

1 *Original Article*

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3 **Genetic distance and social compatibility in the aggregation behavior of Japanese**

4 **toad tadpoles**

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6 Running title:

7 **Genetic influence in tadpole's aggregation**

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26 **Abstract**

27 From microorganism to vertebrates, living things often exhibit social aggregation. One of
28 anuran larvae, dark-bodied toad tadpoles (genus *Bufo*) are known to aggregate against
29 predators. When individuals share genes from a common ancestor for whom social
30 aggregation was a functional trait, they are also likely to share common recognition cues
31 regarding association preferences, while greater genetic distances make cohesive aggregation
32 difficult. In this study, we conducted quantitative analyses to examine aggregation behavior
33 among three lineages of toad tadpoles: *Bufo japonicus japonicus*, *B. japonicus formosus*, and
34 *B. gargarizans miyakonis*. To determine whether there is a correlation between cohesiveness
35 and genetic similarity among group members, we conducted an aggregation test using 42
36 cohorts consisting of combinations drawn from a laboratory-reared set belonging to distinct
37 clutches. As genetic indices, we used mitochondrial DNA (mtDNA) and major
38 histocompatibility complex (MHC) class II alleles. The results clearly indicated that
39 aggregation behavior in toad tadpoles is directly influenced by genetic distances based on
40 mtDNA sequences and not on MHC haplotypes. Cohesiveness among heterogeneous
41 tadpoles is negatively correlated with the geographic dispersal of groups. Our findings
42 suggest that social incompatibility among toad tadpoles reflects phylogenetic relationships.

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45 **Keywords**

46 *Bufo gargarizans*, *Bufo japonicus*, genetic distance, social aggregation, species recognition,
47 tadpole

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51 **Introduction**

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53 Many species exhibit aggregation behavior to avoid predation threat and/or to improve
54 foraging efficiency, indicating that the benefits inherent in group living outweigh the costs.
55 Anuran larvae (tadpoles) commonly aggregate into groups and, similar to fish, their schooling
56 behavior varies between centralized for predator avoidance and non-centralized for foraging
57 (Beiswenger, 1975; Watt *et al.*, 1997; Lardner, 2000). Cohesive aggregation is conducive to
58 higher survival potential (Watt *et al.*, 1997), and individuals stand to benefit from collective
59 foraging (Beiswenger, 1975). Nevertheless, the mechanisms by which genetic polymorphism
60 affects the formation of tadpole social aggregation remain unclear (Wells, 2010). In other
61 words, we do not understand how simple association preference is derived via individual
62 genetic background.

63 For grouping behavior to initiate, recognition and discrimination of association
64 partners must occur. Several studies have reported kin-biased association of anuran tadpoles
65 across taxa (e.g., Waldman & Adler, 1979; Blaustein & O'Hara, 1981, 1987; Waldman,
66 1982, 1984). Kin recognition ability is significant in terms of evolution of altruism (Hamilton
67 1964), which is supported by several reports of kin preference behavior across taxa (Hepper,
68 2005; Breed, 2014). In *Bufo* (the common toad), kin-biased high cohesiveness may result in
69 high tadpole survival rates (Watt *et al.*, 1997). Aggregations consisting of dark-bodied
70 tadpoles have also been considered a warning signal of distastefulness to predators (Waldman
71 & Adler, 1979; Glandt, 1984). Although no incontestable evidence for kin preference has
72 been identified (Wells, 2010), the aforementioned studies offer positive evidence in support
73 of a hypothesis of kin selection, as opposed to the selfish herd hypothesis, regarding the
74 social aggregation of toad tadpoles.

75 A key question is how the mechanisms of association preference among
76 amphibians evolved? The capacity for recognition between kin and non-kin, or between
77 closely and distantly related individuals, carries ecological significance, not only as a device
78 for predator avoidance but also with regard to territoriality, parental care, and mate choice,
79 including inbreeding avoidance and optimal outbreeding (Hepper, 2005; Breed, 2014).
80 Studies have reported that anuran tadpoles discriminate using visual and olfactory cues (e.g.,
81 Blaustein & O'Hara, 1987; K Hase & N Kutsukake *unpubl.data*). When the mechanisms that
82 guide association preference are derived from assortative preference, it is likely that the
83 recognition cue will consist of multiple traits and will have evolved via conspecific
84 recognition. This gives rise to the prediction that a negative relationship will be discerned
85 between genetic distance and cohesiveness in the social aggregation of toad tadpoles.

86 *Bufo japonicus* (hereafter *japonicus*), *B. japonicus formosus* (*formosus*), and
87 *B. gargarizans miyakonis* (*miyakonis*) are native Japanese toads belonging to the true toad
88 family, Bufonidae. Although the distribution of these toads is geographically isolated across
89 Japan's islands (*japonicus* and *formosus* are found in the western and eastern regions of the
90 mainland and *miyakonis* inhabits the Miyako Islands), their ecological features are relatively
91 common (Matsui, 1984). The genetic differentiations of these Japanese toads are indicative of
92 isolation based on geographic distance (Kawamura *et al.*, 1990). According to classification
93 based on mitochondrial DNA (mtDNA) sequences, they belong to three distinct clades
94 (Igawa *et al.*, 2006). In contrast to the similarities between *japonicus* and *formosus*, adult
95 *miyakonis* exhibits clear differences in morphology, being smaller in size with fewer bumps.
96 However, the larvae of all three toads have similar morphologies. All tadpoles are herbivores
97 and exhibit aggregation behavior.

98 In this study, to determine whether there is a relationship between genetic distance
99 and social aggregation in the wild toad tadpole, we conducted a quantitative analysis of

100 aggregation behavior of tadpoles in the three distinct lineages, *japonicus*, *formosus*, and
101 *miyakonis*. We compared the degrees of aggregation behavior among admixture cohorts,
102 assembled by combining laboratory-reared tadpole groups derived from different clutches. As
103 indices of genetic distance in these tadpole groups, we assessed the mtDNA genotypes of the
104 tested tadpoles, which reflects phylogenetic and geographic distances. We also conducted
105 assessment of haplotypes of major histocompatibility complex (MHC) class II. Molecular
106 markers encoded by MHC loci are responsible for generating the individual odors involved in
107 self/non-self-discrimination (Medzhitov & Janeway, 2002; Piertney & Oliver, 2005). MHC
108 gene families encode genes governing immune recognition and function in vertebrates. MHC
109 class I genes express glycoproteins on the nucleated cell surface, whereas class II genes are
110 mainly found on antigen-presenting immune cells. Furthermore, MHC genes not only play a
111 central role in the immune system, but also play a genetic role in kin recognition (e.g.,
112 Manning et al. 1992; Yamazaki et al. 2000; Olsson et al. 2003; Villinger and Waldman 2008,
113 2012).

114 We report herein the first evaluation of genetic influences on social aggregation
115 behavior in wild tadpoles. We postulate that group formations consisting of
116 heterogeneous tadpoles will be controlled by overall group kinship levels.

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118

119 **Methods**

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121 1. Experimental animals

122

123 Between February and March 2013, we sampled 18 clutches from eight reproductively

124 isolated sites in Japan (Figure 1A; detailed locality and coordinate data are presented in Table

125 S1). The clutches belonged to three different lineages of Japanese toad: *japonicus* ($n = 7$),
126 *formosus* ($n = 8$), and *miyakonis* ($n = 3$).

127 Clutches spawned by amplexant pairs consisting of a single male, as far as we
128 observed until spawning finished, were specifically chosen to avoid polyandry. After the
129 amplexant pair had left the spawned egg strings, we collected approximately 0.4 m of egg
130 strings containing about 200–400 embryos and transported them to the laboratory. Embryos
131 belonging to the same clutch were divided into two sets before hatching (100–150 individuals
132 per set) and transferred into a container (220 mm width \times 310 mm diameter \times 40 mm height)
133 in a large incubator at 18°C under a 12:12 h dark:light cycle. All tadpoles were raised with
134 respect to each set with a sufficient supply of fish food pellets containing vegetable stock as
135 the main component (PLECO; Kyorin, Hyogo, Japan). Individuals of each set were colour-
136 marked by immersion in either a 0.00025% solution of neutral red for 24 h or a 0.00025%
137 solution of methylene blue for 12–24 h before the aggregation test, as described previously
138 (Waldman 1984). Thus, sib tadpoles from the same clutch were randomly distributed into
139 different colour sets (red or blue) without harm or the addition of exogenous odour. This
140 treatment was done to simply identification of clutch type in the duplication of the
141 aggregation test and genotyping. After the aggregation test and genotyping, the tested
142 tadpoles were released into their native ponds when possible, excluding *miyakonis*, which is
143 an artificially introduced alien species.

144

145 2. Genotyping

146

147 The total DNA content was extracted from the tail-tips of all tadpoles included in the
148 aggregation tests. The samples (tadpole's tail tip) were left to digest overnight in
149 ethylenediaminetetraacetic acid-sodium dodecyl sulphate (EDTA-SDS) solution (0.3% SDS,

150 400 mmol/L NaCl, 5 mmol/L EDTA, 20 mmol/L Tris-HCl, pH 8.0) containing 200 µg/mL
151 proteinase K at 55°C. For sequencing and TA cloning, phenol/chloroform extraction and
152 ethanol precipitation were used.

153 The mtDNA of all clutches was analysed using DNA sequencing were amplified by
154 polymerase chain reaction (PCR) using locus as described in Hase et al. 2013 with specific
155 primers developed previously – cytbf1/Bufo (5'-ATCTGCCGAGATGTAAACAACGG-3')
156 and cytbf177731R (5'-TCTGYTRAGYTG GGYWAGTTTGTTC-3') –; the sequence was
157 used to build a tree in MEGA 5.2 (Tamura et al. 2011). Mitochondrial cytochrome b
158 sequences, 0.8 kb in length from 18 clutches, were aligned and deposited in the GenBank
159 nucleotide database [Acc. nos. AB597917, AB597928 from ref. 31; AB713512 from ref. 33;
160 LC071519 – LC071529]. The phylogenetic analyses showed that mtDNA sequences of 18
161 clutches diverged into three lineages with high bootstrap support (Figure 2A). All values
162 were used to estimate the genetic differences among the mtDNA lineages as a genetic index.
163 In these three Bufo species, the differences in the mtDNA cytochrome b sequences reflected
164 their geographic distribution in Japan (Igawa *et al.*, 2006; Hase *et al.*, 2013, Figures 1A and
165 2A).

166 MHC class II antigen (partial exon) allele sequences were amplified by PCR using
167 locus-specific primers developed previously (May et al. 2011) – 2F347 (5'-
168 GTGACCCTCTGCTCTCCATT-3') and 2R307b (5'-
169 ATAATTCAGTATATACAGGGTCTCACC-3') – using KOD FX Neo (Toyobo, Osaka,
170 Japan) in a final reaction volume of 10 µL. The PCR conditions consisted of an initial
171 denaturation step of 2 minutes at 94°C followed by a touchdown program consisting of three
172 cycles of 10 s at 98°C, 30 s at 62°C and 20 s at 74°C, three cycles of 10 s at 98°C, 30 s at
173 60°C and 20 s at 72°C and 24 cycles of 10 s at 98°C, 30 s at 58°C and 20 s at 70°C. To
174 identify the original alleles within each clutch, 4 –15 individuals per clutch were sequenced.

175 TA cloning was performed for heterozygous individuals using a pGEM-T vector system
176 (Promega, Madison, WI, USA) and Ligation high (Toyobo) was used for ligation.
177 Transformation was performed using Competent high DH5 (Toyobo). Finally, 13 alleles of
178 the 0.3-kb MHC class II locus were detected in 127 individuals from 18 clutches, which were
179 used to construct a phylogenetic tree using MEGA 5.2 (Tamura *et al.*, 2011). All MHC
180 alleles have been deposited in the GenBank nucleotide database (Accession Nos. LC065649
181 – LC065661). From these clutches, 13 MHC alleles presented complex relations with the
182 mtDNA lineages; the BJ1 allele was distributed throughout all lineages (Figure 2B). The
183 amino acid alignment of this allele is presented in Figure S1. We then proceeded to genotype
184 all of the tadpoles included in the test except including polyandrous clutch samples (NK1, see
185 below). Hence, Individuals belonging to 42 cohorts were genotyped using restriction
186 fragment length polymorphism analysis (RFLP) of PCR-amplified fragments (PCR-RFLP).
187 For individuals belonging to *B. japonicus formosus* clutches, we used the reverse primer
188 R_BjMHCII (CCATAGTTG TRTTTACAGWATSTCTCC) during PCR instead of 2R307b,
189 which was developed using already identified alleles. All PCR-amplified MHC sequences
190 were subsequently digested using more than one restriction enzyme, such as BmeT1101,
191 BseRI, BsgI or XhoII, chosen to distinguish haplotypes among all the possible combinations
192 of alleles identified in the clutches (Tables S1 and S2). The MHC haplotypes of all 50
193 individuals in each cohort could be identified from PCR-RFLP data. However, in cases where
194 the haplotype of more than two individuals could not be identified using this method, the
195 entire cohort was excluded from the genetic diversity analysis. A total of 1,550 individuals
196 (31 of 42 cohorts) were finally included in the analysis.

197 To check polyandry, we selected four microsatellite loci by PCR: 1) Bbufu23; 2)
198 Bbufu39; 3) Bbufu62 (Brede *et al.* 2011); and 4) Bjap14 (Hase *et al.*, 2013). Following the
199 method described previously (Hase *et al.*, 2013), the loci were amplified with two multiplex

200 reactions using a Multiplex PCR Kit (QIAGEN, Valencia, CA, USA) according to the
201 manufacturer's protocol. PCR amplification consisted of an initial denaturation step at 95°C
202 for 15 minutes, followed by 25–35 cycles of 30 s at 94°C for denaturation, primer-specific
203 touchdown annealing for 90 s at 46–61°C (Hase *et al.* 2013) and an extension step at 72°C
204 for 1 minute. The fragment sizes of the PCR products from each microsatellite locus were
205 analysed using a CEQ8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA,
206 USA) with a Genomelab Size Standard Kit 400 (60 – 420 bp; Beckman Coulter). Overall, we
207 genotyped 1,200 individuals belonging to 18 clutches. A polyandry check performed by
208 GERUD2.0 (Jones, 2005) indicated that 17 of 18 clutches had a single sire. One clutch, NK1,
209 was sired by at least two males; the relative paternity results for NK1 showed that the first
210 male sired 84% of the clutch, and the second male sired the remainder. Hence, we eliminated
211 clutch NK1 from the genotyping of the MHC haplotype.

212

213 3. Aggregation behavior test

214

215 An aggregation behavior test was conducted on 68 sets generated from 50 randomly chosen
216 tadpoles from the different laboratory-reared sets (25 red- and 25 blue-dyed tadpoles were
217 chosen from two containers, respectively). The tadpoles were transferred into plastic
218 containers (230 mm width × 350 mm depth) containing 1 L dechlorinated tap water (20 mm
219 in height). Pairs of red- and blue-dyed tadpoles were characterized as sib (i.e., derived from
220 the same clutch but raised separately) or non-sib (i.e., intra-/inter-lineages). The aggregation
221 behavior test comprised three trials using the same cohort of 50 tadpoles. The selection
222 process lasted 5 days. The aggregation tests were performed according to the procedure
223 described below (also detailed in Figure 1B). Tadpoles were chosen for a trial cohort
224 according to their developmental stage to ensure that each cohort consisted of tadpoles of a

225 similar age between stages 27 and 37 (Gosner, 1960). All trials began at night in a large
226 closed incubator, and lasted for 24 h. After 50 tadpoles had been individually and evenly
227 distributed throughout the container using a spoon, for pre-conditioning purposes the
228 container was kept in the dark for 12 h. Observation of the aggregation behavior began the
229 following morning. Images of each tadpole's aggregation behavior were recorded under
230 normal light conditions using a Pentax Optio W80 camera (Ricoh, Tokyo, Japan) in interval
231 shooting mode. Photographs were captured at 1-min intervals, yielding a total of 720 shots
232 per trial. The trial was repeated three times with two intermissions. During the trials, the
233 containers with the tadpoles were maintained in large incubators at 18°C for 24 h. Following
234 completion of the first trial, the tested cohort was divided into two sets based on the color of
235 the dye (25 red tadpoles and 25 blue tadpoles) and fed, then allowed to acclimate for 24 h
236 (intermission process). The tadpoles were subsequently returned to the experimental
237 container for a repeat aggregation trial, as described above. Figure 1B presents an outline of
238 the experimental process. Aggregation tests were conducted in 42 cohorts, each consisting of
239 two laboratory-reared set pairs.

240

241 4. Aggregation index

242

243 The degree of aggregation was measured using the aggregation index based on nearest-
244 neighbor analysis (Clark & Evans, 1954; Heupel & Simpfendorfer, 2005). We ascertained
245 the positions of 50 individuals in the two-dimensional arena (230 mm × 350 mm), after
246 which we calculated the mean distance r from each individual to its nearest neighbor. For
247 randomly distributed individuals, the mean distance (E_r) from the nearest individual was
248 analytically described (Heupel & Simpfendorfer, 2005). However, to incorporate the
249 effects of the arena corners, we conducted stochastic simulations, scattering 50 random

250 points throughout the arena. After 1,000 iterations, we obtained the $E_r \pm$ standard
251 deviation (SD) = 21.11 ± 1.7 (mm), with a lower 5% confidence limit of 18.28 (mm).
252 Aggregation index may be defined as the ratio of the measured value: $R = r / E_r$. When r
253 was below 18.28 (mm), the distribution of the tadpoles could be ascribed to statistically
254 significant aggregation behavior ($R < 1$). It should be noted that lower aggregation index
255 values indicate higher degrees of aggregation. Figure 1C shows a sample raw data-
256 obtained image.

257

258 5. Genetic indices and data analyses

259

260 We used two genetic indices to compare gene diversity among the tested cohorts: mtDNA
261 pairwise distance and MHC (class II antigen) amino acid sequence difference. Having
262 analyzed the phylogenetic relationships (Figure 2AB), we quantified the genetic distances
263 of each marker.

264 The evolutionary divergence of the mtDNA lineages for each experimental clutch
265 pair was estimated using the number of base substitutions per site. These analyses were
266 conducted based on the maximum composite likelihood model in MEGA 5.2 (Tamura *et*
267 *al.*, 2011). The MHC genotypes were derived from the MHC class II antigen (partial
268 exon, 0.3 kb) haplotypes for each cohort based on 12 alleles that were identified by
269 genotyping (Tables S1 and S2, Figure S1). We calculated the amino acid sequence
270 differences of the MHC genotypes in each cohort as an additional genetic index using
271 MEGA 5.2 (Tamura *et al.*, 2011).

272 To verify the effect of genetic influence on aggregation behavior, the data were
273 analyzed using R ver. 3.4.3 software (R Core Team, 2017). We evaluated the relationship
274 between the genetic indices (mtDNA pairwise distances and MHC class II amino acid

275 differences) and the degree of aggregation using Pearson's correlation test. We also
276 applied generalized linear mixed models (GLMMs) with Gaussian distribution. In the
277 GLMM analyses, each mtDNA and MHC was treated as a fixed variable and the clutch
278 was treated as a random variable. Additionally, to verify whether intra-lineage pair
279 cohorts scored higher in the aggregation test than inter-lineages irrespective of genetic
280 indices, we conducted the GLMMs by treating intra-/ inter-lineage as a fixed variable and
281 clutch as a random variable.

282

283 **Results**

284

285 Table 1 presents the results of the aggregation test. We confirmed that tadpoles could
286 aggregate across distinct lineage boundaries. In all tests, the tadpoles exhibited significant
287 levels of aggregation behavior: the mean value of the aggregation index (over 720 shots
288 and three trials) was 0.894 ± 0.049 ($n = 42$, mean \pm SD; range: 0.776–0.959).

289 We detected a distinct negative correlation between phylogenetic distance and
290 tadpole cohesiveness, where genetic distance based on mtDNA was significantly correlated
291 with the degree of aggregation ($n = 42$, $t = 3.888$, Pearson's $r = 0.524$, $P < 0.001$; Figure 2C).
292 Conversely, no correlation was discerned between the MHC amino acid differences and
293 cohesiveness ($n = 31$, $t = 0.811$, Pearson's $r = 0.149$, $P = 0.424$; Figure 2D). The results of the
294 GLMMs corroborated this. mtDNA pairwise distance exhibited a significant effect on
295 aggregation behavior ($t = 3.49$, $P < 0.001$), whereas MHC amino acid difference was not
296 associated with any significant influence ($t = 1.13$, $P = 0.281$). This indicates that shorter
297 phylogenetic distances are correlated with higher cohesiveness in the toad tadpole's
298 aggregation behavior. Moreover, intra-lineage pair cohorts aggregated significantly more than
299 inter-lineage pair cohorts (GLMMs: $t = -3.2$, $P = 0.002$; Figure 3). This indicates that the toad

300 tadpoles possessed the ability to perform lineage discrimination, which corresponds with the
301 taxonomic relationship between the three Japanese toads (*japonicus*, *formosus*, and *miyakonis*).

302

303 **Discussion**

304

305 Our findings suggest that tadpoles discriminate distantly related individuals and
306 that this has an influence on aggregation behavior (Fig. 2C). Tadpoles can discriminate other
307 lineages (Fig. 3), which suggests that *japonicus*, *formosus*, and *miyakonis* are capable of
308 species recognition in relation to one another. Species recognition has been investigated in
309 the context of mate choice, including its general implications for speciation (e.g., Taylor *et*
310 *al.*, 2000; Hauber *et al.*, 2001). In the case of mate choice in the toads, in particular
311 *miyakonis*, species recognition seems not to be significant due to geographically isolation. In
312 addition, male-male competition is a central factor rather than female preference in the
313 reproductive behavior of *B. japonicus* (Hase & Shimada 2014). An exploration of the
314 ecological function of the species recognition abilities we detected lies beyond the scope of
315 this study. However, the findings suggest that toad tadpoles can not only aggregate across
316 lineages, but can also discriminate one another. We can infer from the data that the genetic
317 background to the social discrimination phenomenon among these species consists of a
318 process rooted in a common ancestor.

319 The GLMM analysis suggested that all individuals, whether *japonicus*, *formosus*,
320 or *miyakonis*, used similar cues (phenotype- or odor-matching) regardless of lineage type,
321 because the degree of aggregation was influenced by genetic distance but not by cohort type
322 ($P < 0.001$). These findings are relevant to the mechanisms of kin recognition in these
323 species. It is likely that the recognition cues have evolved via species recognition. That is, the
324 traits that contribute to discrimination may derive from an ancestor of *Bufo* that developed the

325 properties of distastefulness in the tadpole's black body; therefore, although there were
326 variations in selective pressure across the breeding sites, the warning signals derived from
327 distastefulness should be common to all three lineages (*japonicus*, *formosus*, and *miyakonis*).
328 Cohesive aggregation increases survival potential for tadpole groups (Watt *et al.*, 1997).
329 Thus, we may hypothesize that our findings partially corroborate previous reports on the
330 social behavior of microorganisms that detected a correlation between genetic variation and
331 social incompatibility (Ostrowski *et al.*, 2008; Mehdiabadi *et al.*, 2009; Chaine *et al.*, 2010).
332 Moreover, it can be suggested that social aggregation in toad tadpoles is also fundamentally
333 controlled by the genetic similarities among group members. However, the evolutionary
334 process of the kin recognition system of toad tadpoles and its adaptive significance lie beyond
335 the scope of this study, and several unanswered questions concerning toads' capacity for
336 social discrimination remain.

337 Previous studies have emphasized the importance of MHC genes to social
338 discrimination in the anuran (Villinger & Waldman, 2008, 2012). However, we found no
339 evidence for an MHC-mediated recognition system across the three lineages of tadpoles
340 in the present study, and MHC amino acid differences did not correlate with the
341 aggregation index values ($r = 0.149$, $P = 0.424$, Figure 2D). This may be attributed to our
342 genetic index data's inadequacy in determining the genetic background, and the
343 assessment of the MHC gene offered only a partial explanation, that the identified MHC
344 class II allele was partial (324 bp; details provided in Figure S1). Furthermore, genetic
345 diversity derived from mtDNA yields no information regarding the genetic mechanisms
346 governing group member preference. To ascertain which traits are essential elements for
347 this, genome-wide investigation is required. Further study is now underway to obtain a
348 more detailed appreciation of genetic influence on social discrimination among toad
349 tadpoles. While MHC-mediated social discrimination is likely to work among closely

350 related individuals (Hase *et al.*, unpubl. data), it might not work among distantly related
351 individuals. There follows a discussion regarding the kin recognition system in
352 aggregation behavior and our conclusion regarding the efficacy or influence of MHC in
353 this mechanism.

354 In anurans, although previous studies have verified the occurrence of kin
355 recognition among wild tadpoles, there is no information regarding related genetics
356 (Wells, 2010). In general, it seems that overall kinship level acts to form the cohesiveness
357 in the social aggregation of the toad tadpoles: as genetic distances decreases, degree of the
358 aggregation decreases. The present study constitutes the first step toward determining the
359 genetic background to social discrimination in these species.

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375 **Data accessibility**

376 Sequence data are available from GenBank (accession Nos. LC071519–LC071529,
377 LC065649–LC065661). The raw data for aggregation tests and haplotype data for all tested
378 tadpoles will be available from the Dryad Digital Repository after manuscript acceptance.

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489

490

491 **Table 1**

492 Results of the aggregation tests. The trial cohort information provided is as follows:
493 relatedness, cohort ID, clutch affiliation for tested sets (detailed information is presented
494 in Table S1 and Figure S1), lineage of tested clutch pairs (*j. japonicus*; *f. formosus*; *m*:
495 *miyakonis*) and average developmental stage of the tested tadpoles (Gosner 1960).

496 Genetic diversity and aggregation test scores: mtDNA, pairwise distances; MHC, amino
 497 acid differences by cohort. Aggregation index, mean value of aggregation index over 720
 498 shots over three trials.

Lineage		Cohort			Genetic diversity		Aggregation index
		ID	clutch	Dev	mtDNA	MHC	mean ± SE
Intra-lineage	<i>j+j</i>	320-1R	KM1 + KM2	27	0.005	7.429	0.878±0.004
		404-1R	KM2 + YW1	30.5	0.007	9.518	0.853±0.006
		416-1R	YW3 + YW2	28	0.000	4.500	0.836±0.002
		423-2L	YW1 + YW4	32	0.010	2.528	0.929±0.009
		429-2L	YW3 + OM1	29.5	0.007	9.192	0.928±0.005
	<i>f+f</i>	429-1L	CB1 + RS1	32	0.013	14.114	0.807±0.013
		429-2R	CB1 + NK1	30	0.052	–	0.932±0.005
		430-1R	RS1 + RS2	32	0.000	3.328	0.814±0.003
		506-2R	RS1 + NK1	31	0.042	–	0.919±0.003
		507-1L	ES2 + NK1	28	0.052	–	0.855±0.006
		513-1L	NK1 + NK2	30	0.011	–	0.837±0.006
		514-1R	ES1 + ES2	30	0.002	3.122	0.841±0.006
		514-2L	NK2 + ES1	30	0.049	6.441	0.896±0.011
	<i>m+m</i>	521-1R	ES1 + NK1	37	0.049	–	0.822±0.015
		320-2R	DT1 + DT2	27	0.004	5.495	0.908±0.010
321-2L		DT1 + DT4	27	0.001	3.884	0.776±0.030	
520-1R		DT1 + DT2	32	0.000	7.913	0.929±0.002	
Ave. (±SD)			30.2 (2.51)	0.018 (0.021)	7.189 (3.990)	0.868 (0.0503)	
Inter-lineage	<i>j+f</i>	404-2L	KM2 + CB1	30.5	0.096	NA	0.927±0.003
		416-1L	RS1 + YW2	27.5	0.090	4.040	0.877±0.005
		422-2L	CB1 + YW4	30	0.099	16.644	0.920±0.003
		423-1R	YW1 + RS1	30.5	0.090	4.944	0.856±0.011
		423-2R	YW1 + CB1	32	0.094	14.870	0.917±0.006
		429-1R	CB1 + YW3	33	0.095	15.734	0.819±0.005
		430-2L	YW3 + NK1	30.5	0.104	–	0.872±0.005
		506-1L	CB1 + OM	31	0.096	14.843	0.877±0.013
		507-2R	ES2 + YW3	29	0.097	7.512	0.930±0.008
		514-2R	OM1 + ES2	30	0.099	7.601	0.946±0.006
		521-1L	OM1 + NK1	37	0.111	–	0.861±0.019
		<i>j+m</i>	321-1R	KM1 + DT1	27	0.151	8.242
	321-2R		KM2 + DT2	27	0.145	NA	0.940±0.005
	328-1R		DT4 + KM1	30.5	0.150	0.000	0.850±0.003
	328-2R		DT2 + KM1	30.5	0.149	0.698	0.951±0.008
	329-2R		KM3 + DT1	30.5	0.148	4.135	0.950±0.008
	404-1L		DT4 + YW1	30	0.150	0.698	0.859±0.003
	404-2R		DT1 + CB1	27	0.163	15.276	0.959±0.006
	520-1L		OM1 + DT1	32	0.002	9.010	0.908±0.006
	<i>f+m</i>		422-1L	DT1 + RS1	28	0.147	9.109
		422-1R	DT2 + CB1	29	0.162	NA	0.905±0.005
430-2R		DT2 + NK1	28	0.151	–	0.907±0.007	
507-2L		ES2 + DT2	28.5	0.160	10.187	0.956±0.006	
513-2L		DT1 + NK2	30	0.142	6.010	0.943±0.010	
513-2R		DT1 + ES2	30	0.161	11.030	0.940±0.007	
Ave. (±SD)			30.0 (2.18)	0.128 (0.028)	8.602 (5.470)	0.911 (0.041)	
			29.26 (5.14)	0.0834 (0.060)	8.007 (4.887)	0.894 (0.049)	

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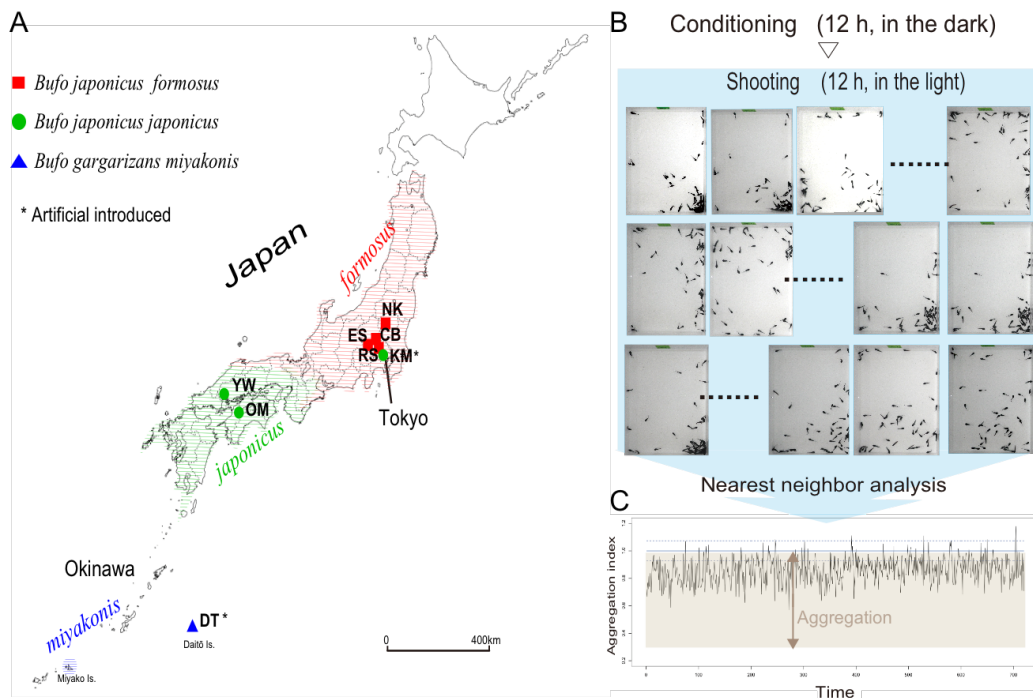
501

502 **Figure 1**

503 Diagrammatic illustration of the experiments: (A) Map of sampling sites. (B) Practical

504 example of the aggregation test. This diagram depicts one trial, which was conducted

505 three times (720×3 shots). (C) An example of the analysis of the 720 images.



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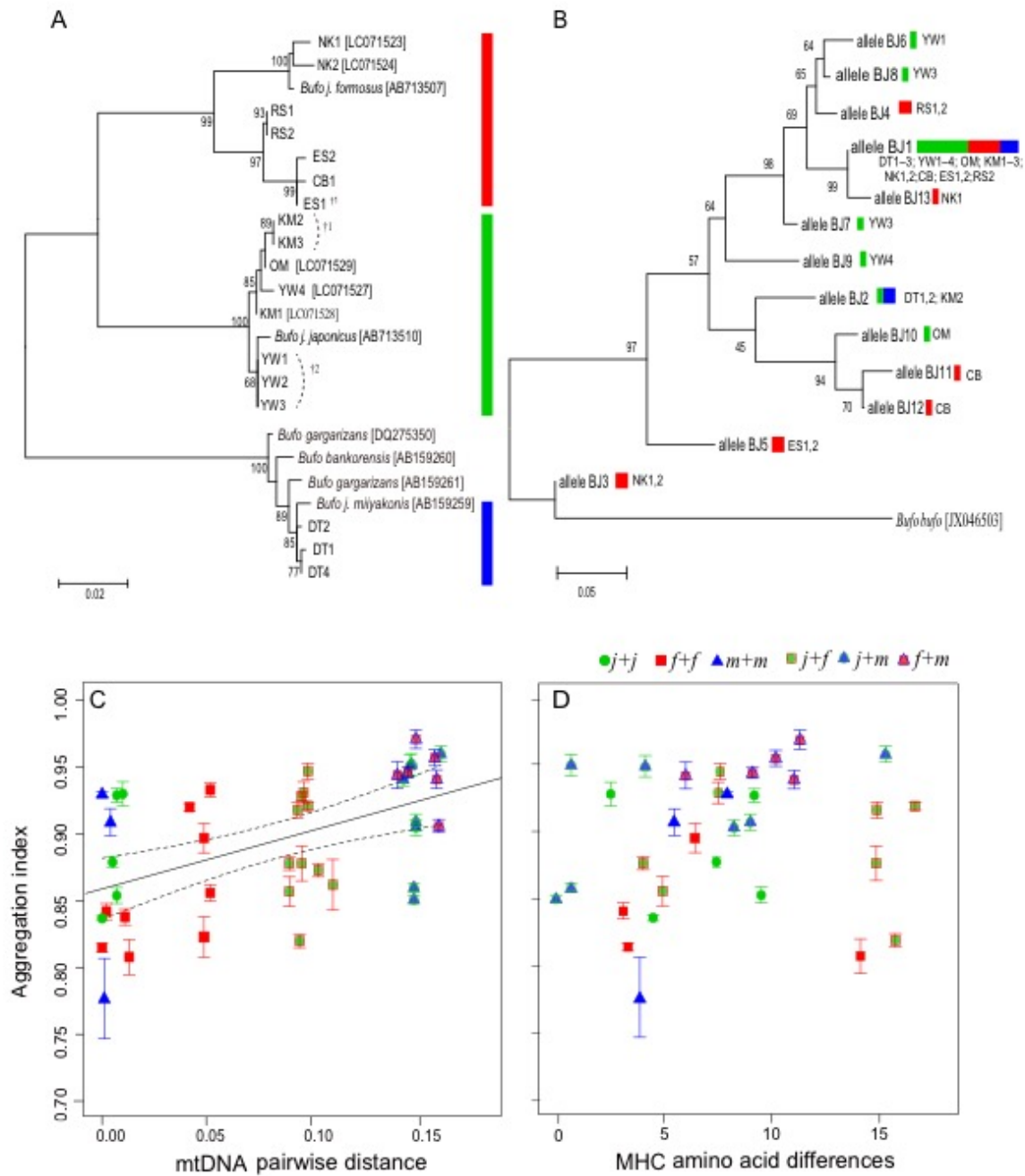
507 **Figure 2**

508 (A) Maximum likelihood (ML) trees of the mitochondrial DNA (mtDNA) lineage of 18
509 clutches based on 831 bp of Cytb sequences. (B) ML tree of major histocompatibility
510 complex (MHC) alleles based on 324 bp of class II exon partial sequences. The width of the
511 colored bar on the right side of each allele ID corresponds to the number of identified
512 clutches. The color represents the attribution of the mtDNA lineage of the identified clutches
513 corresponding to the mtDNA tree, where green, red, and blue indicate the *japonicus*,
514 *formosus*, and *miyakonis* lineages, respectively. From the 18 clutches, 13 MHC alleles
515 presented complex relations with the mtDNA lineages; the BJ1 allele was distributed
516 throughout all lineages. Both trees were estimated using the GTR + Γ model, selected
517 according to the Akaike information criterion value in MEGA 5.0. Support values for the
518 internal nodes were inferred from 1,000 bootstrap replicates. ^{†1} and ^{†2} correspond to the
519 haplotypes from Hase et al. (2012) and (2013), respectively.
520 and genetic diversity. (C) Relationship between aggregation behavior (mean of
521 aggregation index) and mtDNA pairwise distances in the tested cohorts ($n = 42$, $r = 0.524$;
522 $P < 0.001$; $y = 0.429x + 0.858$). Regression lines were calculated for significant
523 Spearman's rank correlation coefficients and the dashed lines indicate two-sided 95%
524 confidence intervals. (D) Aggregation index and MHC class II allele amino acid
525 differences in non-sib cohorts ($n = 31$, $r = 0.149$; $P = 0.424$). Error bars represent standard
526 errors within each cohort for repeated trials. The plot marks indicate the lineage attributes
527 for each cohort of laboratory-reared sets (see Table 1).

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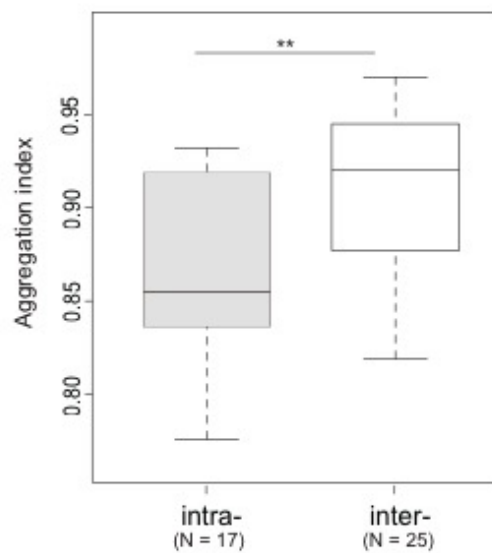
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534 **Figure 3**

535 Boxplots of the aggregation index values of the intra-lineage cohorts (median = 0.872,
536 mean \pm SD = 0.878 ± 0.050) and inter-lineage cohorts (median = 0.920, mean \pm SD =
537 0.911 ± 0.04). The classification of intra- and inter-lineages correspond to those in Table
538 1. ** $P < 0.001$ (GLMMs).



540 **Electronic supplemental material file**

541 Supplementary Tables S1 and S2, and Figure S1.