

**A peer-reviewed version of this preprint was published in PeerJ on 10 November 2015.**

[View the peer-reviewed version](https://doi.org/10.7717/peerj.1397) (peerj.com/articles/1397), which is the preferred citable publication unless you specifically need to cite this preprint.

Slijepčević M, Galis F, Arntzen JW, Ivanović A. 2015. Homeotic transformations and number changes in the vertebral column of *Triturus* newts. PeerJ 3:e1397 <https://doi.org/10.7717/peerj.1397>

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

3  
4                    Maja D. Slijepčević<sup>1</sup>, Frietson Galis<sup>2</sup>, Jan W. Arntzen<sup>2</sup> and Ana Ivanović<sup>2,3</sup>

5  
6     <sup>1</sup>Institute for Biological Research “Siniša Stanković”, University of Belgrade, Serbia

7     <sup>2</sup>Naturalis Biodiversity Center, Leiden, The Netherlands

8     <sup>3</sup>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 formulae, more specifically the variation in the  
16    number of thoracic vertebrae and frequencies of transitional sacral vertebrae in *Triturus* newts  
17    (Caudata: Salamandridae). Within salamandrid salamanders this monophyletic group shows the  
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    which states that structures with a large number of serially homologous repetitive elements are  
22    more variable than structures with smaller numbers, we hypothesized that the variation in  
23    vertebral formulae increases in more elongated species with a larger number of thoracic  
24    vertebrae. We furthermore hypothesized that the frequency of transitional vertebrae will be  
25    correlated with the variation in the number of thoracic vertebrae within the species. We also  
26    investigated potential effects of species hybridization on the vertebral formula. The proportion of  
27    individuals with a number of thoracic vertebrae different from the modal number and the range of  
28    variation in number of vertebrae significantly increased in species with a larger number of  
29    thoracic vertebrae. Contrary to our expectation, the frequencies of transitional vertebrae were not  
30    correlated with frequencies of change in the complete vertebrae number. The frequency of  
31    transitional sacral vertebra in hybrids did not significantly differ from that of the parental species.  
32    Such a pattern could be a result of selection pressure against transitional vertebrae and/or a bias  
33    towards the development of full vertebrae numbers. Although our data indicate relaxed selection

34 for vertebral count changes in more elongated, aquatic species, more data on different selective  
35 pressures in species with different numbers of vertebrae in the two contrasting, terrestrial and  
36 aquatic environments are needed to test for causality.

37

### 38 **Introduction**

39 The vertebral column consists of repetitive, serially homologous skeletal elements – vertebrae.  
40 Along the anterior-posterior axis, vertebrae are classified into regions, with a conserved order  
41 and specific sizes and shapes (e.g. Starck, 1979). The strong regionalization of the vertebral  
42 column is already present early in the evolution of tetrapods (Ahlberg, Clack & Blom, 2005). In  
43 the early tetrapod *Ichthyostega*, five regions (cervical, thoracic, lumbar, sacral and caudal) can be  
44 recognized as in many extant amniotes.

45 Vertebrae develop from embryonic segments (somites) that are generated from the presomitic  
46 mesoderm in a sequential head-to tail order. This process involves a molecular oscillator, the  
47 segmentation clock, that regulates the periodicity of segment formation (Cooke & Zeeman, 1976;  
48 Palmeirim et al., 1997; Dequéant & Pourquié, 2008; Gomez & Pourquié, 2009). The duration of  
49 segment formation (somitogenesis) and the speed of the segmentation clock determine the total  
50 number of segments formed, and hence, the total number of vertebrae (Gomez et al., 2008, 2009).  
51 The determination of the identity of the vertebrae (e.g. cervical or thoracic) occurs as part of the  
52 early head-to-tail patterning of the presomitic mesoderm and early somites. This head-to-tail  
53 patterning involves complex genetic mechanisms that include various signaling molecules, with  
54 an essential mediating role for the well-known Hox genes (e.g. Dubrulle, McGrew & Pourquié,  
55 2001; Diezdel-Corral et al., 2003; Aulehla & Pourquié, 2010; Mallo, Wellik & Deschamps, 2010;  
56 Woltering, 2012; Wong et al., 2015). It is thought that the segmentation process and the head-to-  
57 tail patterning of the segments by the Hox genes can be dissociated and that this dissociation has  
58 allowed for the spectacular evolutionary diversification of vertebral formulae (Carapuço et al.,  
59 2005; Gomez & Pourquié, 2009; Schroeter et al., 2010; Harima et al., 2013; Wong et al., 2015).  
60 When there are shifts of vertebral boundaries, e.g. the cervico-thoracic boundary, these shifts  
61 involve changes in the Hox patterning of the somites along the head-to-tail axis. If indeed the  
62 segmentation process and the head-to-tail patterning of the segments are dissociated, the shifts of  
63 vertebral boundaries necessarily involve homeotic transformations of vertebrae. The involvement  
64 of homeotic transformations is further supported by the observation that in humans, xenarthra and

65 afrotherians, intraspecific changes of the cervico-thoracic or thoraco-lumbar boundary almost  
66 always involve transitional vertebrae, i.e. partial homeotic transformations, also when the number  
67 of presacral or total vertebrae is changed (Galis et al., 2006; Varela-Lasheras et al., 2011; Ten  
68 Broek et al., 2012). This also confirms that initial mutations for homeotic transformations usually  
69 lead to incomplete homeotic transformations, resulting in transitional vertebral identities.

70 In mammals, changes in the number of cervical vertebrae are associated with deleterious  
71 pleiotropic effects that lead to selection against such homeotic transformations (Galis & Metz,  
72 2003; Varela-Lasheras et al., 2011). In two mammalian groups (sloths and manatees) with low  
73 activity and metabolic rates, the exceptional numbers of cervical vertebrae most likely resulted  
74 from the effective breaking of pleiotropic constraints due to a relaxation of stabilizing selection  
75 against the pleiotropic effects (known as congenital abnormalities, Varela-Lasheras et al., 2011).  
76 Furthermore, Galis et al. (2014) concluded that biomechanical problems associated with initial  
77 homeotic transformations (transitional vertebrae) in fast running mammals result in strong  
78 selection against changes of the presacral vertebral counts in these species.

79 In other tetrapods the number of vertebrae in different regions can be more variable. Well-known  
80 examples are the variable number of cervical vertebrae in birds (Woolfenden, 1961) and the  
81 variable number of presacral vertebrae in squamates (Carroll, 1997; Müller et al., 2010). In both  
82 cases, these regions have a large number of vertebrae. Geoffroy St. Hilaire has postulated that as  
83 a rule - structures with a large number of serially homologous repetitive elements are more  
84 variable than structures with smaller numbers (Geoffroy St. Hilaire, 1932). This notion was  
85 supported by Darwin (Darwin, 1860). Along the same line, Bateson (1894) concluded that series  
86 containing large numbers of undifferentiated parts are more variable than series made up of a  
87 few, more differentiated parts.

88 In tailed amphibians, the presacral vertebrae vary in their number but only little in shape. A  
89 single, sacral vertebra is morphologically very similar to the vertebrae from the thoracic or trunk  
90 region, with more robust processes for attachment of the sacral ribs which are also thicker than  
91 regular thoracic ribs. There is considerable intraspecific variation in the number of thoracic  
92 vertebrae in many species of salamanders (Adolphi, 1898; Gerecht, 1929; Peabody & Brodie,  
93 1975; Jockusch, 1997; Litvinchuk & Borkin, 2003). Intraspecific variation originally results from  
94 homeotic transformations that are subsequently maintained in the population. In salamanders,  
95 transitional vertebrae at the thoraco-sacral boundary have been frequently reported (Adolphi,

1898; Gerecht, 1929; Highton, 1960; Worthington, 1971; Jockush, 1997; Arntzen et al., 2015). Such transitional vertebrae with partial thoracic and partial sacral identity result from incomplete homeotic transformations. Therefore, the frequencies of transitional vertebrae could be related to the amount of variation in the number of thoracic vertebrae within species. Data on changes in axial patterning and homeotic transformations in amphibians are relatively scarce and more data are necessary for understanding the evolution of axial patterning in amphibians and the tetrapods. In this study we explore the relationship between variation in the number of thoracic vertebrae and transitional sacral vertebrae using eight species of the monophyletic genus *Triturus* newts as a model system. Within the family Salamandridae, which is the second most diverse group of tailed amphibians, *Triturus* newts are the most disparate in the number of thoracic vertebrae (Arntzen et al., 2015). *Triturus* species form a morphocline from the predominantly terrestrial *T. marmoratus* and *T. pygmaeus* with a short and stout body and 12 thoracic vertebrae to the slender and elongated, largely aquatic *T. cristatus* and *T. dobrogicus* with 15-17 thoracic vertebrae (Arntzen, 2003). *Triturus* species also display considerable intraspecific variation in vertebral numbers (Gerecht, 1929; Crnobrnja et al., 1997; Arntzen et al., 2015). Moreover, there is a well-documented, extensive hybridization in the area of sympatry of *T. marmoratus* (12 thoracic vertebrae) and *T. cristatus* (15 thoracic vertebrae). The hybridization of these two species leads to sterile F1 hybrids with intermediate morphologies and number of thoracic vertebrae (Vallée, 1959; Arntzen et al., 2009). Interspecific hybridization at contact zones also occurs between other *Triturus* species with parapatric distributions (Mikuliček et al., 2012; Arntzen, Wielstra & Wallis, 2014), providing the opportunity to investigate the relationship between vertebral number and frequencies of transitional sacral vertebrae. Here, we compared variation in the number of thoracic vertebrae and transitional sacral vertebrae among *Triturus* species, *T. marmoratus* × *T. cristatus* F1 hybrids with parental species, and populations from contact zones with populations away from contact zones. More specifically, we explored the intra- and interspecific variation in number of the thoracic vertebrae and frequencies of transitional vertebrae at the thoraco-sacral boundary to test the following hypotheses:

1. Species with more vertebrae in the thoracic region are more variable in the number of thoracic vertebrae than those with fewer vertebrae in the thoracic region.
2. The higher the variation in the number of thoracic vertebrae, the higher the frequencies of transitional vertebrae are. In hybrids, we would expect that the range of variation in the number

127 of vertebrae overlaps the ranges of parental species' variation. Also, we would expect the higher  
128 frequencies of transitional vertebrae in comparison with parental species. In species with  
129 parapatric distributions we would expect the higher variation in the number of vertebrae and the  
130 higher frequencies of transitional vertebrae in populations from contact zones in comparison with  
131 populations away from contact zones.

132

### 133 **Materials and Methods**

#### 134 *Triturus newts and their characteristics*

135 The vertebral column in *Triturus* newts is differentiated in: the cervical region– consisting of a  
136 single anterior vertebra, the atlas; the thoracic (trunk) region,–consisting of a rib-bearing  
137 vertebrae; the sacral region – usually a single vertebra with well-developed stout transverse  
138 processes for the attachment of sacral ribs and pelvic girdle; the caudosacral region – up to three  
139 vertebrae that continue from the caudal to the sacral vertebra and are associated with the pelvic  
140 musculature and cloaca and the caudal region – the remaining vertebrae in the tail (Fig. 1). The  
141 body elongation in *Triturus* species appears to be correlated with the length of the aquatic phase –  
142 more terrestrial species have a short and stout trunk with relatively longer legs compared to  
143 species with a more aquatic life style which have a more elongated trunk and relatively shorter  
144 legs. Body elongation involves a larger number of thoracic vertebrae. More specifically, the  
145 number of thoracic vertebrae in the vertebral formulae varies from 12 in *T. marmoratus* and *T.*  
146 *pygmaeus*, which have a short aquatic phase (*T. marmoratus* only two months), 13 in *T. karelinii*  
147 and *T. ivanbureschi*, 14 in *T. macedonicus* and *T. carnifex*, 15 in *T. cristatus* to 16 or 17 in *T.*  
148 *dobrogicus*, the most aquatic species which has six months long aquatic phase (Arntzen, 2003)  
149 (Fig. 2).

150 The distribution of the genus *Triturus* is well documented (Arntzen, Wielstra & Wallis, 2014).  
151 *Triturus cristatus* and *T. marmoratus* have an area of range overlap in France and can often be  
152 found in syntopy (Arntzen & Wallis, 1991; Lescure & de Massary, 2012). Other *Triturus* species  
153 contact zones are generally narrow and show a weak but significant negative relationship  
154 between the level of hybridization and the genetic distance of species pairs (Arntzen, Wielstra &  
155 Wallis, 2014).

156

#### 157 *Material analysed*

158 We analysed axial skeletons of 1436 adult newts that originate from 126 populations of all eight  
159 species of *Triturus* newts (Fig. 3). For this study we used X-ray images of good quality and  
160 cleared and stained skeletons. The X-ray images were obtained on a Faxitron 43855C/D with an  
161 exposure of 20–40s at 3 mA and 70kV. Other material was cleared with trypsin and KOH and  
162 stained with Alizarin Red S for bone deposition (Dingerkus & Uhler, 1977) and stored in  
163 glycerine. Analyzed specimens are from the batrachological collection of the Institute for  
164 Biological Research “Siniša Stanković”, Belgrade, Serbia (N = 601) and from the collection of  
165 the Naturalis Biodiversity Center, Leiden, The Netherlands (N = 835). Our material covers the  
166 geographic, taxonomic and molecular genetic diversity of the genus. On the basis of well  
167 documented species distributions (Mikuliček et al., 2012; Arntzen, Wielstra & Wallis, 2014)  
168 populations were assigned as “central” or “fringe” based upon their geographical position away  
169 ( $\geq 50$ km) or close to ( $< 50$  km) congeneric species. For localities and sample size per population  
170 see Supplementary Table S1.

171  
172 *Scoring vertebral formulae and transitional thoraco-sacral vertebrae*  
173 We determined the vertebral formula by counting the number of cervical (C), thoracic (T) and  
174 sacral vertebrae (S). The caudosacral and caudal regions are excluded from our formula as the  
175 detailed inspection of cleared and stained specimens showed that a variable number of  
176 caudosacral vertebrae frequently bear small, much reduced ribs which could be fused with  
177 transverse processes and cannot always be distinguished on X-ray images. The number of tail  
178 vertebrae was available only for a subset of specimens; in most specimens tails had been removed  
179 for enzyme electrophoretic analyses or were broken or damaged.  
180 Homeotic transformations of thoracic vertebra into sacral vertebra, or *vice versa* (transitional  
181 sacral vertebra having half of the identity of thoracic vertebra and half of the identity of sacral  
182 vertebra) were assigned 0.5 and this score was added to the number of complete thoracic  
183 vertebrae. Only complete changes of identity on one side of the vertebrae (on one side thoracic  
184 and on one side sacral) were declared transitional. Right side asymmetry of a sacral vertebra is  
185 scored when the thoracic rib is present on the right side and the sacral rib on the left side of  
186 transitional thoraco-sacral vertebra and vice versa for left side asymmetry For a 3D model of  
187 regular and transitional thoraco-sacral vertebra obtained by CT-scanning see Supplementary data.  
188 We assumed that the frequency of transitional vertebrae with a complete change of identity at one



189 side of vertebra reflects the frequency of all homeotic transformations, including more gradual  
190 ones, which could not always be scored.

191

### 192 *Statistical analyses*

193 The Spearman correlation coefficient ( $r_s$ ) was used to quantify correlation between species modal  
194 numbers of thoracic vertebrae ( $T_n$ ) and 1) the percentage of individuals with the number of  
195 complete thoracic vertebrae different from the modal number ( $T_{var}$ ) and 2) the range of variation  
196 in the number of thoracic vertebrae ( $T_{range}$ ) within species. The same measure was used to  
197 quantify the relationship between percentages of transitional sacral vertebrae ( $S_{tr}$ ) and  $T_{var}$  and  
198  $T_{range}$ . To test for differences between hybrids and parental species across fringe and central  
199 populations we used the G-test of independence. To analyse interspecific variation in a  
200 phylogenetic context, we used a well resolved time-calibrated phylogeny of genus *Triturus*  
201 (Arntzen et al., 2015) shown in Figure 2. Associations derived from common ancestry were  
202 evaluated by calculating the strength of the phylogenetic signal for analysed variables ( $T_n$ ,  $T_{var}$ ,  
203  $T_{range}$  and  $S_{tr}$ ). The procedure involves the random permutation of the variables over the terminal  
204 units of the phylogenetic tree (10,000 iterations), in which the test statistic is the total amount of  
205 squared change summed over all branches of the tree. We applied the phylogenetic independent  
206 contrasts approach (Felsenstein, 1985) to obtain a set of independent contrasts. The regression of  
207 1)  $T_{var}$  independent contrasts on  $T_n$  independent contrasts and 2)  $T_{range}$  independent contrasts on  
208  $T_n$  independent contrasts were used to explore the relationship between evolutionary change in  
209 the number of the thoracic vertebra in vertebral formula and amount of variation in the number of  
210 thoracic vertebrae. The regressions of  $T_{var}$  and  $T_{range}$  independent contrasts on  $S_{tr}$  were used to  
211 explore changes and frequencies of transitional vertebrae, taking the similarity due to shared  
212 ancestry into account.

213

## 214 **Results**

### 215 *Vertebral formula and transitional sacral vertebra in Triturus newts*

216 The most common vertebral formulae were 1C 12T 1S in *T. marmoratus* and *T. pygmaeus*, 1C  
217 13T 1S in *T. karelinii* and *T. ivanbureschi*, 1C 14T 1S in *T. macedonicus* and *T. carnifex*, 1C 15T  
218 1S in *T. cristatus* and 1C 17T 1S in *T. dobrogicus* (See Fig. 2 and Table 1). The percentage of  
219 individuals with a number of complete thoracic vertebrae different from the modal number ( $T_{var}$ )



220 varied among species, from 3.6% in *T. pygmaeus* to 24.6% in *T. dobrogicus*. The range of  
221 variation in the number of thoracic vertebrae is 12 – 13 observed in *T. marmoratus* and *T.*  
222 *pygmaeus*, 12 – 14 in *T. ivanbureschi*, 12 – 14 in *T. karelinii*, 13 – 15 in *T. macedonicus*, 13 – 16  
223 in *T. carnifex*, 13 – 16 in *T. cristatus* and 15 – 18 in *T. dobrogicus* (see Table 1). The variation in  
224 the vertebrae number per population is shown in Supplementary Table S2.  
225 Frequencies of recorded homeotic transformations in *Triturus* newt species are listed in Table 2.  
226 The least common is the homeotic transformation of cervical vertebra with the thoracic rib  
227 attached to one or both sides of the vertebra, recorded six times (0.41%) and in four out of eight  
228 species. Two types of transitional vertebrae at the thoraco-sacral boundary were recorded. The  
229 first type involves changes of two succeeding vertebrae - transitional sacral vertebra with thoracic  
230 rib at one side and sacral rib at the other side, followed by transitional vertebra having sacral rib  
231 at one side (opposite than previous vertebra) and no rib attached on the other side (see Figs 1 and  
232 4). The second type of transitional sacral vertebra involves transitional thoraco-sacral vertebra,  
233 with a thoracic rib at one side and a sacral rib at the other, followed by regular sacral vertebra.  
234 The transitional changes involving two adjacent vertebrae, thoracic and sacral (here termed  
235 transitional sacral) are more frequent than transitional changes of thoracic to sacral vertebra  
236 without changes of sacral vertebra. Excluding F1 hybrids, we recorded a transitional sacrum in 70  
237 out of 1368 specimens (5.1%). Both, right side and left side asymmetries were recorded (Table  
238 2). We found that  $T_n$  and  $T_{var}$  are significantly positively correlated ( $r_s = 0.75$ ,  $p = 0.023$ ),  
239 indicating that species with more vertebrae in the thoracic region are more variable in the number  
240 of vertebrae. A significant correlation was also found between  $T_n$  and  $T_{range}$  ( $r_s = 0.90$ ,  $p = 0.002$ ),  
241 indicating that the range of variation was significantly higher in species with more thoracic  
242 vertebrae. We did not find a correlation between  $S_{tr}$  and  $T_{var}$  ( $r_s = 0.31$ ,  $p = 0.46$ ) or between  $S_{tr}$   
243 and  $T_{range}$  ( $r_s = 0.13$ ,  $p = 0.76$ ).

244

#### 245 *Phylogenetic comparative analyses*

246 We found a statistically significant phylogenetic signal in  $T_n$  ( $p = 0.013$ ) and  $T_{range}$  ( $p = 0.033$ )  
247 and no significant phylogenetic signal in  $T_{var}$  ( $p = 0.730$ ) and  $S_{tr}$  ( $p = 0.970$ ). The regression of 1)  
248  $T_{var}$  independent contrasts on  $T_n$  independent contrasts ( $p = 0.018$ ) and 2) the regression of  $T_{range}$   
249 independent contrasts on  $T_n$  independent contrasts revealed a significant relationship between the  
250 increase in the number of thoracic vertebrae and the amount of variation in the number of

251 vertebrae ( $p = 0.006$ ). We found no significant relationship between  $T_{\text{range}}$  independent contrasts  
252 and  $S_{\text{tr}}$  independent contrasts ( $p = 0.413$ ).

253  
254 *Hybridization and variation in vertebral formula*  
255 There were statistically significant differences in changes in vertebral formulae between central'  
256 and fringe populations (G-test for independence,  $G = 18.61$ ,  $p = 0.001$ ). For fringe populations,  
257 the observed range of variation in number of thoracic vertebrae is 12-15 in *T. ivanbureschi*, 13-16  
258 in *T. macedonicus*, 13-15 in *T. carnifex*, 13-17 in *T. cristatus* and 14-18 in *T. dobrogicus*. In *T.*  
259 *dobrogicus* and *T. ivanbureschi* fringe populations differed significantly from central populations  
260 in the frequencies of individuals with non-modal vertebrae formulae. For other species no  
261 significant differences between central and fringe populations were found (Table 4). In *T.*  
262 *cristatus*  $\times$  *T. marmoratus*, sixty F1 hybrids (88.2%) have a vertebral formula with an  
263 intermediate number of thoracic vertebrae (Table 3). Six hybrids (8.8%) possess an incomplete  
264 homeotic transformation. Among these, one has an incomplete transformation of a cervical into a  
265 thoracic vertebra. The most frequent incomplete homeotic transformation involves an  
266 asymmetrical sacrum. The frequencies of transitional sacral vertebra in hybrids and parental  
267 species are similar (G-test for independence,  $G = 1.07$ ,  $p = 0.59$ ).

## 268 269 **Discussion**

270 Our study shows a substantial variation in the number of thoracic vertebrae in *Triturus* newts,  
271 suggesting the absence of strong selection against change in the number of thoracic vertebrae. In  
272 agreement with the postulations of Geoffroy St. Hilaire, Darwin and Bateson, the variation in the  
273 vertebral column is positively correlated with the number of vertebrae. Using independent  
274 contrasts we observed a statistically significant, correlated evolutionary change between an  
275 increase in the modal number of thoracic vertebrae and variation in vertebrae number. The range  
276 of variation in the number of thoracic vertebrae is also significantly higher in species with a  
277 larger modal number of thoracic vertebrae. In *T. marmoratus* and *T. pygmaeus* the variation is  
278 limited to one extra thoracic vertebra, while in crested newt species the variation range is up to  
279 four vertebrae. Although such a pattern of intraspecific variation is in agreement with Geoffroy  
280 St. Hilaire's and Darwin's rules, the observed pattern may also represent an association between  
281 variation in vertebral number and differences in selection in different habitats that *Triturus*

282 species occupy, in particular the amount of time they spend in the terrestrial versus the aquatic  
283 environment. During the terrestrial phase the limbs support the weight of the body and provide  
284 forward propulsion by the synchronous use of diagonal limb pairs. In water where the body  
285 weight does not need to be supported by the limbs the newts move by tail propulsion with the  
286 limbs tightly held against the body to reduce hydrodynamic drag (Gvoždik & Van Damme,  
287 2006). Therefore, the selection pressures related to specific biomechanical requirements are  
288 probably different with respect to the duration of the terrestrial and aquatic phase. The larger  
289 variation in the number of thoracic vertebrae may indicate relaxed selection in more aquatic  
290 species, but more data on different selective pressures in terrestrial versus aquatic environments  
291 are needed to find out whether this is the case.

292

### 293 *Frequencies of transitional vertebrae*

294 In *Triturus* newts, the frequency of transitional changes at the cervico-thoracic boundary is more  
295 than ten times lower than changes at the thoraco-sacral boundary. This is also observed in other  
296 salamanders (Wake & Lawson, 1973) and mammals (Galis et al., 2006). This pattern may be  
297 explained by stronger interactivity and low modularity of developmental processes during the  
298 early organogenesis, or phylotypic stage, when the cervical vertebra is determined (Galis et al.,  
299 2006). At later stages, development is increasingly less interactive and more modular, such that  
300 changes are expected to be associated with fewer pleiotropic effects. The hypothesis that  
301 mutations with an effect during early organogenesis stage lead to more pleiotropic effects and as  
302 a consequence to more vulnerability and mortality than earlier or later stages was tested and  
303 strongly supported in rodents (Galis & Metz 2001). In amphibians indirect support for this  
304 hypothesis is discussed by Galis, Wagner & Jockusch (2003). We do not know the cause of the  
305 constraint on the number of cervical and sacral vertebra in tailed amphibians, but further studies  
306 in various amphibian groups that will consider survival rates of individuals with changes in the  
307 cervical and sacral region across ontogenetic stages should provide valuable data to solve this  
308 issue.

309 Although we hypothesized that frequencies of transitional vertebrae at the thoraco-sacral  
310 boundary should be correlated to the range of variation in the number of thoracic vertebrae as in  
311 mammals (Ten Broek et al., 2012), no correlation was found. Available literature data indicate  
312 that incomplete homeotic transformation of sacral vertebrae are relatively common, with up to

313 10% across the various salamander lineages: 4.5% in *Batrachoseps attenuatus* (Jockusch, 1997),  
314 5.7% in *Rhyacotriton olympicus* (Worthington, 1971), 6% in *Plethodon cinereus* (Highton,  
315 1960), up to 9% for newt genera *Lissotriton* and *Ichthyosaura* (Arntzen et al., 2015) and between  
316 1.9% and 9.0% in *Triturus* newts (this study). The lower than expected incidence of transitional  
317 vertebrae could result from developmental mechanisms favoring complete numbers of thoracic  
318 vertebrae and/or from selection against transitional sacral vertebrae due to associated problems  
319 related to an asymmetric sacrum (c.f. Galis et al., 2014). Potential problems associated to  
320 asymmetrical sacrum might arise due to asymmetrical muscle attachments, blood vessels and  
321 innervation, or biomechanical problems during locomotion. In salamanders, the selection  
322 pressures related to specific biomechanical requirements are probably different in fully aquatic  
323 larvae and metamorphosed individuals that spend most of their time on land. Furthermore,  
324 selection pressures may vary with respect to the duration of annual aquatic and terrestrial phase.  
325 More detailed morphological and functional studies of locomotion of larval and metamorphic  
326 stages could shed more light on the functional significance of variation in the axial skeleton in  
327 *Triturus* newts. However, it is possible that our results are biased as we have not included the full  
328 range of transitional vertebrae. We scored only easily identifiable transitional vertebrae with  
329 complete morphological transformations of one side of the vertebra under the assumption that the  
330 frequency of these transitional vertebrae reflects the total amount of homeotic transformations.  
331 Nonetheless, initial mutations for homeotic transformations can lead to a whole series of  
332 gradually transitional homeotic transformations; in the case of thoraco-sacral vertebrae ranging  
333 from predominantly thoracic and only slightly sacral to predominantly sacral and slightly  
334 thoracic. Inclusion of all transitional vertebral morphologies might change the observed  
335 relationship between incomplete homeotic transformations and changes in the number of thoracic  
336 vertebrae in newts.

337

### 338 *Hybridization, marginality and homeotic transformations*

339 Hybridization and marginality significantly increase variability in the number of thoracic  
340 vertebrae but there is no change in the frequency of transitional vertebrae. Crosses between *T.*  
341 *cristatus* (15 vertebrae, range 13-16) and *T. marmoratus* (12 vertebrae, range 12-13) produced  
342 phenotypes with 13 thoracic vertebrae, an intermediate number. It is interesting to note that 13  
343 thoracic vertebrae is the only number that is shared by both parental species. In *T. cristatus* × *T.*

344 *marmoratus* offspring there is considerable mortality and almost all of F1 hybrids (~90%) had *T.*  
345 *cristatus* as mother. The *marmoratus*-mothered specimens were all male, due to low survival of  
346 female embryos (Arntzen et al., 2009). Developmental anomalies in *T. cristatus* × *T. marmoratus*  
347 crosses, including more digital anomalies compared with parental species (hybrids 16.9%,  
348 parental species pooled 5.4%) (Vallée, 1959; more data in Arntzen & Wallis, 1991) are observed,  
349 and therefore, the higher number of changes in the axial skeleton may be related to a generally  
350 higher number of anomalies. The high mortality may also influence the incidence of the  
351 variability and transitional vertebrae.

352 Significantly higher frequency of changes in vertebral formula in fringe populations of *T.*  
353 *ivanbureschi* and *T. dobrogicus* species may well have to do with the confirmed presence of  
354 hybridization in the contact zones of *T. cristatus* and *T. dobrogicus* populations (Mikulíček et al.,  
355 2012), of *T. carnifex* and *T. dobrogicus* populations (Wallis & Arntzen, 1989) and of *T.*  
356 *ivanbureschi* and *T. macedonicus* populations (Arntzen, Wielstra & Wallis, 2014). However, the  
357 effect of the various genotype combinations on the survival rate and morphology of these species  
358 remains to be studied.

359 In conclusion, *Triturus* newts have a relatively large amount of variation in the number of  
360 thoracic vertebrae, both with respect to the frequency of non-modal numbers and the range of  
361 variation. In agreement with Geoffroy St. Hilaire's rule, variation was larger in species with a  
362 larger number of thoracic vertebrae. The absence of a correlation between the frequency of  
363 homeotic change (transitional sacral vertebrae,  $S_{tr}$ ) and variation in the number of vertebrae ( $T_{var}$ ,  
364  $T_{range}$ ) could be a result of developmental mechanisms that favour complete numbers of presacral  
365 vertebrae and/or selection against transitional vertebrae in this group of tailed amphibians.

366

## 367 **Acknowledgements**

368

369 We thank Hans Metz and Joost Woltering for discussions, Ben Wielstra for providing a  
370 distribution map and Marieke Vinkenoog for help with X – ray imaging.

371

## 372 **References**

373

374 **Adolphi H. 1898.** Über das Wandern der Extremitätenplexus und des Sacrum bei Triton  
375 *taeniatus*. *Morphologisches Jahrbuch* **25**: 544–554

- 376 **Ahlberg PE, Clack JA, Blom H. 2005.** The axial skeleton of the Devonian tetrapod  
377 *Ichthyostega*. *Nature* **437**: 137–140 DOI:10.1038/nature.03893
- 378 **Arntzen JW, Wallis GP. 1991.** Restricted gene flow in a moving hybrid zone of the newts  
379 *Triturus cristatus* and *T. marmoratus* in western France. *Evolution* **45** (4): 805–826
- 380 **Arntzen JW. 2003.** *Triturus cristatus* Superspezies - Kammolch-Artenkreis. (*Triturus cristatus*  
381 (Laurenti, 1768) – Noërdlicher Kammolch, *Triturus carnifex* (Laurenti, 1768) –  
382 Italienischer Kammolch. *Triturus dobrogicus* (Kiritzescu, 1903) – Donau-  
383 Kammolch, *Triturus karelinii* (Strauch, 1870) – Süedlicher Kammolch). In: K.  
384 Grossenbacher, B. Thiesmeier, eds. *Handbuch der Reptilien und Amphibien Europas*,  
385 Band 4/IIA: Schwanzlurche (Urodela) IIA. Wiebelsheim: Aula-Verlag, 421–514
- 386 **Arntzen JW, Jehle R, Bardakci F, Burke T, Wallis GP. 2009.** Asymmetric viability of  
387 reciprocal-cross hybrids between crested and marbled newts (*Triturus cristatus* and *T.*  
388 *marmoratus*). *Evolution* **63** (5): 1191–202 DOI: 10.1111/j.1558-5646.2009.00611.x
- 389 **Arntzen JW, Wielstra B, Wallis GP. 2014.** The modality of nine *Triturus* newt hybrid zones  
390 assessed with nuclear, mitochondrial and morphological data. *Biological Journal of the*  
391 *Linnean Society* **113** (2): 604–622 DOI: 10.1111/bij.12358
- 392 **Arntzen JW, Beukema W, Galis F, Ivanović A. 2015.** Vertebral number is highly evolvable in  
393 salamanders and newts (family Salamandridae) and variably associated with climatic  
394 parameters. *Contributions to Zoology* **84** (2): 87–116
- 395 **Aulehla A, Pourquié O. 2010.** Signaling gradients during paraxial mesoderm development. *Cold*  
396 *Spring Harbor Perspectives in Biology* **2** (2): a000869 DOI:10.1101/cshperspect.a000869
- 397 **Bateson W. 1894.** *Materials for the Study of Variation*. London: MacMillan
- 398 **Carapuço M, Nóvoa A, Bobola N, Mallo M. 2005.** Hox genes specify vertebral types in the  
399 presomitic mesoderm. *Genes & Development* **19** (18): 2116–2121
- 400 **Carroll RL. 1997.** *Patterns and Processes of Vertebrate Evolution*. Cambridge: Cambridge  
401 University Press
- 402 **Cooke J, Zeeman ECA. 1976.** Clock and wavefront model for control of the number of repeated  
403 structures during animal morphogenesis. *Journal of Theoretical Biology* **58** (2): 455–476
- 404 **Crnobrnja J, Džukić G, Krstić N, Kalezić ML. 1997.** Evolutionary and paleogeographic  
405 effects on the distribution of the *Triturus cristatus* superspecies in the central Balkans.  
406 *Amphibia-Reptilia* **18** (4): 321–332 DOI: 10.1163/156853897X00378



- 407 **Darwin CR. 1860.** *The Origin of Species*. New York: D. Appleton and Company
- 408 **Dequéant ML, Pourquié O. 2008.** Segmental patterning of the vertebrate embryonic axis.  
409 *Nature Review Genetics* **9**: 370–382 DOI: 10.1038/nrg2320
- 410 **Diezdel-Corral R, Olivera-Martinez I, Goriely A, Gale E, Maden M, Storey K. 2003.**  
411 Opposing FGF and retinoid pathways control ventral neural pattern, neuronal  
412 differentiation, and segmentation during body axis extension. *Neuron* **40** (1): 65–79
- 413 **Dingerkus G, Uhler LD. 1977.** Enzyme clearing of alcian blue stained whole small vertebrates  
414 for demonstration of cartilage. *Stain Technology* **52** (4): 229–232
- 415 **Dubrulle J, McGrew MJ, Pourquié O. 2001.** FGF signaling controls somite boundary position  
416 and regulates segmentation clock control of spatiotemporal Hox gene activation. *Cell* **106**  
417 (2):219–32
- 418 **Felsenstein J. 1985.** Phylogenies and the comparative method. *American Naturalist* **125** (1): 1–  
419 15
- 420 **Galis F, Metz JAJ. 2001.** Testing the vulnerability of the phylotypic stage: on modularity and  
421 evolutionary conservation. *Journal of Experimental Zoology Part B: Molecular and*  
422 *Developmental Evolution* **291** (2): 195–204
- 423 **Galis F, Metz JAJ. 2003.** Anti-cancer selection as a source of developmental and evolutionary  
424 constraints. *Bioessays* **25** (11): 1035–1039
- 425 **Galis F, Wagner GP, Jockusch E. 2003.** Why is limb regeneration possible in amphibians but  
426 not in reptiles, birds and mammals? *Evolution & Development* **5** (2): 208–220
- 427 **Galis F, Van Dooren TJ, Feuth JD, Metz JA, Witkam A, Ruinard S, Steigenga MJ,**  
428 **Wijnaendts LC. 2006.** Extreme selection in humans against homeotic transformations of  
429 cervical vertebrae. *Evolution* **60** (12): 2643–2654
- 430 **Galis F, Carrier DR, Alphen J, Van der Mije SD, Doorn TV, Metz JAJ, Ten Broek CMA.**  
431 **2014.** Fast running restricts evolutionary change of the vertebral column in mammals. .  
432 *Proceedings of the National Academy of Sciences of the United States of America* **111**  
433 (31): 11401–11406 DOI: 10.1073/pnas.1401392111
- 434 **Geoffroy SHI. 1832.** *Histoire Générale et Particulière des Anomalies de L'organisation chez*  
435 *L'homme et les Animaux*. Paris: J.-B. Baillière, Libraire de l' Academie Royale de  
436 Médecine



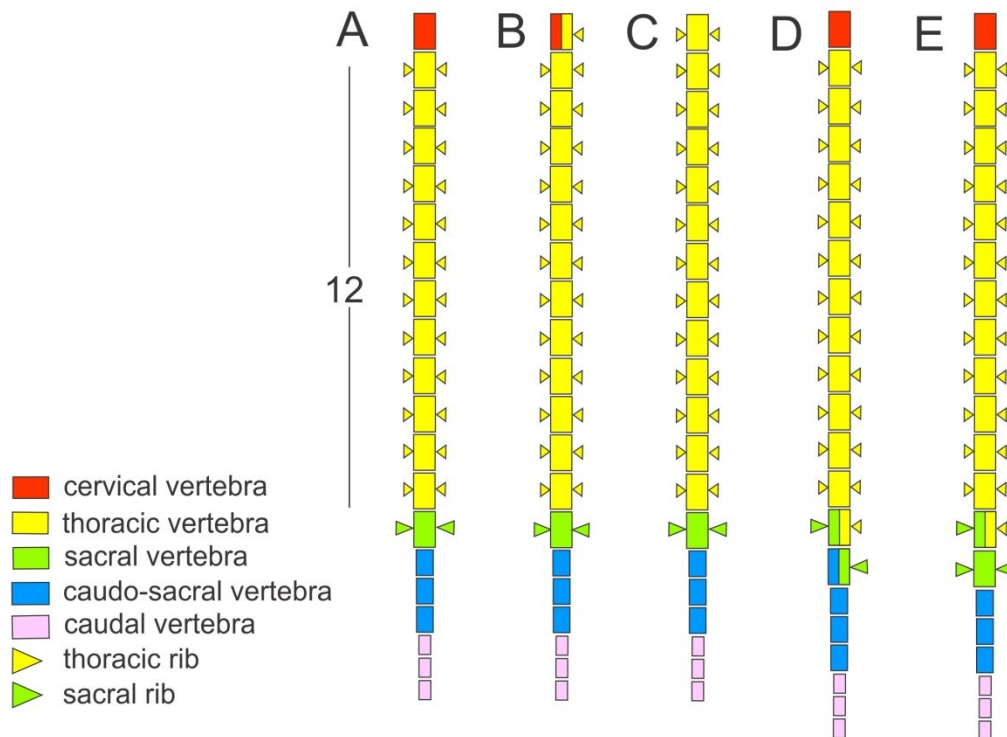
- 437 **Gerecht A. 1929.** Über die Richtung der phyletischen Wanderung der Sakralregion bei *Triton*  
438 *cristatus* und *Triton taeniatus*. *Bulletin de la Société de biologie de Lettonie* **1**(1): 9–19
- 439 **Gomez C, Özbudak EM, Wunderlich J, Baumann D, Lewis J, Pourquoié 2008.** Control of  
440 segment number in vertebrate embryos. *Nature* **454** (7202): 335–339
- 441 **Gomez C, Pourquoié O. 2009.** Developmental control of segment numbers in vertebrates.  
442 *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution.* **312**  
443 (6): 533–544 DOI: 10.1002/jez.b.21305
- 444 **Gvoždik L, Van Damme R. 2006.** *Triturus* newts defy the running-swimming dilemma.  
445 *Evolution* **60** (10): 2110–2121
- 446 **Harima Y, Takashima Y, Ueda Y, Otsuka T, Kageyama R. 2013.** Accelerating the tempo of  
447 the segmentation clock by reducing the number of introns in the *Hes7* Gene. *Cell Reports*  
448 **3** (1): 1–7 DOI: 10.1016/j.celrep.2012.11.012
- 449 **Highton R. 1960.** Heritability of geographic variation in trunk segmentation in the red-backed  
450 salamander, *Plethodon cinereus*. *Evolution* **14** (3): 351–360
- 451 **Jockusch EL. 1997.** Geographic variation and phenotypic plasticity of number of trunk vertebrae  
452 in slender salamanders, *Batrachoseps* (Caudata: Plethodontidae). *Evolution* **51** (6): 1966–  
453 1982 DOI:10.2307/2411017
- 454 **Lescure J, de Massary J-C. 2012.** *Atlas des Amphibiens et Reptiles de France*. Paris: Muséum  
455 national d'Histoire naturelle
- 456 **Litvinchuk SN, Borkin LJ. 2003.** Variation in number of trunk vertebrae and in count of costal  
457 grooves in salamanders of the family Hynobiidae. *Contributions to Zoology* **72** (4): 195–  
458 209
- 459 **Mallo M, Wellik D, Deschamps J. 2010.** Hox genes and regional patterning of the vertebrate  
460 body plan. *Developmental Biology* **344** (1): 7–15 DOI: 10.1016/j.ydbio.2010.04.024
- 461 **Mikulíček P, Horák A, Zavadil V, Kautman J, Piálek J. 2012.** Hybridization between three  
462 crested newt species (*Triturus cristatus* superspecies) in the Czech Republic and  
463 Slovakia: comparison of nuclear markers and mitochondrial DNA. *Folia Zoologica* **61** (3-  
464 4): 202–218
- 465 **Müller J, Scheyer TM, Head JJ, Barrett PM, Werneburg I, Ericson PGP, Pol D, Sanchez-  
466 Villagra MR. 2010.** Homeotic effects, somitogenesis and the evolution of vertebral

- 467 numbers in recent and fossil amniotes. *Proceedings of the National Academy of Sciences*  
468 *of the United States of America* **107** (5): 2118–2123
- 469 **Palmeirim I, Henrique D, Ish-Horowicz D, Pourquoié O. 1997.** Avian hairy gene expression  
470 identifies a molecular clock linked to vertebrate segmentation and somitogenesis. *Cell* **91**  
471 (5): 639–48
- 472 **Peabody RB, Brodie ED. 1975.** Effect of temperature, salinity and photoperiod on the number  
473 of trunk vertebrae in *Ambystoma maculatum*. *Copeia* **1975** (4): 741–746
- 474 **Schroeter C, Oates AC. 2010.** Segment number and axial identity in a segmentaion clock period  
475 mutant. *Current Biology* **20** (14): 1254–1258
- 476 **Starck D. 1979.** *Vergleichende Anatomie der Wirbeltiere*. Berlin: Springer Verlag
- 477 **Ten Broek CM, Bakker AJ, Varela-Lasheras I, Bugiani M, Van Dongen S, Galis F. 2012.**  
478 Evo-Devo of the human vertebral column: on homeotic transformations, pathologies and  
479 prenatal selection. *Evolutionary Biology* **39**(4): 456–471
- 480 **Vallée L. 1959.** Recherches sur *Triturus blasii* de l'Isle, hybride naturel de *Triturus cristatus*  
481 Laur. x *Triturus marmoratus* Latr. Mém. *Bulletin de la Société zoologique de France* **31**:  
482 1–95
- 483 **Varela-Lasheras I, Bakker A, Mije S, Metz J, Alphen J, Galis F. 2011.** Breaking evolutionary  
484 and pleiotropic constraints in mammals: On sloths, manatees and homeotic mutations.  
485 *EvoDevo* **2**: 11 DOI:10.1186/2041-9139-2-11
- 486 **Wallis GP, Arntzen JW. 1989.** Mitochondrial-DNA variation in the crested newt superspecies:  
487 limited cytoplasmic gene flow among species. *Evolution* **43** (1): 88–10 DOI:  
488 10.2307/2409166
- 489 **Wake DB, Lawson R. 1973.** Developmental and adult morphology of the vertebral column in  
490 the plethodontid salamander *Eurycea bislineata*, with comments on vertebral evolution in  
491 the amphibian. *Journal of Morphology* **139** (3):251–299 DOI: 10.1002/jmor.1051390302
- 492 **Woltering JM. 2012.** From lizard to snake; behind the evolution of an extreme body plan.  
493 *Current Genomics* **13** (4): 289–299 DOI:10.2174/138920212800793302
- 494 **Wong SFL, Agarwal W, Mansfield JH, Denans N, Schwartz MG, Prosser HM, Pourquoié O.**  
495 **2015.** Independent regulation of vertebral number and vertebral identity by microRNA-  
496 196 paralogs. *Proceedings of the National Academy of Sciences of the United States of*  
497 *America* **112** (35): E4884–93 DOI: 10.1073/pnas1512655112

- 498 **Woolfenden GE. 1961.** Postcranial osteology of the waterfowl. *Bulletin of the Florida State*  
499 *Museum* 6: (1)–129
- 500 **Worthington RD. 1971.** *Postmetamorphic changes in the vertebrae of the marbled salamander*  
501 *Ambystoma opacum Gravenhorst (Amphibia, Caudata)*. El Paso: Texas Western Press  
502

503 Figure 1.

504 The regionalization of the vertebral column in *Triturus* newts and schematic presentation of  
 505 scored homeotic transformations (example of *T. marmoratus*). A) vertebral column without  
 506 homeotic transformation and regular number of vertebrae – the first three caudal vertebrae are  
 507 shown; B) incomplete homeotic transformations of cervical vertebra into thoracic; C) complete  
 508 homeotic transformation of cervical into thoracic vertebra; D) transitional sacral vertebra with  
 509 thoracic rib at one side and sacral rib at the other side followed by transitional vertebra with  
 510 sacral rib at the one side and no rib at the other; E) transitional thoraco-sacral vertebra, with  
 511 thoracic rib at one side and sacral rib at the other, followed by regular sacral vertebra.



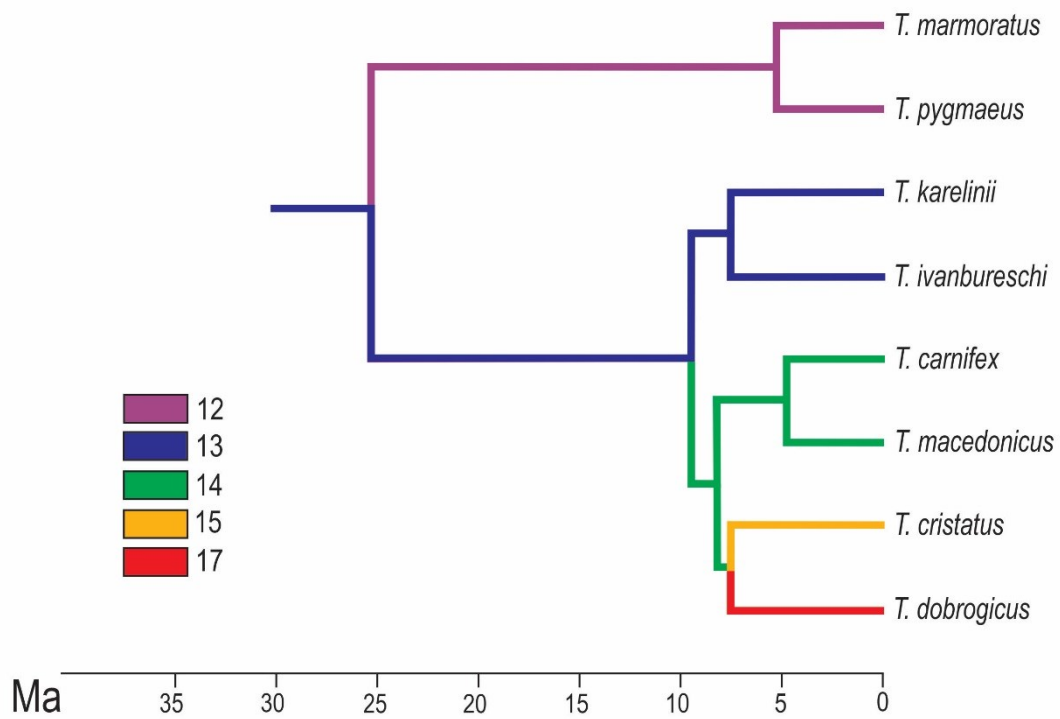
512

513

514 Figure 2.

515 Calibrated phylogeny for the genus *Triturus* with the modal number of vertebrae indicated by  
516 colour code (after Arntzen et al., 2015).

517



518

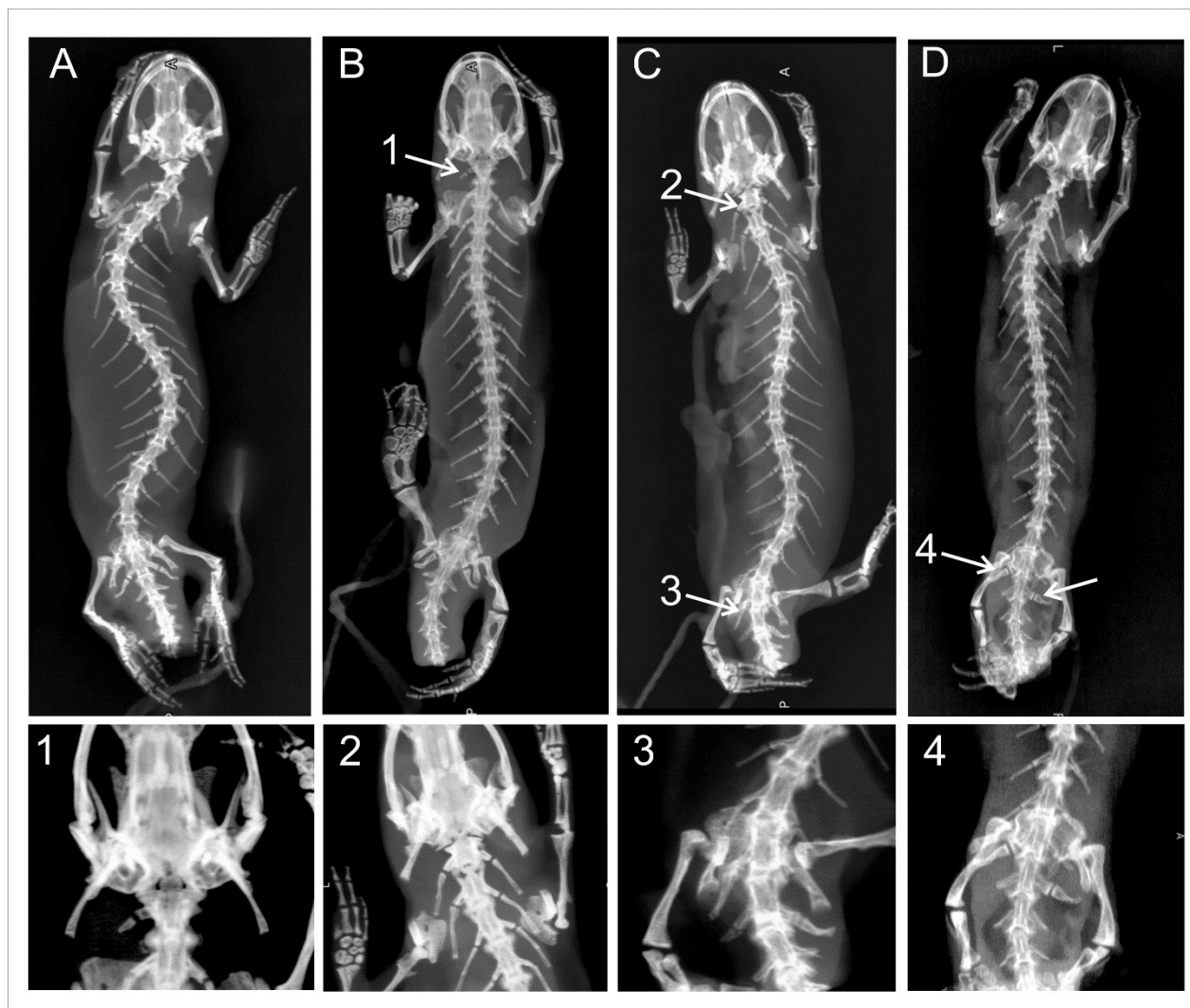
519 Figure 3.

520 Distribution of eight *Triturus* species across Europe and adjacent Asia. Studied populations are  
521 marked by solid dots (central populations) and open dots (fringe populations). For detailed  
522 information see Supplementary Table S1.

523

524 Figure 4.

525 Homeotic transformations recorded: A) complete vertebral column without homeotic  
526 transformations and transitional vertebrae. B) transitional cervical vertebra (cervical into  
527 thoracic) (1); C) complete homeotic transformation of cervical into thoracic vertebra (2) and  
528 transitional sacral vertebra with thoracic rib on the right side and sacral rib on the other side  
529 followed by sacral vertebra (3); D) Transitional sacral vertebra - first vertebra with thoracic rib on  
530 the right side and sacral rib on the left side, followed by second transitional vertebra, with sacral  
531 rib on the right side and no rib attached on the left side (transitional sacral vertebra with a left  
532 side asymmetry) (4); Transformations are marked by arrows and showed in close-up at the  
533 bottom of the Figure.



534

535



536 Table 1.

537 The number of thoracic vertebrae in *Triturus* species (central populations only). Modal numbers  
 538 of thoracic vertebrae in vertebral formulae are shown in bold.  $S_{tr}$  – percentage of individuals with  
 539 transitional vertebrae at thoraco-sacral boundary.  $T_{var}$  – percentage of individuals with the  
 540 complete number of thoracic vertebrae different from the modal number.

Species	Sample size	Number of thoracic vertebrae												$S_{tr}$ (%)	$T_{var}$ (%)	
		12	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5			18
<i>T. marmoratus</i>	58	<b>46</b>	4	8											6.9	13.8
<i>T. pygmaeus</i>	55	<b>52</b>	1	2											1.8	3.6
<i>T. ivanbureschi</i>	175	1	3	<b>150</b>	4	17									4.0	10.3
<i>T. karelinii</i>	43		1	<b>40</b>	2										2.3	4.7
<i>T. carnifex</i>	66			4	5	<b>53</b>	3	1							7.6	12.1
<i>T. macedonicus</i>	67			9	5	<b>51</b>	1	1							9.0	14.9
<i>T. cristatus</i>	122			1		6	1	<b>98</b>	5	11					4.9	14.8
<i>T. dobrogicus</i>	57							2	11	1	<b>42</b>	1			1.8	24.6

541

542

543 Table 2.

544 Overview of homeotic transformations observed in *Triturus* species. Number and percentage of  
 545 individuals with transitional vertebrae are given. Left and right asymmetries of transitional sacral  
 546 vertebra are shown separately.

Species	Sample size	Cervical to thoracic			Transitional sacral			Thoracic to sacral			
		Transitional	%	Complete	Incomplete	%	Left	Right	%	Incomplete	%
<i>T. marmoratus</i>	58	5	8.6	0	1	1.7	1	3	6.9	0	
<i>T. pygmaeus</i>	55	1	1.8	0	0		1	0	1.8	0	
<i>T. ivanbureschi</i>	361	25	6.9	1	3	1.1	6	12	5.0	3	0.8
<i>T. karelinii</i>	43	1	2.3	0	0		0	1	2.3	0	
<i>T. carnifex</i>	123	8	6.5	0	0		6	2	6.5	0	
<i>T. macedonicus</i>	226	14	6.2	0	1	0.4	8	4	5.3	1	0.4
<i>T. cristatus</i>	286	16	5.6	2	1	1.0	10	3	4.5	0	
<i>T. dobrogicus</i>	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

547

548

549 Table 3.

550 The number of thoracic vertebrae in *T. cristatus* × *T. marmoratus* F<sub>1</sub> hybrids and parental species.  
 551 Modal numbers of thoracic vertebrae in vertebral formulae are shown in bold. S<sub>tr</sub> – percentage of  
 552 individuals with transitional vertebrae at thoraco-sacral boundary. T<sub>var</sub> – percentage of individuals  
 553 with the complete number of thoracic vertebrae different from the modal number.

Species	Sample size	Number of thoracic vertebrae												S <sub>tr</sub> (%)	T <sub>var</sub> (%)	
		12	12.5	13	13.5	14	14.5	15	15.5	16	16.5	17	17.5			18
<i>T. marmoratus</i>	58	<b>46</b>	4	8											6.9	13.8
<i>T. cristatus</i>	122			1		6	1	<b>98</b>	5	11					4.9	14.8
<i>Hybrids</i>	68	2	1	<b>39</b>	4	16		6							7.4	35.3

554

555

Table 4.

The number of individuals with regular and changed vertebral formulae in “central” and “fringe” populations.

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

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