Homeotic transformations and number changes in the vertebral column of *Triturus* newts

Maja Slijepčević, Frietson Galis, Jan W Arntzen, Ana Ivanović

We explored intraspecific variation in vertebral formula, more specifically the variation in number of thoracic vertebrae and frequencies of transitional sacral vertebrae in *Triturus* newts (Caudata: Salamandridae). Within salamandrid salamanders this monophyletic group shows highest disparity in the number of thoracic vertebrae and considerable intraspecific variation in the number of thoracic vertebrae. *Triturus* species also differ in their ecological preferences, from predominantly terrestrial to largely aquatic. Following Geoffroy St. Hilaire's and Darwin's rule that structures with a large number of serially homologous repetitive elements are more variable than structures with smaller numbers, we hypothesized that variation in vertebral formula increase in more elongated species with larger number of thoracic vertebrae. As transitional vertebrae at thoraco-sacral boundary are initial changes toward a complete homeotic transformations resulting in number changes, we furthermore hypothesized that the frequency of transitional vertebrae will be correlated with the variation of the number of thoracic vertebrae within species i.e., we expected a higher frequency of transitional vertebrae in elongated species. We also investigated potential effects of hybridization on the vertebral formula. Percentage of individuals with the number of thoracic vertebrae different from the modal number and range of variation in number of vertebrae significantly increased in species with larger number of thoracic vertebrae. Contrary to our expectation, the frequencies of transitional vertebrae were not correlated with frequencies of change in the complete vertebrae number. The frequency of transitional sacral vertebra in hybrids did not significantly differ from that of the parental species. Such a pattern could be a result of selection pressure against transitional vertebrae and/or bias towards the development of full vertebrae numbers. Although our data indicate relaxed selection for vertebral count changes in more elongated, aquatic species, more data on different selective pressures in species with different numbers of vertebrae in the two contrasting, terrestrial and aquatic environments are needed to test for causality.

1   **Homeotic transformations and number changes in the vertebral column of**

2   ***Triturus* newts**

3

4   Maja D. Slijepčević[1], Frietson Galis[2], Jan W. Arntzen[2] and Ana Ivanović[2,3]

5

6   [1]Institute for Biological Research "Siniša Stanković", University of Belgrade, Serbia
7   [2]Naturalis Biodiversity Center, P. O. Box 9517, 2300 RA Leiden, The Netherlands
8   [3]Institute of Zoology, Faculty of Biology, University of Belgrade, Serbia
9
10   Corresponding author
11   Ana Ivanović,
12   ana@bio.bg.ac.rs
13

14   Abstract

15   We explored intraspecific variation in vertebral formula, more specifically the variation in

16   number of thoracic vertebrae and frequencies of transitional sacral vertebrae in *Triturus* newts

17   (Caudata: Salamandridae). Within salamandrid salamanders this monophyletic group shows

18   highest disparity in the number of thoracic vertebrae and considerable intraspecific variation in

19   the number of thoracic vertebrae. *Triturus* species also differ in their ecological preferences, from

20   predominantly terrestrial to largely aquatic. Following Geoffroy St. Hilaire's and Darwin's rule

21   that structures with a large number of serially homologous repetitive elements are more variable

22   than structures with smaller numbers, we hypothesized that variation in vertebral formula

23   increase in more elongated species with larger number of thoracic vertebrae. As transitional

24   vertebrae at thoraco-sacral boundary are initial changes toward a complete homeotic

25   transformations resulting in number changes, we furthermore hypothesized that the frequency of

26   transitional vertebrae will be correlated with the variation of the number of thoracic vertebrae

27   within species i.e., we expected a higher frequency of transitional vertebrae in elongated species.

28   We also investigated potential effects of hybridization on the vertebral formula. Percentage of

29   individuals with the number of thoracic vertebrae different from the modal number and range of

30   variation in number of vertebrae significantly increased in species with larger number of thoracic

31   vertebrae. Contrary to our expectation, the frequencies of transitional vertebrae were not

32   correlated with frequencies of change in the complete vertebrae number. The frequency of

33   transitional sacral vertebra in hybrids did not significantly differ from that of the parental species.

34   Such a pattern could be a result of selection pressure against transitional vertebrae and/or bias

35   towards the development of full vertebrae numbers. Although our data indicate relaxed selection

36   for vertebral count changes in more elongated, aquatic species, more data on different selective

37   pressures in species with different numbers of vertebrae in the two contrasting, terrestrial and

38   aquatic environments are needed to test for causality.

39   **Introduction**

40   The vertebral column consists of repetitive, serially homologous skeletal elements – vertebrae.

41   Along the anterior-posterior axis, vertebrae are grouped in regions, with a conserved order and

42   specific sizes and shapes (e.g. Starck, 1979). The strong regionalization of the vertebral column is

43   already found early in the evolution of tetrapods (Ahlberg, Clack & Blom, 2005). In the early

44   tetrapod *Ichthyostega*, five regions (cervical, thoracic, lumbar, sacral and caudal) can be

45   recognized as in many extant amniotes.

46   The determination of the number of vertebrae and their identity is mediated by *Hox* genes

47   through complex genetic mechanisms that include various signaling molecules (e.g. Dubrulle,

48   McGrew & Pourquié, 2001; Diezdel-Corral et al., 2003; Aulehla & Pourquié, 2010; Mallo,

49   Wellik & Deschamps, 2010; Woltering, 2012). The generation of somites occurs in a sequential

50   head-to-tail order and is coupled to the simultaneous head-to-tail patterning of these somites, both

51   under the influence of head-to-tail signaling gradients. Therefore, changes in the number and

52   identity of the vertebrae necessarily involve homeotic transformations, except in the last-formed

53   tail region (Varela-Lasheras et al., 2011; Ten Broek et al., 2012). Initial mutations for homeotic

54   transformations usually lead to incomplete homeotic transformations, resulting in transitional

55   vertebral identities.

56   In mammals, changes of the number of cervical vertebrae are associated with deleterious

57   pleiotropic effects that lead to selection against such homeotic transformations (Galis & Metz,

58   2003; Varela-Lasheras et al., 2011). In two mammalian groups (sloths and manatees) with

59   extremely low activity and metabolic rates, the exceptional numbers of cervical vertebrae most

60   likely resulted from the effective breaking of pleiotropic constraints due to a relaxation of

61   stabilizing selection against the pleiotropic effects (congenital abnormalities, Varela-Lasheras et

62   al., 2011). Furthermore, Galis et al. (2014) concluded that biomechanical problems associated

63   with initial homeotic transformations (transitional vertebrae) in fast running mammals result in

64   strong selection against changes of the presacral vertebral count in these species.

65   In other tetrapods the number of vertebrae in different regions can be more variable. A well-

66   known example is the variable count of cervical vertebrae in birds (Woolfenden, 1961), or

67   variable number of presacral vertebrae in squamates (Carroll, 1997; Müller et al., 2010). In both

68   cases, these regions have a large number of vertebrae. Isidore Geoffroy St. Hilaire has postulated

69   that as a rule - structures with a large number of serially homologous repetitive elements are more

70   variable than structures with smaller numbers (Isidore Geoffroy St. Hilaire, 1932). This notion

71   was supported by Darwin (Darwin, 1860). Along the same line, Bateson (1894) concluded that

72   series containing large numbers of undifferentiated parts are more variable than series made up of

73   a few, more differentiated parts.

74   In tailed amphibians, the presacral vertebrae vary in their number but only little in shape. A

75   single, sacral vertebra is morphologically very similar to the vertebrae from the thoracic region,

76   with more robust processes for attachment of the sacral ribs which are also thicker than regular

77   thoracic ribs. There is considerable intraspecific variation in the number of thoracic vertebrae in

78   many species of salamanders (Adolphi, 1898; Gerecht, 1929; Peabody & Brodie, 1975; Jockusch,

79   1997; Litvinchuk & Borkin, 2003). Intraspecific variation originally results from homeotic

80   transformations that are subsequently maintained in the population. Frequently, transitional

81   vertebrae at the thoraco-sacral boundary have been reported (Adolphi, 1898; Gerecht, 1929;

82   Highton, 1960; Worthington, 1971; Jockush, 1997; Arntzen et al., 2015). Such transitional

83   vertebrae with partial thoracic and partial sacral identity result from incomplete homeotic

84   transformations. Therefore, the frequencies of transitional vertebrae could be related to the

85   amount of variation in the number of thoracic vertebrae within species. Data on changes in axial

86   pattering and homeotic transformations in amphibians are relatively scarce; yet data on

87   amphibians are necessary for understanding the evolution of axial pattering in tetrapods.

88   In this study we explore the relationship between variation in the number of thoracic vertebrae

89   and transitional sacral vertebrae using eight species of the monophyletic genus *Triturus* newts as

90   a model system. Within the family Salamandridae, which is the second most diverse group of

91   tailed amphibians, *Triturus* newts are the most disparate in the number of thoracic vertebrae

92   (Arntzen et al., 2015). *Triturus* species form a morphocline from predominantly terrestrial *T.*

93   *marmoratus, T. pygmaeus* with a short and stout body and 12 thoracic vertebrae to slender and

94   elongated largely aquatic *T. cristatus* and *T. dobrogicus* with 15-17 thoracic vertebrae (Arntzen,

95   2003). *Triturus* species also display considerable intraspecific variation in vertebrae numbers

96 (Gerecht, 1929; Crnobrnja et al., 1997; Arntzen et al 2015). Moreover, there is a well-

97 documented hybridization of two sympatric *Triturus* species, *T. marmoratus* (12 thoracic

98 vertebrae) and *T. cristatus* (15 thoracic vertebrae) (Arntzen et al., 2009). The hybridization of

99 these two species leads to sterile F1 hybrids with intermediate morphologies and numbers of

100 thoracic vertebrae (Vallée, 1959). Interspecific hybridization at contact zones also occurs

101 between other *Triturus* species with parapatric distributions (Mikulíček et al., 2012; Arntzen,

102 Wielstra & Wallis, 2014), providing the opportunity to investigate relationship between number

103 changes and frequencies of transitional sacral vertebrae. Here, we compared variation in the

104 number of thoracic vertebrae and transitional sacral vertebrae among *Triturus* species, *T.*

105 *marmoratus* × *T. cristatus* F1 hybrids with parental species, and populations from contact zone

106 with central populations.

107 More specifically, we explored the intra- and interspecific variation in number of the thoracic

108 vertebrae and frequencies of transitional vertebrae at thoraco-sacral boundary to test the

109 following hypothesis:

110 1. Species with more vertebrae in the thoracic region are more variable in the number of thoracic

111 vertebrae than those with fewer vertebrae in the thoracic region.

112 2. Transitional vertebrae are more frequent in species with more variable number of thoracic

113 vertebrae than in less variable species. In hybrids, we expected that range of variation in

114 vertebrae number will overlap with the ranges of variation in parental species and higher

115 frequencies of transitional vertebrae comparing to parental species. In species with parapatric

116 distribution we expected higher variation in vertebrae number and higher frequencies of

117 transitional vertebrae in populations from contact zone comparing to the central populations.

118

119 **Materials and Methods**

120 *Triturus newts and their characteristics*

121 The vertebral column in *Triturus* newts is differentiated in: cervical region– consisting of a single

122 anterior vertebra (atlas); thoracic region –rib-bearing thoracic vertebrae; sacral region – usually a

123 single vertebra with well-developed stout transverse processes for the attachment of sacral ribs

124 and pelvic girdle; caudosacral region – up to three vertebrae that continues caudal to the sacral

125 vertebra that are associated with the pelvic musculature and cloaca; and caudal region – the

126 remaining vertebrae in the tail (Fig. 1). The body elongation in *Triturus* species appears to be

127 correlated with the length of the aquatic phase – more terrestrial species have a short and stout

128 trunk with relatively longer legs compared to species with a more aquatic life style which have a

129 more elongated trunk and relatively shorter legs. Body elongation involves a larger number of

130 thoracic vertebrae. More specifically, the number of thoracic vertebrae in the vertebral formula

131 varies from 12 in *T. marmoratus* and *T. pygmaeus*, which have a short aquatic phase (*T.*

132 *marmoratus* only two months), 13 in *T. karelinii* and *T. ivanbureschi*, 14 in T. *macedonicus* and

133 *T. carnifex*, 15 in *T. cristatus* to 16 or 17 in *T. dobrogicus,* the most aquatic species which have

134 six months long aquatic phase (Arntzen, 2003) (Fig. 2).

135 The distribution of the genus *Triturus* is well documented (Arntzen, Wielstra & Wallis, 2014).

136 *Triturus cristatus* and *T. marmoratus* have a broader contact zone in France and these two species

137 could be found widely in syntopy (Arntzen & Wallis, 1991; Lescure & de Massary, 2012).

138 Between other species hybrid zones are generally narrow, and a weak negative relationship

139 between the level of hybridization and genetic distance for species pairs (Arntzen, Wielstra &

140 Wallis, 2014).

141 *Material analysed*

142 We analysed axial skeletons of 1436 adult newts that originate from 126 populations of all eight

143 species of *Triturus* newts (Fig. 3). For this study we analysed X-ray images of good quality and

144 cleared and stained skeletons. The X-ray images were obtained on a Faxitron 43855C/D with an

145 exposure of 20–40s at 3 mA and 70kV. Other material was cleared with trypsin and KOH and

146 stained with Alizarin Red S for bone deposition (Dingerkus & Uhler, 1977) and stored in

147 glycerine. Analyzed specimens are from the batrachological collection of the Institute for

148 Biological Research "SinišaStanković", Belgrade, Serbia (N = 601) and from the collection of the

149 Naturalis Biodiversity Center, Leiden, Netherlands (N = 835). Our material covers the

150 geographic, taxonomic, and molecular genetic diversity of the genus. On the basis of well

151 documented species distributions (Mikulíček et al., 2012; Arntzen, Wielstra & Wallis, 2014)

152 populations were assigned as "central" or "fringe" based upon their geographical position away

153 (>= 50km) or close to (< 50 km) congeneric species. For localities and sample size per population

154 see Supplementary Table S1.

155

156 *Scoring vertebral formula and transitional thoraco-sacral vertebrae*

We determined the vertebral formula by counting the number of cervical (C), thoracic (T) and sacral vertebrae (S). The caudosacral and caudal regions are excluded from our formula as the detailed inspection of cleared and stained specimens showed that variable number of caudosacral vertebrae frequently bear small, much reduced ribs which could be fused with transverse processes, and cannot always be distinguished on X-ray images. The number of tail vertebreae was available only for the subset of specimen as in the most of the specimens tail tip was removed for enzyme electrophoretic analyses, or it was broken or damaged.

Homeotic transformations of thoracic vertebra into sacral vertebra, or vice versa (transitional sacral vertebra having half of the identity of thoracic vertebra and half of the identity of sacral vertebra) was scored as 0.5 and added to the number of complete thoracic vertebrae. The transitional vertebrae were restricted to those vertebrae which showed a complete change of identity on one side of the vertebrae (on one side thoracic and on one side sacral) i.e. only clear and full cases were declared as transitional. Right side asymmetry of a sacral vertebra is when the thoracic rib is present on the right side and the sacral rib on the left side of transitional thoraco-sacral vertebra and vice versa for left side asymmetry (for 3D model of regular and transitional thoraco-sacral vertebra obtained by CT-scanning see Supplementary data).

We assumed that the frequency of these transitional vertebrae with complete change of identity at one side of vertebra reflects the total amount of homeotic transformations which could have a series of transitional morphologies, but could not be precisely scored on X-ray images.

Statistical analyses

The Spearman correlation coefficient (r_s) was used to quantify correlation between species modal numbers of thoracic vertebrae (nT) and 1) the percentage of individuals with the number of complete thoracic vertebrae different from the modal number (varT) and 2) the range of variation in the number of thoracic vertebrae (rangeT) within species. The same measure was used to quantify the relationship between percentages of transitional sacral vertebrae (trS) and varT and rangeT.

To test for differences between hybrids and parental species across fringe and central populations we performed the G-test of independence.

To analyse interspecific variation in a phylogenetic context, we used a well resolved time-calibrated phylogeny of genus *Triturus* (Fig. 2). Associations derived from common ancestry were evaluated by calculating the strength of the phylogenetic signal for analysed variables (nT,

188 varT, rangeT and trS). The procedure involves the random permutation of the variables over the

189 terminal units of the phylogenetic tree (10,000 iterations), in which the test statistic is the total

190 amount of squared change summed over all branches of the tree. We applied the phylogenetic

191 independent contrasts approach (Felsenstein, 1985) to obtain a set of independent contrasts of

192 analysed variables. The obtained independent contrast were regressed to each other, which

193 allowed exploring the association between 1) the evolutionary change in the number of the

194 thoracic vertebra in vertebral formula (nT) and amount of variation in the number of thoracic

195 vertebrae (varT and rangeT) and 2) variation in the number of thoracic vertebrae (varT and

196 rangeT) and frequencies of transitional vertebrae (trS), taking the similarity due to shared

197 ancestry into account.

198

199 **Results**

200 *Vertebral formula and transitional sacral vertebra in <u>Triturus</u> newts*

201 The most common vertebral formulas were 1C 12T 1S in *T. marmoratus* and *T. pygmaeus*, 1C

202 13T 1S in *T. karelinii* and *T. ivanbureschi*, 1C 14T 1S in *T. macedonicus* and *T. carnifex*, 1C 15T

203 1S in *T. cristatus* and 1C 17T 1S in *T. dobrogicus* (See Fig. 2 and Table 1). For the number of

204 thoracic vertebrae within species, varT and rangeT see Table 1. The variation in the vertebrae

205 number per population is shown in Supplementary Table S2.

206 Frequencies of recorded homeotic transformations in *Triturus* newt species are listed in Table 2.

207 The least common is the homeotic transformations of cervical vertebra (the thoracic rib is

208 attached to both sides or to one side of the vertebra), recorded in 6 specimens (0.41%) and in four

209 out of eight species.

210 Two types of transitional vertebrae at the thoraco-sacral boundary were recorded. The first type

211 involves changes of two succeeding vertebrae - transitional sacral vertebra with thoracic rib at

212 one side and sacral rib at the other side, followed by transitional vertebra having sacral rib at one

213 side (opposite than previous vertebra) and no rib attached on the other side (see Figs 1 and 4).

214 The second type of transitional sacral vertebra involves transitional thoraco-sacral vertebra, with

215 a thoracic rib at one side and a sacral rib at the other, followed by regular sacral vertebra.

216 The transitional changes involving two succeeding vertebrae (named as transitional sacral) are

217 more frequent than transitional changes of thoracic to sacral vertebra without changes of sacral

218 vertebra. We recorded a transitional sacrum in 70 out of 1368 specimens (5.1%) excluding F1

219   hybrids. Right as well as left side asymmetries were recorded (Table 2). We found that nT and

220   varT are significantly positively correlated ($r_s = 0.75$, $p = 0.023$), indicating that species with

221   more vertebrae in the thoracic region are more variable in the number of vertebrae. Also, a

222   significant correlation was found between nT and rangeT ($r_s = 0.90$, $p = 0.002$) indicating that

223   range of variation significantly increase in species with more thoracic vertebrae. However, we did

224   not find a correlation between trS and varT ($r_s = 0.31$, $p=0.46$) or between trS and rangeT ($r_s = $

225   0.13, $p = 0.76$).

226   *Phylogentic comparative analyses*

227   We found a statistically significant phylogenetic signal in nT, $p = 0.013$) and rangeT, $p = 0.033$)

228   and no significant phylogenetic signal in varT, $p = 0.730$) and trS, $p = 0.970$). The regression of

229   1) independent contrasts of varT on independent contrasts of nT, $p = 0.018$) and 2) regression of

230   independent contrast of rangeT on independent contrasts of nT ($p = 0.006$) revealed that

231   significant relationship between increase in the number of thoracic vertebrae and amount of

232   variation in vertebrae number exist. We found no statistically significant relationship between

233   independent contrast of rangeT and independent contrast of trS ($p = 0.413$).

234

235   *Hybridization and variation in vertebral formula*

236

237   There were statistically significant differences in changes in vertebral formula between "central"

238   and "fringe" populations (G-test for goodness of fit, $G = 18.61$, $p = 0.001$). For fringe

239   populations, the range of variation in number of thoracic vertebrae is 12-15 for *T. ivanbureschi*,

240   13-16 for *T. macedonicus*, 13-15 for *T. carnifex*, 13-17 for *T. cristatus* and 14-18 for *T.*

241   *dobrogicus*. When "central" and "fringe" populations were compared for species with a

242   sufficiently large sample sizes a statistical significant result was obtained for *T. dobrogicus* and

243   *T. ivanbureschi* (Table 4). In *T.cristatus× T.marmoratus* hybrids, most of the specimens (88.2%)

244   have vertebral formula with intermediate numbers of thoracic vertebrae (Table 3). Six out of 68

245   hybrids (8.8%) possess incomplete homeotic transformations. Of these, one specimen has an

246   incomplete transformation of cervical vertebra into thoracic vertebra, while the most frequent

247   incomplete homeotic transformation involves an asymmetrical sacrum. The frequencies of

248   transitional sacral vertebra in hybrids and parental species are similar (G-test for goodness of fit,

249    $G = 0.95$, $p = 0.33$).

250

Discussion

252 Our study on variation in vertebrae number in *Triturus* newts shows substantial variation in the
253 number of thoracic vertebrae, indicating that there is no strong selection against changes in the
254 number of thoracic vertebrae. In agreement with the postulations of Geoffroy St. Hilaire, Darwin
255 and Bateson, the variation in the vertebral column is positively correlated to the number of
256 vertebrae. With independent contrasts we observed a statistically significant, correlated
257 evolutionary change in increase in the modal number of thoracic vertebrae and variation in
258 vertebrae number. The ranges of variation in the number of thoracic vertebrae also significantly
259 increase in species with larger modal numbers of thoracic vertebrae. In *T. marmoratus* and *T.*
260 *pygmaeus* the variation is limited to one extra thoracic vertebra (12-13) while in crested newt
261 species both increases and decreases in number from the modal number were recorded, with total
262 range up to four vertebrae (see Table 1). Although such a pattern of intraspecific variation
263 coincides with the general rules of "high number" variation, the observed pattern may at the same
264 time represent an association between vertebral number variation and the strength of selection in
265 various habitats in *Triturus* newts. As noted before, species differ in the time they spend on land
266 and in the aquatic environment. During the terrestrial phase limbs support the weight of the body
267 and provide forward propulsion by the synchronous use of diagonal limb pairs, although in most
268 species the amount of walking is quite limited. In an aquatic environment *Triturus* species move
269 by tail propulsion (Gvoždik & Van Damme, 2006) with limbs tightly held against the body to
270 reduce hydrodynamic drag. Longer aquatic phases with locomotion in water where legs (and
271 pelvic girdle) do not support body weight, may relax selection pressures against changes of the
272 axial skeleton. Therefore, the selection pressures related to specific biomechanical requirements
273 may be different with respect to the duration of each phase and this may be causally involved in
274 why more aquatic species with larger number of thoracic vertebrae have higher variation in
275 vertebral numbers comparing to terrestrial ones. Although our data indicate relaxed selection for
276 vertebral count in more aquatic species, more data on different selective pressures in these two
277 contrasting, terrestrial and aquatic environments are needed to understand why this is so.

278 *Frequencies of transitional vertebrae*

279 The frequency of transitional changes at cervico-thoracic boundary is more than ten times lower
280 than changes at thoraco-sacral boundary. This is also observed in for other salamanders (Wake &

281 Lawson, 1973) and mammals (Galis et al., 2006). This pattern may be explained by stronger

282 interactivity of developmental processes at the early developmental stages when the cervical

283 vertebra is determined (Galis et al., 2006). At later stages, development is much less interactive

284 and more modular, such that changes are expected to be associated with fewer pleiotropic effects.

285 We do not know the cause of the constraint on the number of cervical and sacral vertebra in tailed

286 amphibians, but further studies in various amphibian groups that will consider survival rates of

287 individuals with changes in cervical and sacral region across ontogenetic stages should provide

288 valuable data to solve this issue.

289 Although we hypothesized that frequencies of transitional vertebrae at the thoraco-sacral

290 boundary should be correlated to the range of variation in the number of thoracic vertebrae as in

291 mammals (Ten Broek et al., 2012), no correlation was found. Available literature data indicate

292 that incomplete homeotic transformation of sacral vertebra are relatively common, with up to

293 10% across the various salamander lineages: 4.5% in *Batrachoseps attenuates* (Jockusch, 1997),

294 5.7% in *Rhyacotritonolympicus* (Worthington, 1971), 6% in *Plethodoncinereus* (Highton, 1960),

295 up to 9% for newt genera *Lissotriton* and *Ichthyosaura* (Arntzen et al., 2015) and between 1.9%

296 and 9.0% in *Triturus* newts (Table 1). The lower than expected incidence of transitional vertebrae

297 could result from developmental mechanisms favoring complete numbers of thoracic vertebrae

298 and/or from selection against transitional sacral vertebrae due to associated problems related to an

299 asymmetric sacrum (c.f. Galis et al., 2014). Potential problems associated to asymmetrical

300 sacrum might arise due to asymmetrical muscles attachments, blood vessels and innervation, or

301 biomechanical problems during locomotion. In salamanders, the selection pressures related to

302 specific biomechanical requirements are probably different in fully aquatic larvae and

303 metamorphosed individuals that spend most of their time on land. Furthermore, selection

304 pressures may vary with respect to the duration of annual aquatic and terrestrial phase. More

305 detailed morphological and functional studies of locomotion of larval and metamorphic stages

306 could throw more light on the functional significance of variation in axial skeleton in *Triturus*

307 newts.

308 However, it is possible that our results are biased as we have not included the full range of

309 transitional vertebrae. We scored only easily identifiable transitional vertebrae with complete

310 morphological transformations of one side of the vertebra under the assumption that the

311 frequency of these transitional vertebrae reflects the total amount of homeotic transformations.

312   Nonetheless, initial mutations for homeotic transformations can lead to a whole series of

313   gradually transitional homeotic transformations; in the case of thoraco-sacral vertebrae ranging

314   from predominantly thoracic and only slightly sacral to predominantly sacral and slightly

315   thoracic. Inclusion of all transitional vertebral morphologies might change the observed

316   relationship between incomplete homeotic transformations and number changes in newts.

317   Possibly this explains the difference with mammals, where there is a positive relationship

318   between the number of transitional vertebrae and overall variation.

319

320   *The effects of hybridization and marginality on frequencies of homeotic transformations*

321   Hybridization and marginality significantly increase variability in the number of thoracic

322   vertebrae but there is no change in the frequency of transitional vertebrae. Crosses between *T.*

323   *cristatus* (15 vertebrae, range 13-16) and *T. marmoratus* (12 vertebrae, range 12-13) produced

324   phenotypes with 13 thoracic vertebrae, an intermediate number. It is interesting to note that 13

325   thoracic vertebrae is the only number that is shared by both parental species. In *T. cristatus* × *T.*

326   *marmoratus* offspring there is considerable mortality and almost all of F1 hybrids (~90%) had *T.*

327   *cristatus* as mother. The *marmoratus*-mothered specimens were all male, due to low survival of

328   female embryos (Arntzen et al., 2009). Developmental anomalies in *T. cristatus* × *T. marmoratus*

329   crosses, including more digital anomalies compared with parental species (hybrids 16.9%,

330   parental species pooled 5.4%) (Vallée, 1959) are observed, and therefore, the higher number of

331   changes in the axial skeleton may be related to a generally higher number of anomalies. The high

332   mortality may also influence the incidence of the variability and transitional vertebrae.

333   Increased range of variation in the number of thoracic vertebrae was found in fringe populations

334   of *T. ivanbureschi, T. macedonicus, T. cristatus* and *T. dobrogicus*. The only exception is *T.*

335   *carnifex* with an even lower range in variation in the number of thoracic vertebrae in fringe than

336   in central populations. Significantly higher frequency of changes in vertebral formula in fringe

337   populations of *T. ivanbureschi* and *T. dobrogicus* species may well have to do with the confirmed

338   presence of hybridization in the contact zones of *T. cristatus* and *T. dobrogicu*s populations

339   (Mikulíček et al., 2012), of *T. carnifex* and *T. dobrogicus* populations (Wallis & Arntzen, 1989)

340   and of *T. ivanbureschi* and *T. macedonicus* populations (Arntzen, Wielstra & Wallis, 2014).

341   Hence, it is not known whether the higher variation in the fringe populations is entirely due to

342 hybridization or to other factors such as suboptimal ecological factors at the edge of a species

343 distribution.

344 In conclusion, *Triturus* newts have a relatively large amount of variation in the number of

345 thoracic vertebrae, both with respect to the frequency that non-modal numbers of vertebrae occur

346 and the range of variation in the number of vertebrae. In agreement with Geoffroy St. Hilaire's

347 rule the variation was larger in species with larger number of thoracic vertebrae. The absence of a

348 correlation between frequencies of homeotic changes (transitional sacral vertebrae, trS) and

349 variation in the number of vertebrae (varT, rangeT) could be a result of developmental

350 mechanisms favoring complete numbers of presacral vertebrae and/or selection against

351 transitional vertebrae in this group of tailed amphibians.

352

Acknowledgements

358

References

Adolphi H. 1898. Über das Wandern der Extremitätenplexus und des Sacrum bei Triton taeniatus. *Morphologisches Jahrbuch* **25**:115–142.

Ahlberg PE, Clack JA, Blom H. 2005. The axial skeleton of the Devonian tetrapod Ichthyostega. *Nature* **437**: 137-140 DOI:10.1038/nature.03893.

Arntzen JW, Wallis GP. 1991. Restricted gene flow in a moving hybrid zone of the newts *Triturus cristatus* and *T. marmoratus* in western France. *Evolution* **45**: 805-826.

Arntzen JW. 2003. *Triturus cristatus* Superspezies — Kammolch-Artenkreis. (Triturus cristatus (Laurenti, 1768) — Nirdlicherkammolch, Triturus macedonicus (Laurenti, 1768) - Italienischerkammolch. Triturus dobrogicus (Kiritzescu, 1903) — Donau-Kammolch, Triturus karelinii (Strauch, 1870) — Sudlicherkammolch). In: W. Böhme, ed. Handbuch der Reptilien und Amphibien Europas. Wiebelsheim: Aula-Verlag, 421-514.

Arntzen JW, Jehle R, Bardakci F, Burke T, Wallis GP. 2009. Asymmetric viability of reciprocal-cross hybrids between crested and marbled newts (*Triturus cristatus* and *T. marmoratus*). *Evolution* **63**: 1191-202 DOI: 10.1111/j.1558-5646.2009.00611.x.

Arntzen JW, Wielstra B, Wallis GP. 2014. The modality of nine *Triturus* newt hybrid zones assessed with nuclear, mitochondrial and morphological data. *Biological Journal of the Linnean Society* **113**: 604-622 DOI: 10.1111/bij.12358.

Arntzen JW, Beukema W, Galis F, Ivanović A. 2015. Vertebral number is highly evolvable in salamanders and newts (family Salamandridae) and variably associated with climatic parameters. *Contributions to Zoology* **84** (2): 87-116.

Aulehla A, Pourquié O. 2010. Signaling gradients during paraxial mesoderm development. *Cold Spring Harbor perspectives in biology* 2 (2): DOI:10.1101/cshperspect.a000869.

Bateson W. 1894. *Materials for the study of variation*. London: MacMillan.

Carroll RL. 1997. *Patterns and Processes of Vertebrate Evolution*. Cambridge: Cambridge university press.

Crnobrnja J, Džukić G, Krstić N, Kalezić ML. 1997. Evolutionary and palegeographic effects on the distribution of the *Triturus cristatus* superspecies in the central Balkans. *Amphibia-Reptilia* **18** (4): 321-332 DOI: 10.1163/156853897X00378.

Darwin CR. 1860. *The Origin of Species*. New York: D. Appleton and Company.

389 **Diezdel-Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K. 2003**.

390   Opposing FGF and retinoid pathways control ventral neural pattern, neuronal

391   differentiation, and segmentation during body axis extension. *Neuron* **40** (1): 65-79.

392 **Dingerkus G, Uhler LD. 1977**. Enzyme clearing of alcian blue stained whole small vertebrates

393   for demonstration of cartilage. *Stain Technology* **52** (4): 229–232.

394 **Dubrulle J, McGrew MJ, Pourquié O. 2001**. FGF signaling controls somite boundary position

395   and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**

396   (2):219-32.

397 **Felsenstein J. 1985**. Phylogenies and the comparative method. *American Naturalist* **1**: 15.

398 **Galis F, Metz JAJ. 2003**. Anti-cancer selection as a source of developmental and evolutionary

399   constraints. *Bioessays* **25** (11): 1035-1039.

400 **Galis F, Van Dooren TJ, Feuth JD, Metz JA,Witkam A, Ruinard S, Steigenga MJ,**

401   **Wijnaendts LC. 2006**. Extreme selection in humans against homeotic transformations of

402   cervical vertebrae. *Evolution* **60** (12): 2643–2654.

403 **Galis F, Carrier DR, Alphen J, Van der Mije SD, Doorn TV, Metz JAJ, Ten Broek CMA.**

404   **2014**. Fast running restricts evolutionary change of the vertebral column in mammals.

405   *PNAS* **111** (31): 11401-11406 DOI: 10.1073/pnas.1401392111.

406 **Geoffroy SHI. 1832**. *Histoire générale et particulière des anomalies de l'organisation chez*

407   *l'homme et les animaux*. Paris: J. –B. Baillère, Libraire de l' Academie Royale de

408   Médecine.

409 **Gerecht A. 1929**. Über die Richtung der phyletischenWanderung der Sakralregionbei *Triton*

410   *cristatus* und *Triton taeniatus*. *Bulletin de la Société de biologie de Lettonie* 1(1):9-19.

411 **Gvoždik L,Van Damme R. 2006**. *Triturus* newts defy the running-swimming dilemma.

412   *Evolution* **60** (10): 2110-21.

413 **Highton R. 1960**. Heritability of geographic variation in trunk segmentation in the red- backed

414   salamander, *Plethodoncinereus*. *Evolution* **14**: 351-360.

415 **Jockusch EL. 1997**. Geographic variation and phenotypic plasticity of number of trunk vertebrae

416   in slender salamanders, *Batrachoseps* (Caudata: Plethodontidae). *Evolution* **51**(6): 1966

417   DOI:10.2307/2411017.

418 **Lescure J, de Massary J-C. 2012.** *Atlas des Amphibiens et Reptiles de France*. Paris: Muséum

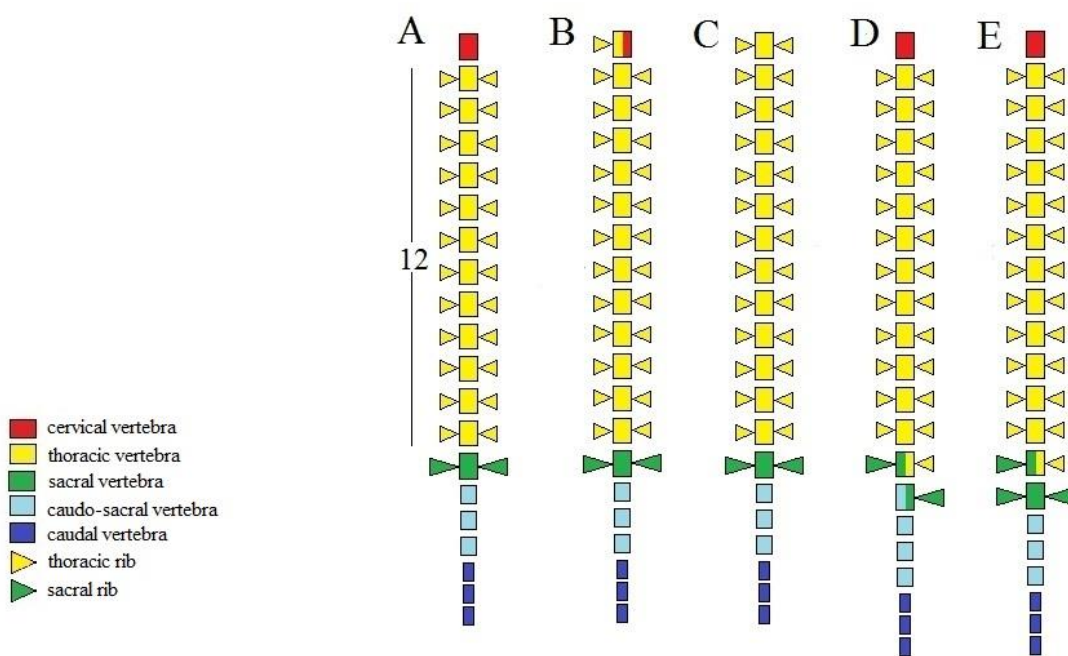419   national d'Histoire naturelle.

420   **Litvinchuk SN, Borkin LJ. 2003**.Variation in number of trunk vertebrae and in count of costal
421   grooves in salamanders of the family Hynobiidae. *Contributions to Zoology* **72** (4):195-
422   209.
423   **Mallo M, Wellik D, Deschamps J. 2010**. Hox genes and regional patterning of the vertebrate
424   body plan. *Developmental biology* **344** (1): 7-15 DOI: 10.1016/j.ydbio.2010.04.024. .
425   **Mikulíček P, Horák A, Zavadil V, Kautman J, Piálek J. 2012**. Hybridization between three
426   crested newt species (*Triturus cristatus* superspecies) in the Czech Republic and
427   Slovakia: comparison of nuclear markers and mitochondrial DNA. *Folia Zoologica* **61** (3-
428   4): 202-218.
429   **MüllerJ, Scheyer TM, Head JJ, Barrett PM, Werneburg I, Ericson PGP, Pol D, Sanchez-**
430   **Villagra MR. 2010**. Homeotic effects, somitogenesis and the evolution of vertebral
431   numbers in recent and fossil amniotes. *PNAS* **107** (5): 2118-2123.
432   **Peabody RB, Brodie ED. 1975**. Effect of temperature, salinity and photoperiod on the number
433   of trunk vertebrae in *Ambystoma maculatum*. *Copeia* **1975** (4): 741-746.
434   **Starck D. 1979**. *Vergleichende Anatomie der Wirbeltiere*. Berlin: Springer Verlag.
435   **Ten Broek CM, Bakker AJ,Varela-Lasheras I, Bugiani M, Van Dongen S, Galis F. 2012**.
436   Evo-Devo of the human vertebral column: on homeotic transformations, pathologies and
437   prenatal selection. *Evolutionary Biology* **39**(4): 456-471.
438   **Vallée L. 1959**. Recherches sur *Triturus blasii* de l'Isle, hybride naturel de *Triturus cristatus*
439   Laur. x *Triturus marmoratus* Latr.Mém. *Bulletin de la Société zoologique de France* **31**:
440   1-95.
441   **Varela-Lasheras I, Bakker A, Mije S, Metz J, Alphen J, Galis F. 2011**. Breaking evolutionary
442   and pleiotropic constraints in mammals: On sloths, manatees and homeotic mutations.
443   *EvoDevo* **2**: 11.
444   **Wallis GP, Arntzen JW. 1989**. Mitochondrial-DNA variation in the crested newt superspecies:
445   limited cytoplasmic gene flow among species. *Evolution* **43**: 88–10 DOI:
446   10.2307/2409166.
447   **Wake DB, Lawson R. 1973**. Developmental and adult morphology of the vertebral column in
448   the plethodontid salamander *Eurycea bislineata*, with comments on vertebral evolution in
449   the amphibian. *Journal of Morphology* **139** (3):251–29 DOI: 10.1002/jmor.1051390302.
450

451   **Woltering JM. 2012**. From lizard to snake; behind the evolution of an extreme body plan.

452   *Current Genomics* **13** (4): 289-299 DOI:10.2174/138920212800793302.

453   **Woolfenden GE. 1961**. Postcranial osteology of the waterfowl. *Bulletin of the Florida State*

454   *Museum* **6**: (1)-129.

455   **Worthington RD. 1971**. *Postmetamorphic changes in the vertebrae of the marbled salamander*

456   *Ambystoma opacum Gravenhorst (Amphibia, Caudata).* El Paso: Texas Western Press.
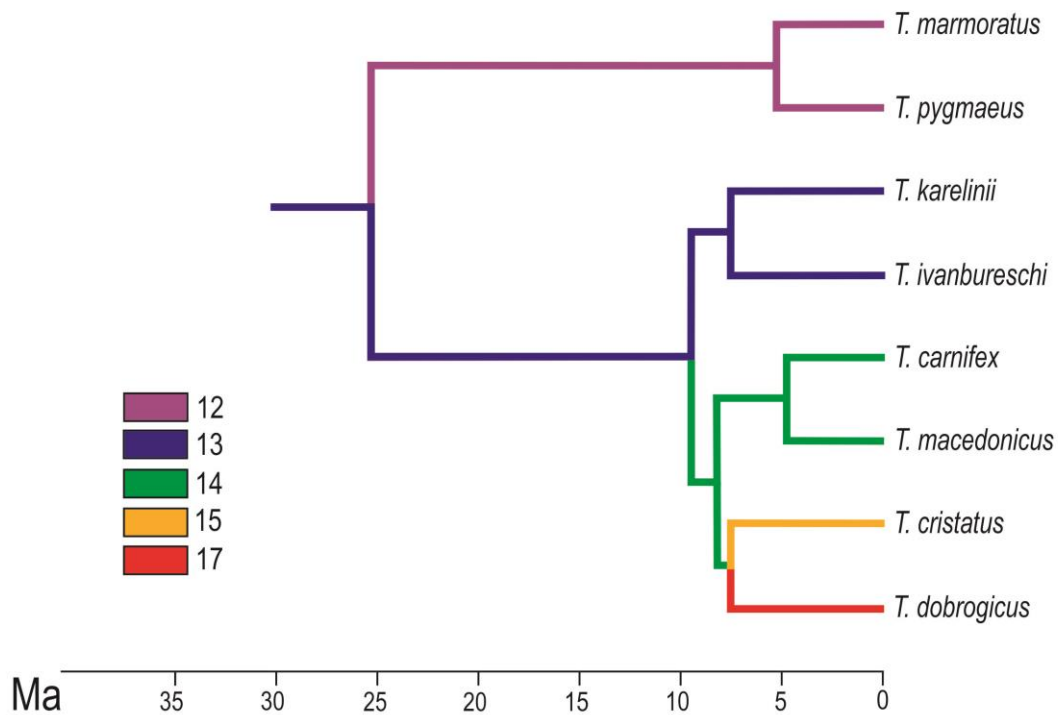
457

458   Figure 1.

459   The regionalization of the vertebral column in *Triturus* newts and schematic presentation of

460   scored homeotic transformations (example of *T. marmoratus*). A) vertebral column without

461   homeotic transformation and regular number of vertebrae - first three caudal vertebrae are shown;

462   B) incomplete homeotic transformations of cervical vertebra into thoracic; C) complete homeotic

463   transformation of cervical into thoracic vertebra; D) transitional sacral vertebra with thoracic rib

464   at one side and sacral rib at the other side followed by transitional vertebra with sacral rib at the

465   one side and no rib at the other; E) transitional thoraco-sacral vertebra, with thoracic rib at one

466   side and sacral rib at the other, followed by regular sacral vertebra.

467

468   Figure 2.

469   Calibrated phylogeny for *Triturus* newts species, lifted out from time calibrated Salamandridae

470   phylogeny (Arntzen et al., 2015). Modal numbers of thoracic vertebrae are color coded as in that

471   paper.

472

473   Figure 3.

474   Distribution of eight *Triturus* species across Europe and adjacent Asia. Studied populations are

475   marked by solid dots (central populations) and empty dots (fringe populations). For detailed

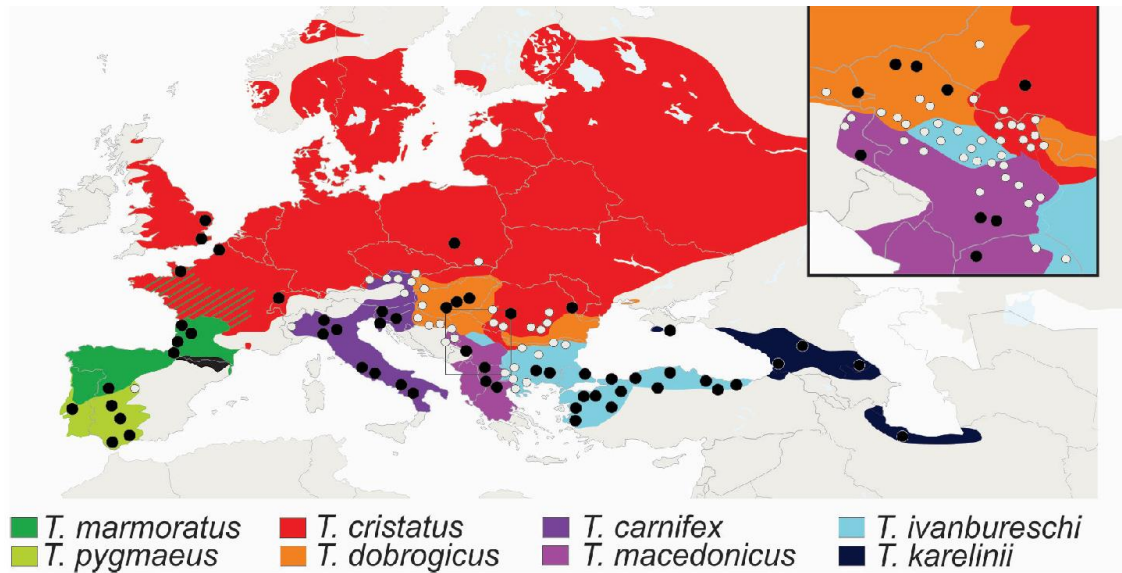476   information see Supplementary Table S1.

477

478   Figure 4.

479   Homeotic transformations recorded: A) transitional cervical vertebra (cervical into thoracic) (1);

480   B) complete homeotic transformation of cervical into thoracic vertebra (2) and transitional sacral

481   vertebra with thoracic rib on the right side and sacral rib on the other side followed by sacral

482   vertebra (3); C) Transitional sacral vertebra - first vertebra with thoracic rib on the right side and

483   sacral rib on the left side, followed by second transitional vertebra, with sacral rib on the right

484   side and no rib attached on the left side (transitional sacral vertebra with a left side asymmetry)

485   (4); D) complete vertebral column without homeotic transformations and transitional vertebrae.

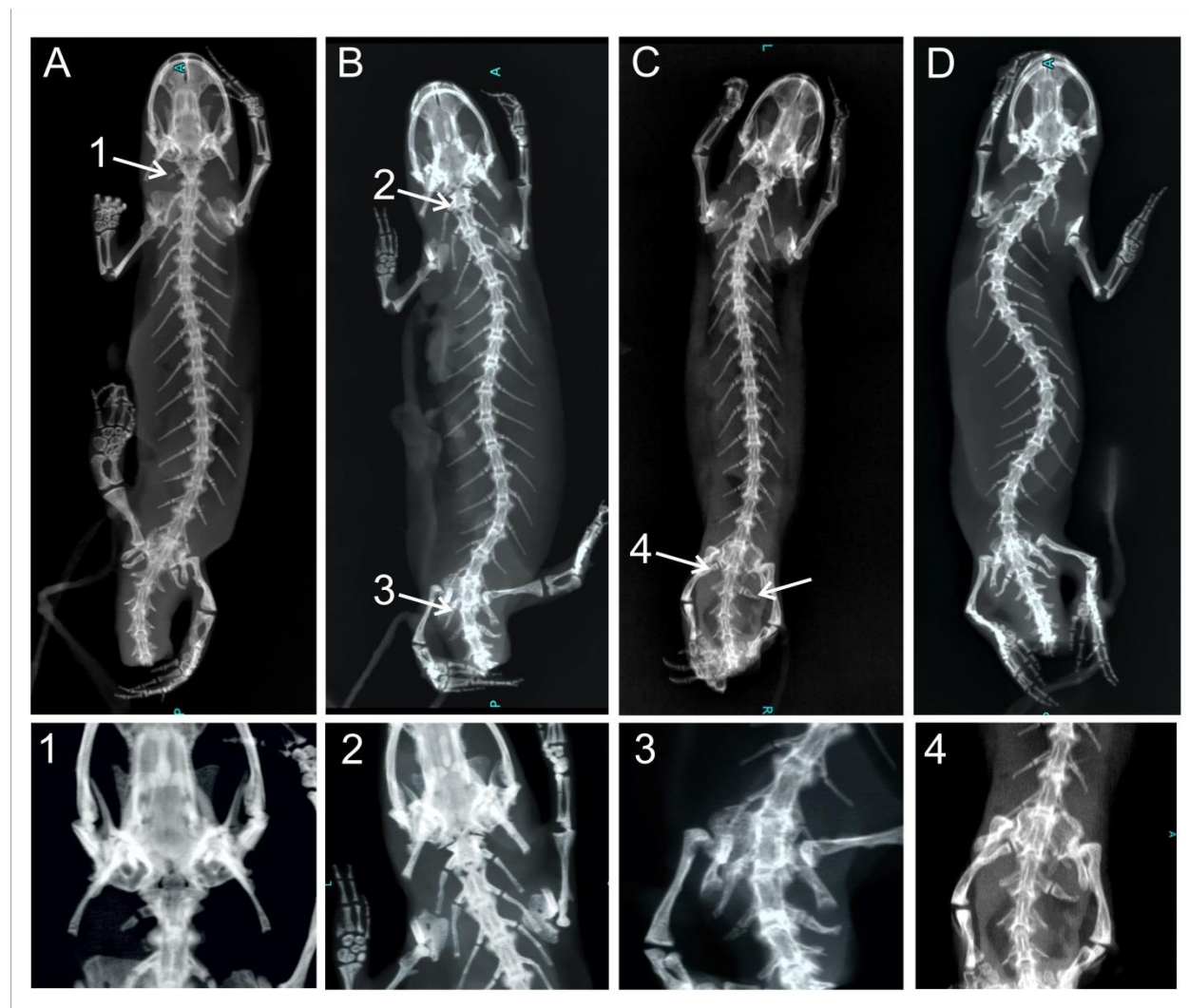486   Transformations are marked by arrows and showed in close-up at the bottom of the Figure.

487

488

489 Table 1.

490 The number of thoracic vertebrae in *Triturus* species (central populations only). Modal numbers
491 of thoracic vertebrae in vertebral formula are shown in bold. trS – percentage of individuals with
492 transitional vertebrae at thoraco-sacral boundary. varT – percentage of individuals with the
493 complete number of thoracic vertebrae different from the modal number.

Species	Sample size	Variation in the number of thoracic vertebrae													trS (%)	varT (%)
		12	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18		
T. marmoratus	58	**46**	4	8											6.9	13.8
T. pygmaeus	55	**52**	1	2											1.8	3.6
T. ivanbureschi	175	1	3	**150**	4	17									4.0	10.3
T. karelinii	43		1	**40**		2									2.3	4.7
T. carnifex	66			4	5	**53**		3		1					7.6	12.1
T. macedonicus	67			9	5	**51**	1	1							9.0	14.9
T. cristatus	122			1		6	1	**98**	5	11					4.9	14.8
T. dobrogicus	57							2		11	1	**42**		1	1.8	24.6

494

495

496 Table 2.

497 Overview of homeotic transformations in *Triturus* newts (central and fringe populations pooled).

498 N - number of individuals per species, number (and percentage) of individuals with transitional

499 vertebrae. Left and right asymmetries of transitional sacral vertebra were given separately.

500

Species	sample size	Transitional	%	Cervical to thoracic			Transitional sacral			Thoracic to sacral	
				Complete	Incomplete	%	Left	Right	%	Incomplete	%
T. marmoratus	58	5	8.6	0	1	1.7	1	3	6.9	0	
T. pygmaeus	55	1	1.8	0	0		1	0	1.8	0	
T. ivanbureschi	361	25	6.9	1	3	1.1	6	12	5.0	3	0.8
T. karelinii	43	1	2.3	0	0		0	1	2.3	0	
T. carnifex	123	8	6.5	0	0		6	2	6.5	0	
T. macedonicus	226	14	6.2	0	1	0.4	8	4	5.3	1	0.4
T. cristatus	286	16	5.6	2	1	1.0	10	3	4.5	0	
T. dobrogicus	216	13	6.0	0	0		7	6	6.0	0	
Total	1368	83	6.1	3	6	0.6	39	31		4	0.3

501   Table 3.

502   The number of thoracic vertebrae in *T. cristatus* x *T. marmoratus* F_1 hybrids and parental species.

503   Modal numbers of thoracic vertebrae in vertebral formula areshown in bold. TrS – percentage of

504   individuals with transitional vertebrae at thoraco-sacral boundary. VarT– percentage of

505   individuals with the complete number of thoracic vertebrae different from the modal number.

Species	Sample size	Variation in the number of thoracic vertebrae													trS (%)	varT (%)
		12	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5	18		
T. marmoratus	58	**46**	4	8											6.9	13.8
T .pygmaeus	55	**52**	1	2											1.8	3.6
Hybrids	56	2	1	**39**	4	16		6							7.4	35.3

506

Table 4.

The number of individuals with regular and changed vertebral formula in "central'' and "fringe" populations.

Species	Number with modal vertebral formula		Number with non-modal vertebral formula		G	*p*
	Cenral	Fringe	Central	Fringe		
T.ivanbureschi	150	110	25	76	18.86	***
T.carnifex	53	37	13	20	2.09	ns
T.macedonicus	51	122	16	37	0.006	ns
T.cristatus	98	130	24	34	0.032	ns
T.dobrogicus	42	67	15	92	6.65	**
Total	394	466	83	259		

ns, not significant; ** *p* < 0.01; *** *p* < 0.001