1 **Absence of heterosis in hybrid crested newts**

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3 Running title – Absence of heterosis in hybrid newts

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Abstract

We investigated relationshipsRelationships between phylogenetic relatedness, hybrid zone spatial structure, the amount of interspecific gene flow and population demography were investigated, with the newt genus *Triturus* as a model system. AIn earlier work, a bimodal hybrid zone of two distantly related species in France combined low interspecific gene flow with hybrid sterility and heterosis, whereas a suite of unimodal hybrid zones in closely related *Triturus* showed more or less extensive introgressive hybridization with no evidence for heterosis. We here report on population demography and interspecific gene flow in two *Triturus* species (*T. macedonicus* and *T. ivanbureschi* in Serbia) that are moderately related, engage in a heterogeneous uni-/bimodal hybrid zone and hence represent an intermediate situation. TheThis study withused 13 diagnostic nuclear genetic markers ofin a population at the species contact zone. This showed that all individuals were hybrids, with no parentals detected. Age, size and longevity and the estimated growth curves are as in purenot exceeding that of the parental species populations, so that we conclude to the absence of heterosis in *T. macedonicus - T. ivanbureschi*. Observations across the genus support the hypothesis that fertile hybrids allocate resources to reproduction and infertile hybrids allocate resources to growth. Several *Triturus* species hybrid zones not yet studied allow the testing of this hypothesis.

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46 **Introduction**

47 Hybrid zones are regions where genetically distinct populations meet and hybridize (Barton and Hewitt,

48 1985; Harrison, 1993). They provide natural settings for the study of speciation. In particular they allow

49 research on the consequences of new, previously untested genetic combinations of differentiated genomes

50 in nature. Hybrid zones may take several forms, from long and narrow zones to large areas of overlap and

51 mosaics (Arnold, 1992). The general positive relationship between the degree to which hybridization

52 proceeds and the phylogenetic relatedness of the species involved is well established (Jiggins and Mallet,

53 2000). In spite of some variation in the strength of reproductive isolation for individuals from spatially

54 isolated populations, or among different species pairs from the same evolutionary lineages (Seehausen et

55 al., 2014; Kearns et al., 2018) accumulated evidence accumulatessuggests that, in diploid animals,

56 reproductive isolation increases and introgression between lineages decreases with divergence (Singhal

57 and Moritz, 2013; Arntzen et al., 2014; Beysard and Heckel, 2014; Dufresnes et al., 2014; Montanari et

58 al., 2014; Taylor et al. 2014). Unfortunately, much less is known about how species' ecological

59 preferences - and with that species distributions and local spatial configurations - may affect hybridization

60 and *vice versa*, and how phylogenetic relatedness affects the interaction with ecology. Yet, such

61 knowledge is relevant for the understanding of hybrid zones and the evolutionary inferences we draw

62 from them. As Barton and Hewitt (1985) proposed '… as soon as the loss of fitness through hybridization

63 inside the zone becomes small enough … the zone will collapse into broad sympatry', ~~so~~. Accordingly,

64 the more closely related hybridizing species are, the more hybridization will take place, over yet smaller

65 areas. A negative relationship between the ~~level~~degree of hybridization and the amount range overlap was

66 indeed noted by Zuiderwijk (1980) in a variety of European amphibian species pairs, but geographical

67 variation and ecological differences remain to be studied.

68 We propose that a good group to investigate if reproductive isolation accumulates gradually

69 would fulfill the following requirements. It would i) be monophyletic and ii) show more or less

70 contiguous species ranges where iii) closely as well as distantly related species engage in hybrid zones

71 and iv) ecological profiles of the species are different. Finally, v) to reduce the impact of spatial scale a

72 low dispersal capability would be an asset. One group that qualifies is the European newt genus *Triturus*.

73 The genus is diverse and has a pan-European distribution, with outer ranges up to the Atlantic and the

74 Mediterranean and reaching into Scandinavia, Russia, the Caucasus and Iran. The species group inner

75 ranges form a patchwork and all nine species are, in one place or the other, involved in intra-generic

76 hybridization (Arntzen et al., 2014; Wielstra et al., 2014b). The genus *Triturus* is composed of three

77 clades, namely the *T. marmoratus* group (or marbled newts) with two species (clade A), the *T. cristatus*

78 group with four species (clade B) and the *T. karelinii* group with three species (clade C). Clades A and B

79 engage in western Europe and clades B and C meet up in southeastern Europe (Arntzen, 2003; Wielstra et

80 al., 2014b). See Figure 1 for species distributions and phylogenetic relationships.

81 The A and B clades are distantly related with an estimated 27.6 Ma period of lineage

82 independence (Wielstra and Arntzen, 2011). At their hybrid zone in France, *T. marmoratus (*clade A) and

83 *T. cristatus* (clade B) interspecies F_1 hybrids are infrequent (ca. 4 % of the total population) and

84 introgression is rare (< 0.1 %, Arntzen et al., 2009). ~~The~~Heterosis is the increased vigour in a cross

85 between two genetically differentiated lines as compared with either of the parental lines. The *T. cristatus*

86 *– T. marmoratus* F_1 hybrids show hybrid vigour in body size and longevity, that is, they get older and

87 larger ~~and older~~ than their parents (Francillon-Vieillot et al., 1990; Figure ~~2~~2A). The *T. cristatus – T.*

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88 *marmoratus* hybrid zone is bimodal, i.e. with predominantly the parental species and few hybrids, with a

89 well-understood ecological differentiation. Forested and hilly areas are mostly occupied by *T.*

90 *marmoratus* and open and flat areas have *T. cristatus* (Schoorl and Zuiderwijk, 1981; Visser et al., 2017).

91 Representatives of clades B and C - together known as crested newts - show an intermediate level of

92 phylogenetic relatedness with 10.4 Ma of lineage independence. TheEuropean species involved in the

93 contact are *T. cristatus, T. dobrogicus* and *T. macedonicus* in clade B and *T. ivanbureschi* in clade C.

94 Among these four, *T. dobrogicus* stands out as a lowland species (Vörös et al., 2016). The species meet

95 up in the Balkan peninsula along the lower Danube and in a wide zone running from Belgrade (Serbia) to

96 Thessaloniki (Greece) (Figure 1). Species within the B and C clades have 8.8 – 5.3 Ma of lineage

97 independence and engage in a variety of more or less unimodal hybrid zones across Europe, i.e. with a

98 majority of hybrids and Asiafew or no parentals (Arntzen et al., 2014 and Wielstra et al., 2017ab), with no

99 evidence for heterosis (hybrid vigor – forso far. For data reviews in *Triturus* see Arntzen, (2000;) and

100 Lukanov and Tzankov, (2016).

101

102 **Materials and methods**

103 Post-metamorphic crested newts were collectedcaught with funnel traps from March to July 2013 in a

104 pond near the village Vlasi in the southeast of Serbia (43.00 N, 22.64 E, altitude 468 m a.s.l.). The focal

105 newt population we henceforth refer to as 'Vlasi'. A morphological classification on the basis ofIn total,

106 336 individuals were measured and marked and a small part of the newt's tail at its tip was taken for

107 genetic analyses (Arntzen et al., 1999). The population sample was classified in five groups determined

108 from external morphology and colouration characteristics (Arntzen andwith documented phenotypes as a starting point

109 (Wallis, 1999) was in five groups as and Arntzen, 1989; Arntzen & Wallis, 1999; Arntzen, 2003; Wielstra et al., 2013b). Classes were

110 as follows: group 1 - *T. macedonicus*-like (group 1, N=26), group 2 - leaning towards *T. macedonicus* (group 2, N=48),

111 angroup 3 - intermediate phenotype (group 3, N=146), group 4 - a phenotype leaning towards *T. ivanbureschi* (group

112 4, N=83) and group 5 - *T. ivanbureschi*-like (group 5, N=33). A small part of the newt's tail at its tip was taken for genetic analyses. Permission for fieldwork for making and to collect

113 tissue samples was obtained from the Ministry of Energy, Development and Environmental Protection of

114 the Republic of Serbia (permit no. 353-01- 35/2013-08).

115 Molecular data were gathered for a panel of nuclear encoded SNP markers (see below). We

116 included reference samples with N=3 for seven populations of *T. ivanbureschi* and seven populations of

117 *T. macedonicus* available from Wielstra et al. (~~2013~~2013a) (Table 1). Reference populations were located

118 to the south of the Vlasi population, ~~to stay away~~distant from other, more northerly distributed *Triturus*

119 species. Wielstra et al. (2014a) produced sequence data for 52 short (ca. 140 bp) nuclear markers

120 positioned in 3'UTR regions of protein-coding genes for three individuals from four populations

121 positioned throughout the ranges of both *T. ivanbureschi* and *T. macedonicus*. We focussed on the subset

122 of 24 nuclear markers with species diagnostic allele variants for *T. ivanbureschi* and *T. macedonicus*.

123 Additionally an mtDNA SNP (*nd4*) was designed from Sanger sequence data taken from Wielstra et al.

124 (~~2013~~2013a). We determined diagnostic SNPs by checking the sequence alignments by eye in MacClade

125 4.08 (Maddison and Maddison, 2005).

126 Genotyping was conducted commercially at the SNP genotyping facility of the Institute of

127 Biology, Leiden University, using the Kompetitive Allele-Specific PCR (KASP) genotyping system

128 (LGC KBioscience, UK). This involves fluorescence-based genotyping using SNP-specific primers. We

129 used the program Kraken to design two forward or reverse primers that are specific for (i.e. with a final

130 base complementary to) one of the two potential SNP variants, in addition to a general reverse or forward

131 primer (Semagn et al., 2014). We genotyped 378 crested newts in total: 21 individuals of both parental

132 species (the eight populations on which SNP identification was based plus an additional six populations)

133 and 336 individuals from Vlasi. Sequence alignments and Kraken input for all markers are available in

134 Suppl. Inf. 1. Raw output of the KASP genotyping protocol is in Suppl. Inf. 2. The Ion Torrent next-

135 generation sequence data of Wielstra et al. (2014a) used for SNP discovery are available from Dryad

136 Digital Repository entry http://dx.doi.org/10.5061/dryad.36775. The genetic and morphological data used

137 in the analysis are presented in Suppl. Inf. 3 and 4.

138 ~~The program GePos analyses of the Wilson plot in (Wallace plot in (D Rannala Rand, 1995)~~The program New HybridLA and Tompo 202) was based upon

139 of the Vlasi population consisting of recently formed hybrids. Reference populations were invoked with

140 the 'z-option'~~.~~, otherwise settings were default. The program HIest was used to estimate heterozygosity

141 (H) and ancestry (S) (Fitzpatrick, 2012). To evaluate the consistency and independence of the genetic data

142 and to assess hybrid zone structure Hardy-Weinberg equilibrium (HW) and linkage disequilibrium (LD)

143 were quantified with GenePop (Raymond and Rousset, 1995).

144 All animals encountered were marked by clipping the middle toe on the right foot for the purpose

145 of a population size estimate by the capture-recapture method, with results not here reported. In a

146 subsample of individuals (N=170, 77 males, 85 females ~~plus eight~~and eight small individuals with

147 secondary sexual characters not expressed, i.e. juveniles) that represented the five morphological groups,

148 a phalanx of the ~~third~~ toe was taken for age determination by skeletochronology. Lines of arrested

149 ~~growh~~growth (LAGs) were counted as in Francillon-Viellot et al. (1990) ~~and we~~Endosteal resorption was

150 estimated by comparing the diameters of eroded marrow cavities of adults with the diameters of non-

151 eroded marrow cavities of juveniles and was observed in 75 males (97.4%), 72 females (91.7 %) and two

152 juveniles. Individual ages were estimated taking endosteal resorption into account. We also determined

153 age at maturity from 'rapprochement'. This is the tightening of LAGs that is associated with the shift of

154 resources from growth to reproduction. ~~Rapprochement was not~~In some cases we observed a line formed

155 at metamorphosis. Some examples are in ~~13 individuals (8.0 %).~~ Figure 3.

156 Body size was measured in mm from the tip of the snout up to the posterior end of the cloaca

157 (snout vent length, SVl). Sexual dimorphism was estimated with the Lovich and Gibbons (1992) sexual

158 dimorphism index SDi = (mean length of the larger sex / mean length of the smaller sex) minus unity, and

159 arbitrarily defined as positive when females are larger than males and negative in the reverse case. Von

160 Bertalanffy growth curves were determined with the function 'growthmodels' in the R package FSA

161 (~~FSA, 2017).~~R Core Team, 2013; FSA, 2017). Since the morphological groups that we distinguished

162 showed no marked genetic differentiation (see below) the data on size, age and growth were pooled for

163 both sexes. The few juveniles in the sample remained unsexed and were excluded from the analyses.

164 Confidence intervals were estimated by bootstrapping with nlstools in 2000 bootstrap iterations (Nlstools,

165 2015). ~~Other statistical analyses were with~~Differences between morphological groups in the level of

166 estimated ancestry and heterozygosity were tested by ANOVA. The relationship between level of

167 heterozygosity and SVl was tested by the Pearson correlation coefficient. ANOVA and correlations were

168 done with SAS software (SAS, 2011).

169

Results

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171 Out of the candidate nuclear markers two (*cnppd* and *kdm3*) were dropped because a diagnostic SNP

172 could not be identified and for four (*ace*, *eif4ebp2*, *ssh2* and *syncrip*) the Kraken software could not

173 design a suitable set of primers. For the 18 remaining diagnostic nuclear markers for which assays could

174 be designed, PCR amplification for one (*amot*) failed. The locus *slc25* yielded a high frequency of

175 missing data (16%) in the reference populations, suggesting it did not amplify the *T. ivanbureschi*-allele

176 well and results for this marker were also discarded. For the remainder missing data amounted to 1.8%.

177 The four loci *ahe*, *ddx17*, *dnaj* and *sre* showed highly significant LD for all six pairwise combinations.

178 This result was interpreted as tight physical linkage in a single linkage group. To increase data

179 independence results for the locus *ahe* (with the fewest missing data) were retained and the others

180 dismissed. Subsequent tests for HW and LD yielded no ~~significances~~significant comparisons under

181 Bonferroni correction. The KASP genotyping output for the remaining 13 markers is summarized in

182 Suppl. Inf. 3. One nuclear SNP marker (*col18*) showed a single instance of heterozygosity in a parental

183 individual, suggesting either a genotyping error or a marker that is not fully diagnostic. With N=82 (21.7

184 %) of missing data the mtDNA marker (*nd4*) performed relatively poorly. All newts from the Vlasi

185 population that could be genotyped possessed the mtDNA haplotype typical for *T. ivanbureschi*, as

186 expected for this marker in this system (for background information see Wielstra et al., 2017a).

187 In NewHybrids none of the individuals from the Vlasi population were allocated to either of the

188 parental species, to the F$_1$ hybrid class or to the class of backcrosses towards *T. macedonicus*. Several

189 individuals were allocated to the 'backcross to *T. ivanbureshi* class' (n=8, 2.4%). The ~~far~~ majority of

190 individuals was classified as F_2 hybrids (n=328, 97.6%). When NewHybrids was instructed to take not

191 two but three generations into account, the majority (99.7%) was allocated to that third generation. HIest

192 grouped all Vlasi individuals in the middle of the ancestry times heterozygosity bivariate plot, somewhat

193 off-center in the direction of *T. ivanbureschi* (Figure 34), with the reference populations in the lower left

194 (pure *T. macedonicus*) and lower right corners (pure *T. ivanbureschi*). To explore possible differences

195 between morphological groups in the level of estimated ancestry (S) and heterozygosity (H) ANOVA was

196 used to test for differences in H and S over five (groups 1-5) orand three morphological groups (groups 1,

197 2-4 pooled, 5) with no statistically significant results (P > 0.05). Pearson correlation coefficients

198 betweenfor S and SVl and the two HIest parametersH and SVl were also not significant, for males as well

199 as for females.

200   The body size distribution showed that females were significantly larger than males (SVl males

201 63.9 mm, SVl females 67.5 mm; Student's t-test P < 0.0001; SDi = 0.056). The age distribution for males

202 and females showed no significant difference (Mann-Whitney U-test, P > 0.05; Table 2). In both sexes >

203 70% of the individuals had an estimated age of 5, 6 or 7 years. Age at maturity estimated by

204 rapprochement was mostly 2, 3 or 4 years in both sexes (average males 3.1, n=75; average for

205 malesfemales 3.0, n=74; Suppl. Inf. 4.). NoThe absence of an observable rapprochement was more

206 frequent in females (1412.9 %) than in males (2.6 %) (*P* < 0.05, G-test of independence), perhaps

207 suggesting a more regularless drastic shift in resource allocation in the transition from growth to

208 reproduction in females. Longevity was 13 years in males and 11 years in females. The parameters that

209 describe the Von Bertalanffy growth curves and thetheir confidence intervals are presented in Table

210 3Suppl. Inf. 5. Results for the Vlasi populations were compared with data from the literature (Cvetković

211 et al., 1996 and A. Ivanović, unpublished data on *T. macedonicus* and Üzüm, 2006 and Üzüm and Olgun,

212 2009 on *T. ivanbureschi*). In three out of four populations females are larger than males of the same age.

213 The other growth curves for populations and species are similar, with no more than 10 mm difference in

214 SVl among the most different groups (Figure 42B). Unlike *T. cristatus* x *T. marmoratus* hybrids, the *T.*

215 *macedonicus* x *T. ivanbureschi* hybrids showed no heterosis. Longevity increased over species aswas

216 higher in *T. cristatus macedonicus* than in *T. marmoratus, T. ivanbureschi* and *T. macedonicus* (Figures 2 and 4), with interspecific hybrids from the Vlasi population

217 taking an intermediate position.

218

Discussion

220 We encountered a population of crested newts near Vlasi in southeastern Serbia that showed

221 largeextensive phenotypic variation and - being close to the territory of both *T. macedonicus* and *T.*

222 *ivanbureschi* - a hybrid nature was assumed. We were, however, unable to determine the extent of

223 hybridization from morphologymorphological and colouration characters and yet wanted to find out if

224 this hybrid zone population has a unimodal character (with a majority of hybrids and few or no parentals),

225 a bimodal character (with predominantly the parental species and few hybrids), or something in between.

226 We were also interested in fitness consequences that hybridization might have on the demography of the

227 population, in particular if heterosis would be combined with hybrid sterility.

228 All individuals from the focal population were allocated to the second generation of hybrids,

229 indicating strong admixture along with the absence of recent crossings between the parental species.

230 When a third generation was an option, individuals where allocated to that third generation, further

231 supporting the absence of recent hybrids. Looking yet deeper into the coalescence would require more

232 genetic markers. AncestryAn ancestry versus heterozygosity plotsplot (Figure 4) further support that

233 genotypes in this Vlasi population result from many generations of admixture. In the absence of selection

234 and banningexcluding physical linkage LD halves every next generation and will barely be measurable after a few

235 generations. (Barton and Gale, 1993). Accordingly, the absence of admixture LD also suggests that

236 hybridization between *T. macedonicus* and *T. ivanbureschi* has been going onongoing for more than a couple of

237 generations.

238 hybridized population was also suggested by the absence of genetically diagnosable morphological

239 groups. These results suggest that *T. macedonicus* and *T. ivanbureschi* engage in a unimodal hybrid zone.

240 The genetic affiliation of the Vlasi population is somewhat closer to *T. ivanbureschi* than to *T.*

241 *macedonicus* (Figure 34). This is in line with the documented distributions of the two species where the

242 Vlasi population is geographically closer to *T. ivanbureschi* than to *T. macedonicus* (Wielstra et al.,

243 2014b). However, the exact position of the center and the width of the cline remain to be documented.

244 This result contrasts with the situation to the northwest, where the two species engage over a wide area of

245 species replacement, including. The mosaic distribution includes a *T. ivanbureschi* enclave that was cut off from theits

246 main distribution by superseding *T. macedonicus* (Arntzen, 2003; Arntzen & Wallis 1999, cf. Figure 1).

247 The sole transmission of the mitochondrial haplotype typical for *T. ivanbureschi*, that we here confirmed

248 for the Vlasi hybrid population, helped to reconstruct thea scenario in which T. *macedonicus* advanced at the

249 expense of species replacement*T. ivanbureschi* (Wielstra et al., 2017a).

250   Some *Triturus* species engage in smooth clinal transitions with rampant hybridization confined to

251 a narrow zone and others overlap in a spatial mosaic with hybridization infrequent. An example of a

252 mosaic (or 'bimodal') hybrid zone is found for the distantly related *T. cristatus* and *T. marmoratus* in

253 France. Narrow zones with rampantNarrow zones with extensive hybridization are found at contacts

254 between crested newts species belonging to clades B and C (Arntzen et al., 2014; Wielstra et al., 2017b,

255 cf. Figure 1). A relationship appears to exist with species relatedness, so that closely related species form

256 clines and more distant species engage in a mosaic distribution. like previously described for *T. cristatus*

257 and *T. marmoratus.* The presence of a unimodal (clinal) as well as bimodal (mosaic) hybrid zone structure

258 in the *T. macedonicus - T. ivanbureschi* contact is in line with an intermediate level of relatedness.

259

260 **Conclusion**

261 As here demonstrated here, the *T. macedonicus - T. ivanbureschi* hybrids are fertile, producing new

262 genetic combinations for testingthat themselves reproduce in nature. Conversely, hybridization in a

263 narrow hybrid zone. This result for a comparison of the B and C clades contrasts with the results obtained

264 earlier for *T. cristatus* and *T. marmoratus* in the A and B clades, where hybridization in is an evolutionary

265 dead-end because their. These hybrids are largely sterile and introgression is near-absent. (Arntzen &

266 Wallis, 1991; Arntzen et al., 2009). We propose that in the genus *Triturus* heterosis is restricted to hybrids

267 that are infertile and that even as adults also in adult stage direct their resources more to growth than

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268 to reproduction. This is more likely to be the case for genetically incompatible than for genetically

269 compatible species. The ~~demonstration of heterosis in any of the six or seven~~testing grounds for this

270 assertion are several unimodal hybrid zones of closely related *Triturus* species~~-~~, i.e. within the A and B

271 clades (Figure 1) where demonstration of heterosis would contradict ~~this explanation~~our hypothesis.

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399   **Suppl. Inf. 1 -** Input consensus sequences for Kraken.

400   **Suppl. Inf. 2 -** Raw output of the KASP genotyping protocol.

401   **Suppl. Inf. 3 -** Genetic data in GenePop format.

402   **Suppl. Inf. 4 -** Skeletochronology results along with morphometric data and individual Structure scores.

403   **Suppl. Inf. 5 -** Parameter values and 95% confidence intervals (CI) in the Von Bertalanffy growth curves

404   for *Triturus* populations in the west and southeast of the genus' range.

405

406   Figure 1. Distribution of nine *Triturus* species over Europe and the Near East (after Wielstra et al., 2011).

407   Major clades are A, the marbled newts, B, the *T. cristatus* species group of crested newts and C, the *T.*

408   *karelinii* species group of crested newts. Note that the spatial contact of clades is limited to central France

409   (clades A and B) and the southern Balkans (B and C). Heterosis in size and longevity was observed in A x

410   B hybrids (Mayenne, France, white dot, Francillon-Vieillot et al., 1990) and not in B x C hybrids (Vlasi,

411   Serbia, asterisk, present paper). The insert shows the species phylogeny.

412

413   Figure 2. Von Bertalanffy growth curves for ~~males and females of the~~ *Triturus* newts. A - *Triturus*

414   *cristatus* (~~red).,~~orange), *T. marmoratus* (green~~).~~) and *Triturus cristatus - T. marmoratus* F$_1$ hybrids (black)

415   from Mayenne, France. Data are from Francillon-Vieillot et al. (1990). Females are larger than males of

416   the same age. The hybrids ~~are~~become larger and ~~in general~~ older than the parental species. No model

417   could be fitted for *T. marmoratus* females. B - *Triturus macedonicus* x *T. ivanbureschi* hybrid population

418   from Vlasi in southeastern Serbia (black lines), *T. macedonicus* from Montenegro (pink) and *T.*

419   *ivanbureschi* populations (blue) at localities Keşan and Klaros. Reference data are from Cvetković et al.

420 (1996); Üzüm (2006); Üzüm and Olgun (2009) and A. Ivanović, unpublished data). Females are larger

421 than males of the same age in three out of four populations. The Vlasi populations of hybrids shows no

422 evidence for hybrid vigour in size, growth or longevity. The parameters that describe the Von Bertalanffy

423 growth curves and the confidence intervals are presented in Suppl. Inf. 5.Data are from Francillon-

424 Vieillot et al. (1990).

425 Figure 3.

426 Figure 3. Cross-sections of a phalange of a juvenile, male and female *Triturus macedonicus* x *T.*

427 *ivanbureschi* crested newt from Vlasi. Abbreviataions are: m.c. - marrow cavity, m.l. - metamorphosis

428 line, e.r. - endosteal resorption, p. - periphery, rap. - rapprochement. Note that the periphery is not

429 regarded as a LAG. Sections are 18 μm thick and taken at the level of the diaphysis. A) Three-year-old

430 juvenile, SVl 48 mm. Three LAGs (arrow heads) are observed in the periosteal bone as well as a

431 metamorphosis line (arrow). B) Five-year old male, SVl 64 mm. Four LAGs are observed in the

432 periosteal bone (arrow heads). Endosteal resorption present (arrow). C) Six-year old female, SVL 64 mm.

433 Five LAGs are observed in the periosteal bone (arrow heads). Endosteal resorption and rapprochement

434 are also marked by arrows.

435

436 Figure 4. Ancestry versus heterozygosity plot based on 13 species diagnostic nuclear genetic markers.

437 Individuals from the Vlasi population are shown by open round symbols (N=336). The left corner of the

438 triangle corresponds to *Triturus macedonicus* (solid square symbols, N=21), the right corner to *T.*

439 *ivanbureschi* (solid triangle symbol, N=21) and the upper corner to (non-observed) F_1 hybrids.