
229 & McDonnell, 2008; Goldberg & Waits, 2010). These results are consistent with established  
230 studies that identified lower genetic differentiation in toad ponds in urban areas (Hitchings &  
231 Beebee, 1998).

232 We detected the least genetic differentiation in a breeding pond pair that were ~ 6 km  
233 apart (Ducklington-Standlake). Despite being separated by this distance these two ponds were  
234 genetically homogenous as inferred from our examination of the seven microsatellite  
235 markers. This result may suggest a greater level of movement of individuals between these  
236 ponds than was possibly expected considering their reported philopatry (Reading *et al.*, 1991)  
237 and the maximum migration distance recorded for toads of 3.6 km (Moore, 1954; Heusser,  
238 1958). Common toads can colonise new ponds when the nearest breeding pond is within 950  
239 m (Baker & Halliday, 1999). Considering the high density of ponds between Standlake and  
240 Ducklington (Fig. 1), we hypothesize that the abundance of ponds in this landscape matrix  
241 function as stepping stones, facilitating movement of individuals or their genetic material  
242 over several generations. Though it is impossible to know the extent to which artificial toad  
243 translocation has affected the genetic structure of these breeding populations, it is unlikely to

244 have been commonplace as most of the ponds in this study were relatively isolated. Possible  
245 exceptions were the ponds at Fowler (accessible and closely monitored by the public as part  
246 of a toad crossing scheme) and Bagley Wood (in the grounds of a school), which were  
247 genetically homogenous despite the relatively large Euclidean and cost distances separating  
248 them (Fig. 1).

249 We failed to detect a significant relationship between common toad genetic  
250 differentiation and Euclidean distance at the local landscape scale. These findings may  
251 suggest that despite concern over recent dramatic population-level declines (Carrier &  
252 Beebee, 2003; Petrovan & Schmidt, 2016), connectivity between rural common toad  
253 populations at the local landscape scale might be maintained with appropriate management.  
254 To encourage a greater level of movement between breeding populations we suggest the  
255 creation of more ponds and woody habitat. Creation of ponds would go some way to redress  
256 the losses experienced in the last century (Williams *et al.*, 2010) and creation of nearby  
257 woody habitat would improve the relative probability of toads using these habitats during the  
258 terrestrial phase. Increasing the number of ponds increases pond density and so reduces  
259 distances between ponds that, though not shown to be limiting at the local landscape scale,  
260 may become more important at a larger scale.

261

262 *Summary.* – We collected common toad genetic material from eight different breeding  
263 populations in rural England to test the effect of isolation by distance and isolation by barrier  
264 on toad genetic differentiation. We detected no significant effect of distance but did identify a  
265 significant and positive relationship between barriers and common toad genetic  
266 differentiation, with breeding populations being more genetically different with increasing  
267 barrier cost distance.

268

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278 conservation (Macdonald and Feber, 2015a,b).

279

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410 **TABLES**

411 **Table 1.** Tissue sample numbers, source ponds, and sampling dates for the collection of  
 412 common toad (*Bufo bufo*) samples in Oxfordshire, England.

Pond name	No. tadpoles	Code	Sampling date
Bagley Wood	26	BW 1-26	25/06/12
Combe	48	Cb 1-48	18/06/12
Cumnor	30	Cu 1-30	27/06/12
Fawler	45	Fa 1-45	27/06/12
Garford	26	Ga 1-26	22/06/12
Standlake	31	St 1-31	29/06/12
Cothill	27	Hi 1-27	04/07/12
Ducklington	26	RC 1-26	26/06/12

413

414 **Table 2.** The seven microsatellite loci and their associated primers used for amplification of the common toad (*Bufo bufo*) samples (Brede et al.  
 415 2001) in Oxfordshire, England, together with the associated Multiplex design, labelling and Polymerase Chain Reaction conditions.

Microsatellite loci (GenBank code)	Primer sequences (5'-3')	
Bbuf54 (AY037820)	FAM-CATTGCGCTGCTGTCAGATTACAC TTAGGGATTGCCGTCCAGTTGTC	
Bbuf14 (AY037811)	NED-CGTGCATGCAAGTGTACCTAACC ATGGAGAGTGAAGGGGAAAGAGTG	
Bbuf47 (AY037818)	VIC-GGATCAAGCCCTCAGACAACCTC CACAGCAGCAGAAATTTTGACCAG	
Bbuf62 (AY037821)	PET-GCACATTCCTGTGTCCGTGTATAG ATTCCGAAAACGAAAAGAAAAGAG	
Bbuf15 (AY037812)	FAM-TCAATATAGGAGTCCCAGAATGTC AATCCCCTAGCGTACACAAGATAC	
Bbuf11 (AY037809)	GTCACATGGATAATAAATGAGACC TCTAATATTGATGACCAGACAACC	
Bbuf65 (AY037823)	PET-GGATCTAAGCGCTGTGAGAGTGA CGGTCCGTGTTACCACTGATGC	
PCR Name	Gene amplified/ Flourescent label	Reaction cycles
Multiplex4	Bbuf $\mu$ 15/Blue, Bbuf $\mu$ 14/Yellow, Bbuf $\mu$ 47/Red, Bbuf $\mu$ 65/Green	95°C for 5min denaturation, 94°C 30sec, 55°C 30sec and 70°C 30sec x25 cycles, 70°C for 30min final elongation step.
Multiplex6	Bbuf $\mu$ 54/Blue, Bbuf $\mu$ 62/Red	95°C for 5min denaturation, 94°C 30sec, 57°C 30sec and 70°C 30sec x23 cycles, 70°C for 45min final elongation step.
Singleplex11	Bbuf $\mu$ 11	94°C for 3min denaturation, 94°C 30sec, 51°C 1min and 72°C 1min x25 cycles, 72°C for 30min final elongation step.

416 **Table 3.** Pairwise Fst values between pairs of breeding patches.

Breeding patches	Bagley Wood	Combe	Cothill	Cumnor	Ducklington	Fawler	Garford	Standlake
Bagley Wood	-							
Combe	<b>0.0280</b>	-						
Cothill	<b>0.0389</b>	<b>0.0305</b>	-					
Cumnor	<b>0.0639</b>	<b>0.0298</b>	<b>0.0576</b>	-				
Ducklington	<b>0.0605</b>	<b>0.0182</b>	<b>0.0410</b>	<b>0.0305</b>	-			
Fawler	0.0137	<b>0.0122</b>	<b>0.0343</b>	<b>0.0453</b>	<b>0.0206</b>	-		
Garford	<b>0.0364</b>	<b>0.0146</b>	0.0078	<b>0.0416</b>	<b>0.0264</b>	<b>0.0229</b>	-	
Standlake	<b>0.0373</b>	<b>0.0181</b>	<b>0.0387</b>	<b>0.0442</b>	0.0033	<b>0.0107</b>	<b>0.0237</b>	-

417 Significant values are indicated in bold.

418

419 **FIGURE LEGENDS**

420 **Figure 1** Locations of eight ponds in Oxfordshire, England from which common toad (*Bufo*  
421 *bufo*) genetic material was collected.

422

423 **Figure 2** The spatial prediction of the resource selection function (RSF) depicting common  
424 toad (*Bufo bufo*) habitat in Oxfordshire, England. The permeable habitat (grey) among eight  
425 Oxfordshire ponds (black dots). The areas in white are those predicted to have a 0%  
426 probability of common toad occurrence

427

Figure 1

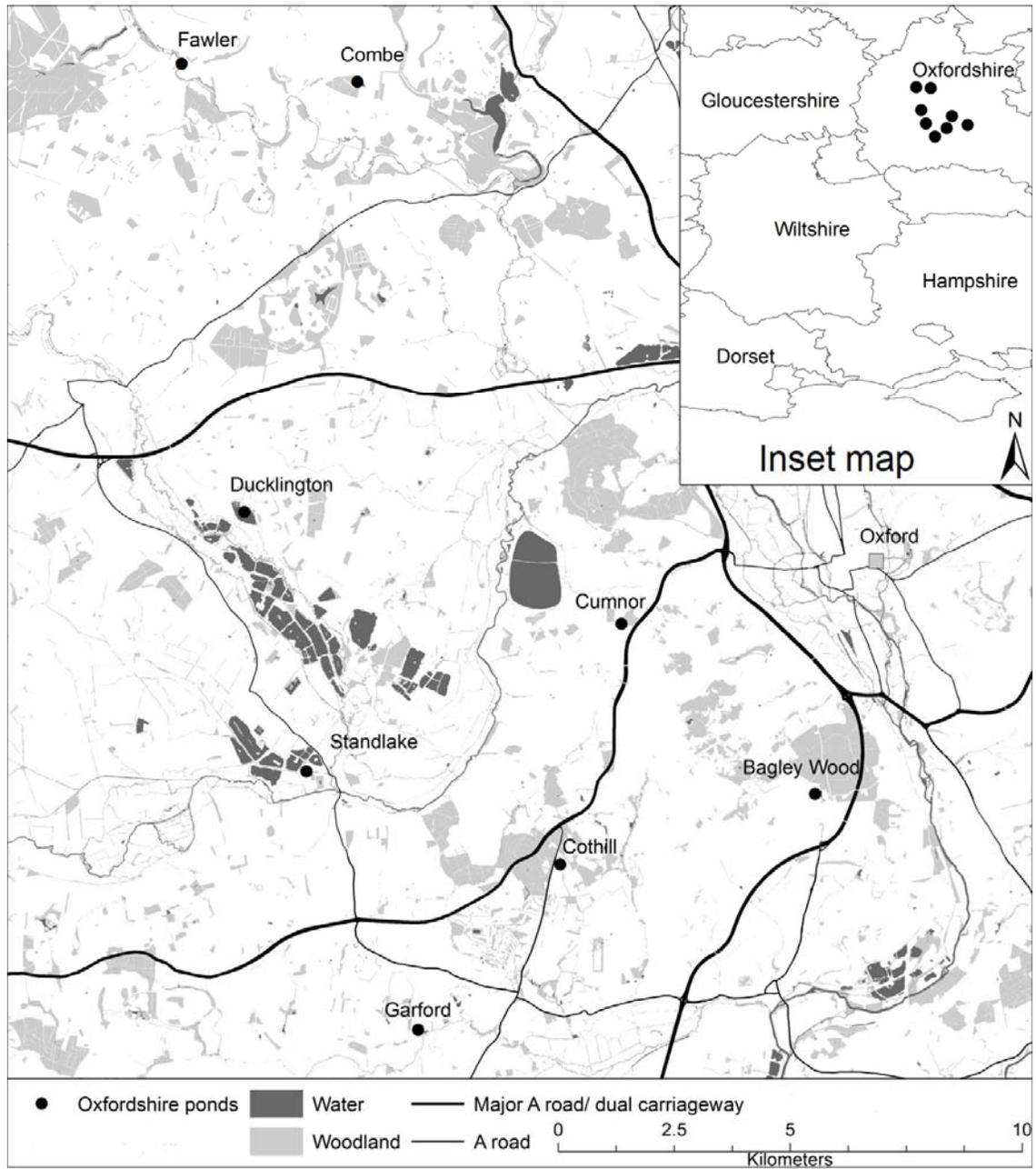


Figure 2

