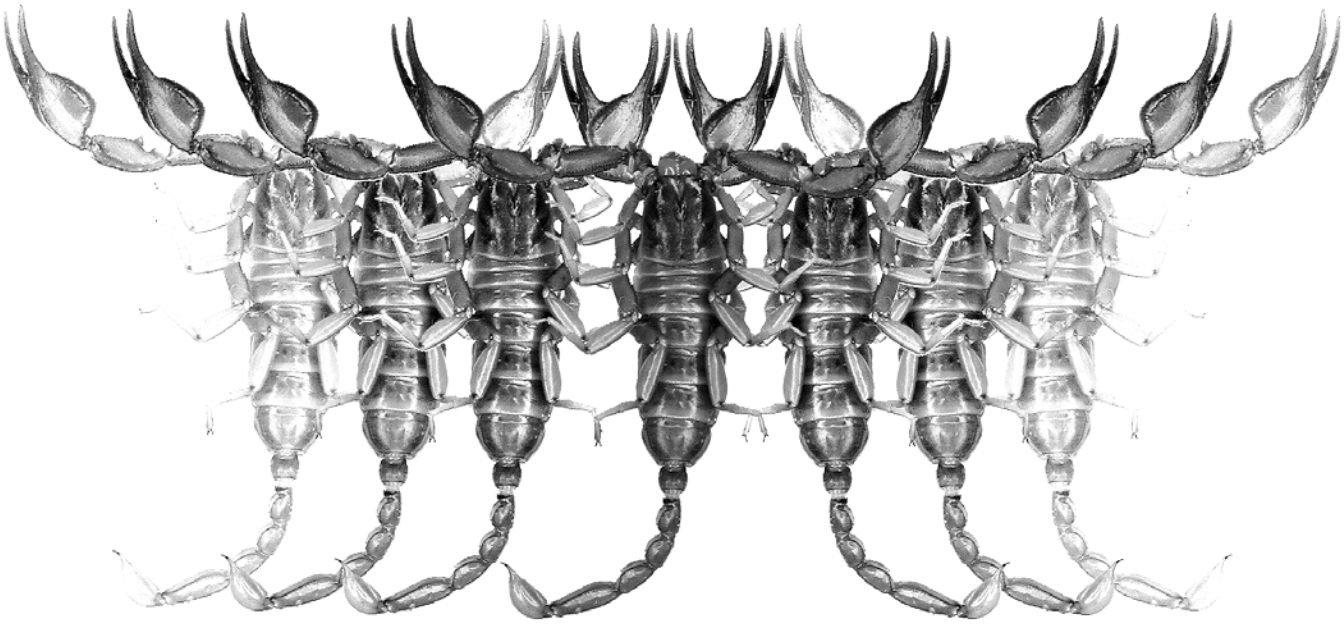
Euscorpius

Occasional Publications in Scorpiology



***Tityus ythieri* Lourenço, 2007 is a Synonym of *Tityus magnimanus* Pocock, 1897 (Scorpiones: Buthidae): a Combined Approach Using Morphology, Hybridization Experiments, Chromosomes, and Mitochondrial DNA**

František Kovařík, František Šťáhlavský, Tereza Kořínková, Jiří Král & Tom van der Ende

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Derivatio Nominis

The name *Euscorpius* Thorell, 1876 refers to the most common genus of scorpions in the Mediterranean region and southern Europe (family Euscorpiidae).

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- **ZISP**, Zoological Institute, Russian Academy of Sciences, St. Petersburg, Russia
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Tityus ythieri Lourenço, 2007 is a synonym of *Tityus magnimanus* Pocock, 1897 (Scorpiones: Buthidae): a combined approach using morphology, hybridization experiments, chromosomes, and mitochondrial DNA

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Summary

Diagnostic morphological differences to distinguish between two scorpion species, *Tityus ythieri* Lourenço, 2007 and *T. magnimanus* Pocock, 1897 do not exist; moreover, the two easily hybridize to produce fertile offspring. Therefore, we suggest that *T. ythieri* is a synonym of *T. magnimanus.* This conclusion is further supported by (1) very similar distribution of relative sizes of chromosome bivalents in both species, (2) regular pairing of homologous chromosomes in meiosis of their hybrids, and (3) identical sequences of mitochondrial DNA (COI) markers.

Introduction

Since the concept of a species can be based not only on morphological similarity but also on other aspects, especially on reproductive isolation and the ability of successive reproduction (Biological Species Concept; Mayr, 1982), it is surprising how rarely these aspects have been considered in taxonomically oriented papers, especially in groups exhibiting conservative morphology, such as scorpions. We decided to use an opportunity of having live specimens of *Tityus magnimanus* Pocock, 1897 and *T. ythieri* Lourenço, 2007 available at the same time to obtain information on the ontogenetic cycle and to attempt cross-breeding in order to find out whether a reproductive barrier exists. To obtain more conclusive results, we employed also cytogenetic and DNA sequence analyses. Comparison of obtained data on morphological variability, development, karyotype, and selected DNA sequences as well as crossing of both forms and regular course of meiosis in their hybrids suggest that *T. ythieri* is a junior synonym of *T. magnimanus*.

Material and Methods

Breeding conditions

Specimens were kept together in sibling groups at temperatures ranging from 22 to 30°C, on a substrate of moistened lignocel and pieces of bark added for hiding. Food consisted exclusively of crickets *Acheta domestica* of suitable size. As soon as a specimen underwent an ecdysis, it was transferred into another similarly furnished enclosure. In this way each of the sibling groups was split into two to three enclosures with a different frequency/volume of feeding (as an ecdysis approaches, the intake of food declines). Individuals were marked with acetone-based paints that beekeepers use to mark queens. We used four colors on different body parts, most often on the legs. In each group we marked only those juveniles that were the first and last to undergo an ecdysis, whereas in mature specimens we marked every individual of which life parameters (longevity, number, and periodicity of clutches) were followed.

Chromosome preparations

Chromosomes were prepared by a spreading technique described by Pekár and Král (2001), with some modifications. The testes of adult males were hypotonised in 0.075 M KCl for 15 min and fixed in two changes (10 and 20 min) of freshly prepared Carnoy fixative (ethanol:chloroform:acetic acid 6:3:1) or ethanol:acetic acid (3:1) fixative. Cell suspension was prepared from a piece of tissue in a drop of 60% acetic acid on a microscope slide by a pair of fine tungsten needles. The preparation was then placed on a histo-

logical plate (surface temperature of 40–45°C). The drop of suspension was allowed to evaporate while keeping it moving constantly using a fine tungsten needle. Preparations were air-dried overnight, stained with 5% Giemsa solution in Sörensen phosphate buffer (pH = 6.8) for 25–30 min, and inspected in a Olympus BX 50 microscope. Black-and-white images of chromosome plates were recorded with a CCD camera (DP 71, Olympus) using immersion objective 100x. Ten metaphase I of both forms were evaluated to construct the karyotype. The relative lengths of particular bivalents were calculated as a percentage of total length of set formed by all bivalents of the karyotype.

DNA analysis

All the specimens used in analysis (see below) were preserved in 96% ethanol and the genomic DNA was extracted using the Qiagen DNeasy Blood & Tissue Kit. A fragment of the cytochrome oxidase I (COI) gene (~600 bp) was amplified by PCR using the standard primers LCO 5'-GGTCCACAAATCATAAAGATATT GG-3' and HCO 5'-TAAACT TCAGGATGACCAAA AAATCA-3'. Following PCR conditions were used: initial denaturation 94°C for 5 min, followed by 45 cycles of 94°C for 35 s, 45°C for 35 s, and 72°C for 45 s. The final extension was at 72°C for 5 min. PCR products were purified by Montage PCR Centrifugal Filter Devices (Millipore, cat. No. UFC7PCR50) and were sent for sequencing to Macrogen Inc. (Republic of Korea) (http://www.macrogen.co.kr/). The obtained DNA sequences were edited and aligned using SeqMan 5.05 program. All new DNA sequences were deposited in GenBank (http://www.ncbi.nlm.nih.gov/), with the following accession numbers: *T. ythieri* Lourenço, 2007 (one male) (Ecuador, Morona-Santiago Province, south of Yaupi; a direct descendant of the paratypes of *T. ythieri* collected in 2005) (FJ525424); three juveniles of *T. magnimanus* Pocock, 1897 (FJ525425–FJ525427) (born from three different females collected in Venezuela, Falcón Province); and congeneric species *Tityus simonsi* Pocock, 1900 (one specimen) (Ecuador, Vilcabamba) (FJ525422) and *T. nematochirus* Mello-Leitão, 1940 (one specimen) (Venezuela, Agua Dulce near San Cristóbal) (FJ525423). One juvenile of *Zabius fuscus* (Thorell, 1876) (Buthidae) (Argentina, Cordoba Province) (FJ525421) was used as an outgroup. A Neighbor-Joining (NJ) algorithm was applied to assess the phylogenetic relationships among studied taxa. A Neighbor-Joining phylogenetic tree (Fig. 22) was created using ClustalX program (Thompson et al., 1997); the statistical support for internal clades within NJ tree was determined by the bootstrap approach with 1000 replicates. Sequence distances (Table 3) were conducted using MEGA Version 4 (Tamura et al., 2007).

Systematics

Tityus* (*Atreus*) *magnimanus Pocock, 1897
Figures 1–19

Tityus magnimanus Pocock, 1897: 514; Fet & Lowe, 2000: 249; Kovařík, 2002: 20; Lourenço & Bruehmueller Ramos, 2004: 285; Lourenço, 2007a: 376.
Tityus (*Atreus*) *magnimanus*: Lourenço, 2006: 61.
= *Tityus falconensis* González-Sponga, 1974: 62; Fet & Lowe, 2000: 244; Rojas-Runjaic & De Sousa, 2007: 287 (syn. by Lourenço & Bruehmueller, 2004: 286).
Tityus (*Atreus*) *falconensis*: Lourenço, 2006: 61; Lourenço, 2007b: 477.
= *Tityus* (*Atreus*) *ythieri* Lourenço, 2007a: 377; Lourenço, 2007b: 477. **Syn. n.**

TYPE LOCALITY AND TYPE DEPOSITORY. Brazil (error, see comments); BMNH (The Natural History Museum, London, United Kingdom).

MATERIAL EXAMINED. **Venezuela**, Falcón Province, 56♂ 42♀ 138 juvs, 2006. **Ecuador**, Morona-Santiago Province, south of Yaupi, 38♂ 24♀ 86 juvs, direct descendants of the of the holotype female and paratype male of *T. ythieri* collected by local Indians. **Hybrids No. 1**, 12♂9♀2juvs. (♀ from Venezuela and ♂ descendants of the holotype female and paratype male of *T. ythieri*), 26 juvs. descendants of the hybrids No. 1. **Hybrids No. 2**, 8♂5♀11juvs. (♂ from Venezuela and ♀ descendants of the of the holotype female and paratype male of *T. ythieri*), 21 juvs. descendants of the hybrids No. 2. All specimens were reared by first and fifth authors in 2004–2008, and are in first author's collection (FKCP).

DIAGNOSIS. Adults 45–72 mm long. Females mature after fifth ecdysis; males mature after fourth or fifth ecdysis, which determines their final size. Base color reddish yellow to dark reddish (depending on age), young spotted. Pectinal teeth number 17–23. Subaculear tooth short and strongly spinoid. Basal middle lamella of female pectines dilated. Fixed and movable fingers of pedipalps with 14/16 rows of granules that include external and internal granules. Movable finger with minute basal tubercle in both sexes. Ventral carinae of third and fourth metasomal segments in Y-shaped configuration. Manus of pedipalp narrower in female than in male. Metasoma slightly longer in male than in female.

Results

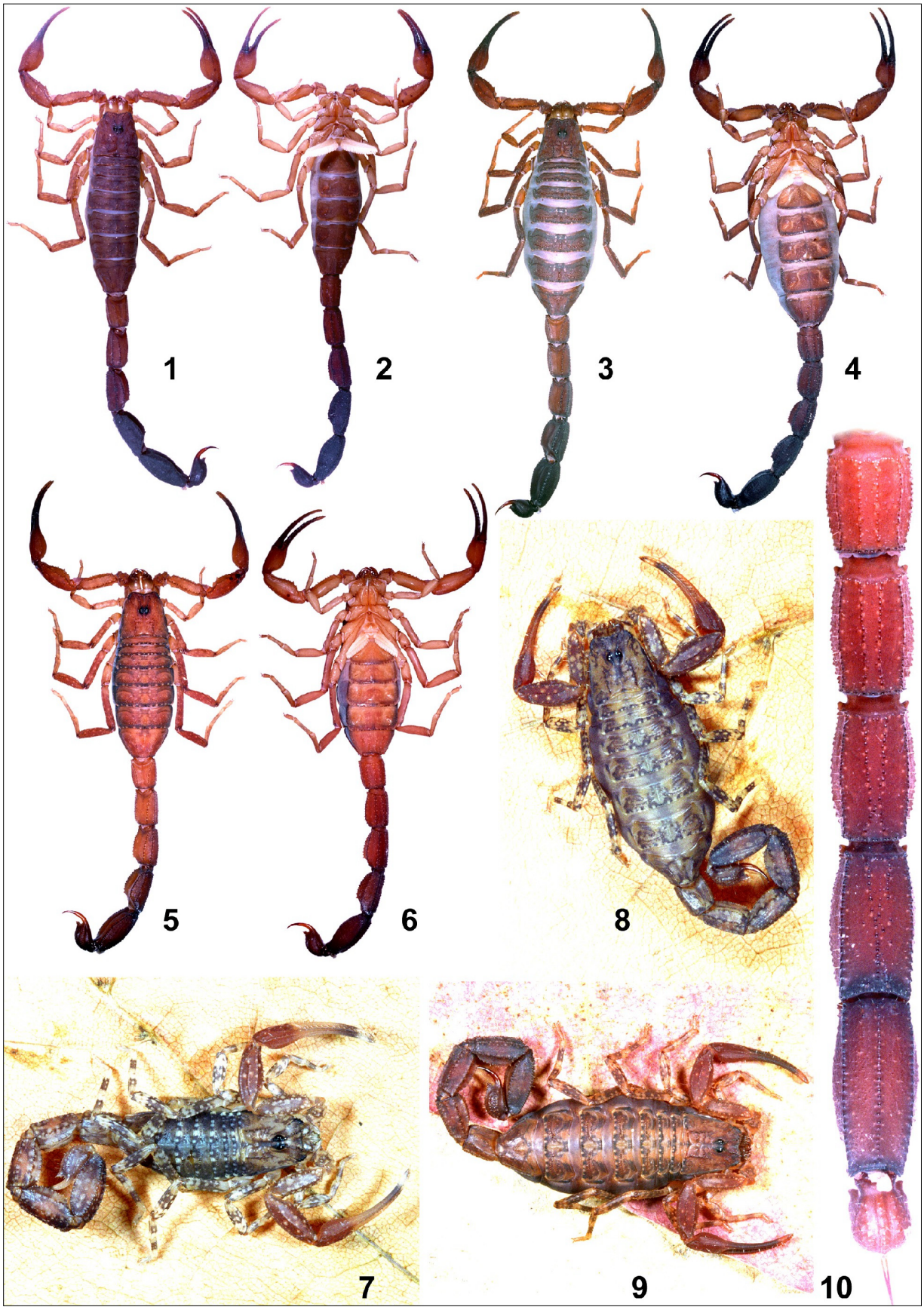
Breeding

Table 1 contains data on the development of captive specimens as well as information on subsequent births

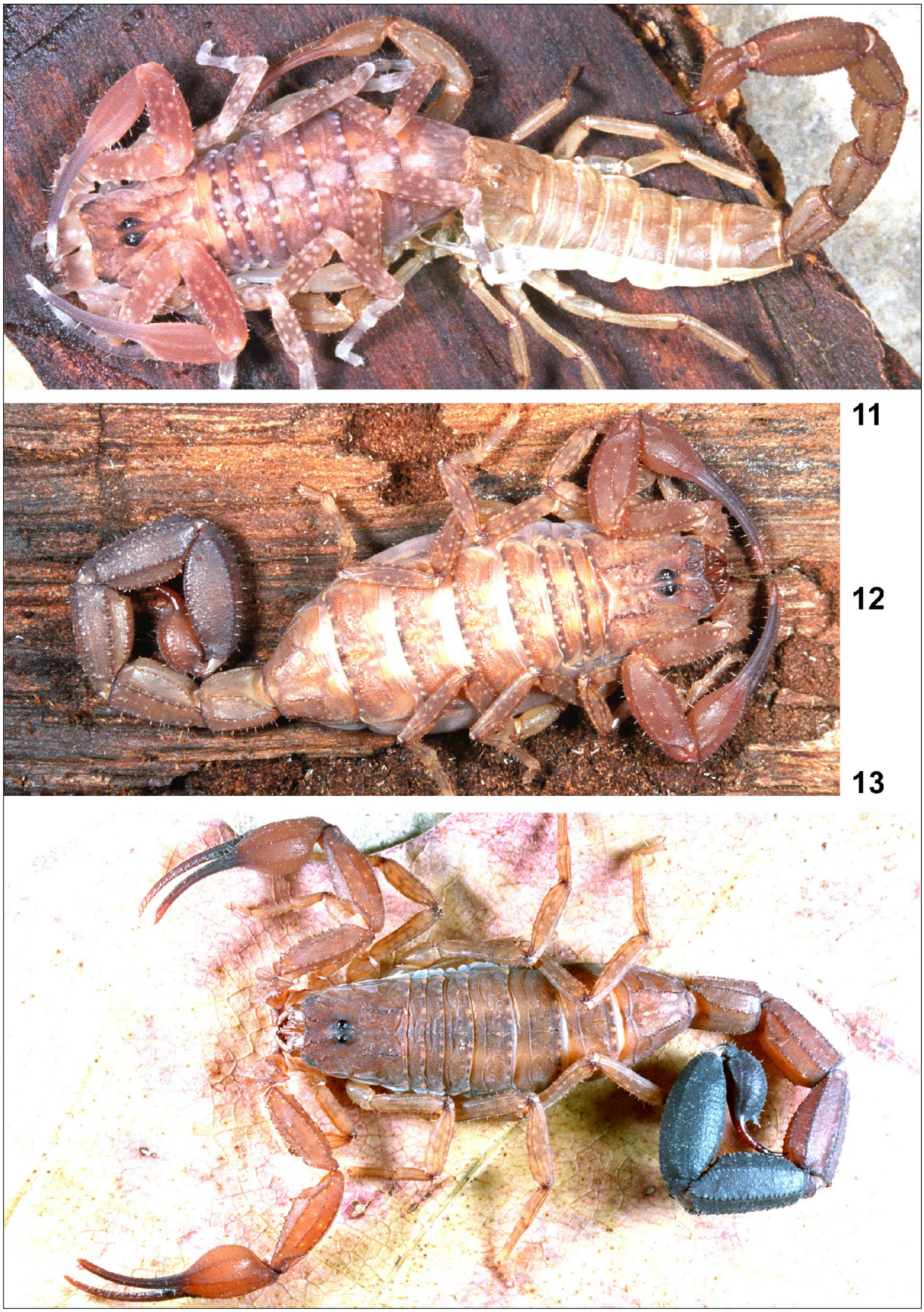
	Time of ecdyses counted in days from date of birth					First Delivery (number of larvae)	Second Delivery (number of larvae)	Third Delivery (number of larvae)	Fourth Delivery (number of larvae)	Fifth Delivery (number of larvae)	Age at Death
	First	**Second**	**Third**	**Fourth**	**Fifth**						
Tityus magnimanus	6	48–53	94 95	124 151	258♂ 231♀	331 (33)	429 (25)	531 (28)	- (-)	- (-)	646 619
Tityus magnimanus	4	37–66	65–118	90–126 108–177♂	134–213♀ - - - - -	252 (14) 255 (27) 261 (29) 269 (27) 273 (24) 310 (33) 315 (29) 317 (30)	357 (14) 357 (19) 361 (26) 366 (24) 371 (20) 406 (28) 404 (25) 410 (26)	446 (25) 442 (26) 454 (23) 459 (25) 463 (21) 494 (29) 494 (26) 494 (24)	544 (18) 539 (25) 551 (19) 555 (25) 562 (-) 609 (24) 618 (12) 606 (18)	- (-) 638 (22) 654 (14) - (-) - (-) 714 (18) 718 (17) - (-)	681 752 754 576 562 950* 728 661 696 707 758 729 950
Tityus ythieri	5	33–54	66-86	101–121 114–141♂	170♀ 190♀ 203♀ - - - 214♂ 236♂	280 (24) 296 (26) 300 (24)	381 (16) 401 (21) 402 (20)	470 (18) 499 (28) 501 (22)	572 (12) 596 (18) 602 (27)	- (-) 701 (16) 710 (20)	615 780 752 690 705 730 705 860
Hybrids No. 1	4	34–64	58–108	106–125 108–127♂	183♀ -	289 (26)					
Hybrids No. 2	4	29–36	53–81	101–118 95♂	176♀ -	287 (21)					

* 950- sixth delivery 814 days (only 3 larvae)

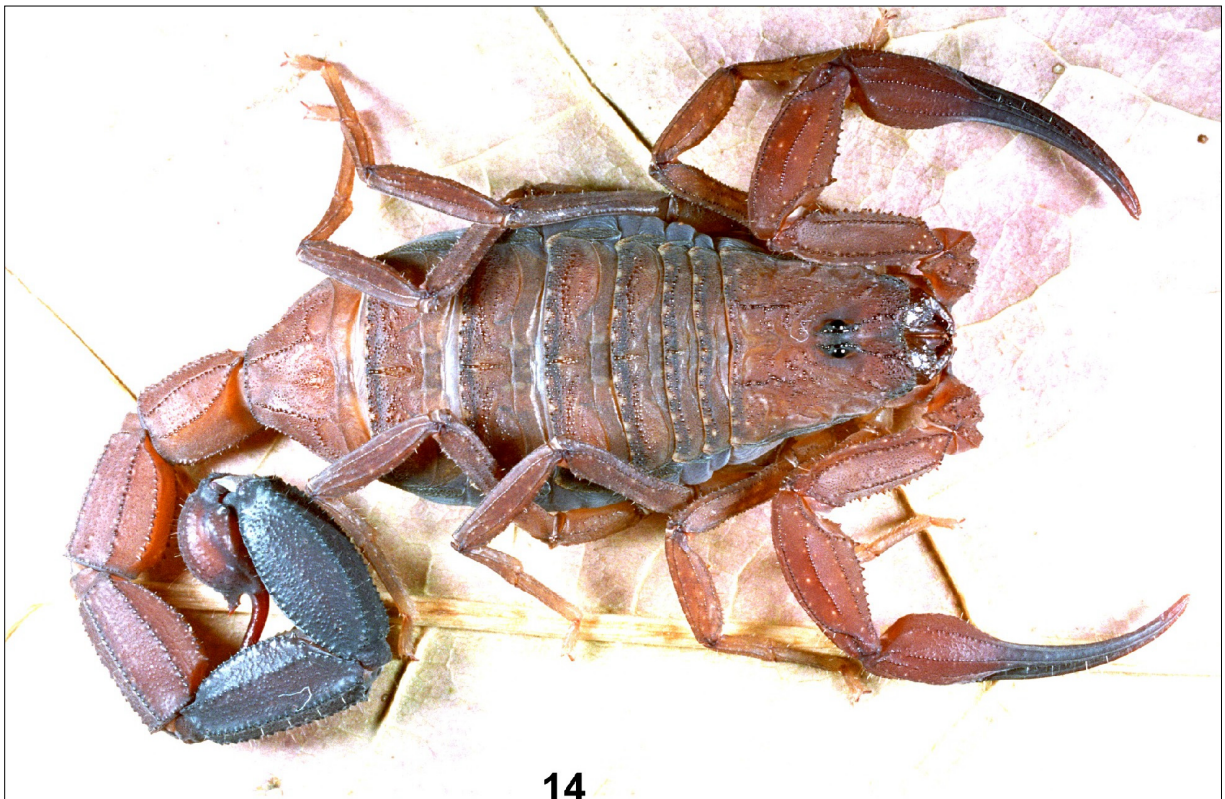
Table 1: Data on development of captive specimens as well as information on subsequent births and longevity of several individuals of *T. magnimanus* and *T. ythieri*.



Figures 1–10: ***Tityus magnimanus***, Venezuela, Falcón Province, FKCP. **1–2**. Dorsal and ventral views, ♂ (65 mm). **3–4**. Dorsal and ventral views, ♀ (65 mm). **5–6, 10**. Dorsal and ventral views, and metasoma ventral, ♀ (65 mm) immediately after last (maturation) ecdysis. **7**. Juvenile after second ecdysis. **8**. Juvenile after third ecdysis. **9**. Juvenile immediately before fourth ecdysis.



Figures 11–13: ***Tityus magnimanus***, Venezuela, Falcón Province, FKCP. **11**. Juvenile in last phase of fourth ecdysis. **12**. Immature female after fourth ecdysis. **13**. Adult male after fourth ecdysis.



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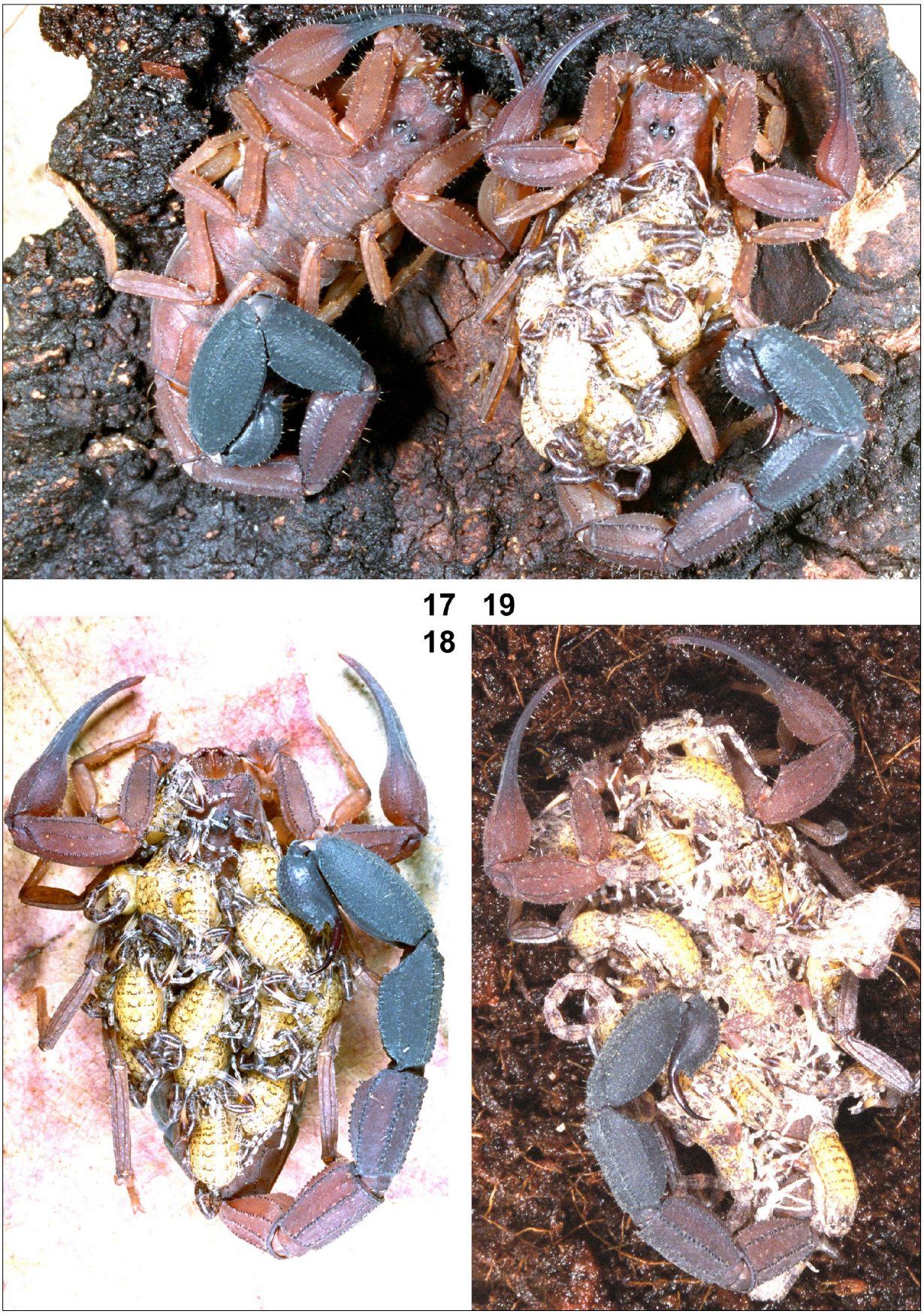
Figures 14–16: ***Tityus magnimanus***, Venezuela, Falcón Province, FKCP. **14**. Adult female after fifth ecdysis. **15**. Female at delivery. **16**. Female with juveniles and eggs.



Figures 17–19: ***Tityus magnimanus***. **17**. Male and female with juveniles. Direct descendants of the female holotype and male paratype of *Tityus ythieri* Lourenco, 2007. **18–19**. Female from Venezuela, Falcón Province. **18**. Female with juveniles before first ecdysis. **19**. Female with juvs after and in first ecdysis.

and longevity of several individuals. The table shows that males of *T. magnimanus* reach adulthood after the fourth (age of 108–177 days) or fifth (age of 214–258 days) ecdysis, whereas females reach adulthood always after the fifth ecdysis (age of 134–231 days), give birth four to six times and their gravidity takes 84–124 days. In captivity both sexes died at the age of 562–950 days.

Specimens born in captivity measured ca. 20 mm after the first ecdysis, ca. 30 mm after the second, ca. 40–45 mm after the third, 50–60 mm after the fourth and 60-75 mm after the fifth ecdysis. It can thus be concluded that in captivity this species reaches the same size as in the wild, regardless of the rate of growth which may be to some extent accelerated by higher temperature.

Similar results were obtained from captive specimens directly descended from the holotype female and paratype male of *T. ythieri*. The two populations were interbreeding without any problems, and gravid and postnatal development of F_1 hybrids (♂ *T. magnimanus* and ♀ *T. ythieri*, ♀ *T. magnimanus* x ♂ *T. ythieri*), and subsequent F_2 hybrids in no way differed from those observed in captive *T. magnimanus*.

Bivalent No.	RL(%)	
	T. magnimanus	***T. ythieri***
1	12.49	12.33
2	11.10	11.30
3	10.60	10.62
4	10.34	10.23
5	10.11	9.87
6	9.90	9.61
7	9.67	9.57
8	9.35	9.35
9	8.53	8.86
10	7.91	8.25

Table 2: *Tityus magnimanus* and *T. ythieri*. Relative lengths (RL) of particular meiotic bivalents of males.

Karyotype

Chromosomes of both forms lacked centromere and showed holocentric structure. During male meiosis, bivalents were achiasmatic (Figs. 20–21). Karyotypes of *T. magnimanus* and *T. ythieri* were formed by 10 chromosome pairs (2n = 20) that gradually decreased in size (Figs. 20–21, Table 2). Distribution of the relative lengths of chromosome bivalents of *T. magnimanus* and *T. ythieri* was very similar (Table 2). We observed no univalents or abnormal pairing of homologous chromosomes in male meiosis of hybrids of ♂ *T. magnimanus* and ♀ *T. ythieri*, as well as in hybrids resulting from reciprocal crossing (♀ *T. magnimanus* x ♂ *T. ythieri*).

DNA analysis

We obtained an alignment of 605 nucleotide pairs (base pairs, bp) for comparison of the fragment of COI gene in seven specimens belonging to five different species. We calculated pairwise absolute p-distances and Kimura distance and we find that all sequences of *T. magnimanus* and *T. ythieri* were absolutely identical, without any variability (Tab. 3). On the other hand, the genetic distance of *T. magnimanus* and *T. ythieri* from other two congeneric species and from an outgroup *Zabius fuscus* was high (16 to 19%). Neighbor Joining phylogeny indicates that *T. magnimanus* and *T. ythieri* are more related to *T. nematochirus* than to *T. simonsi* (Fig. 22).

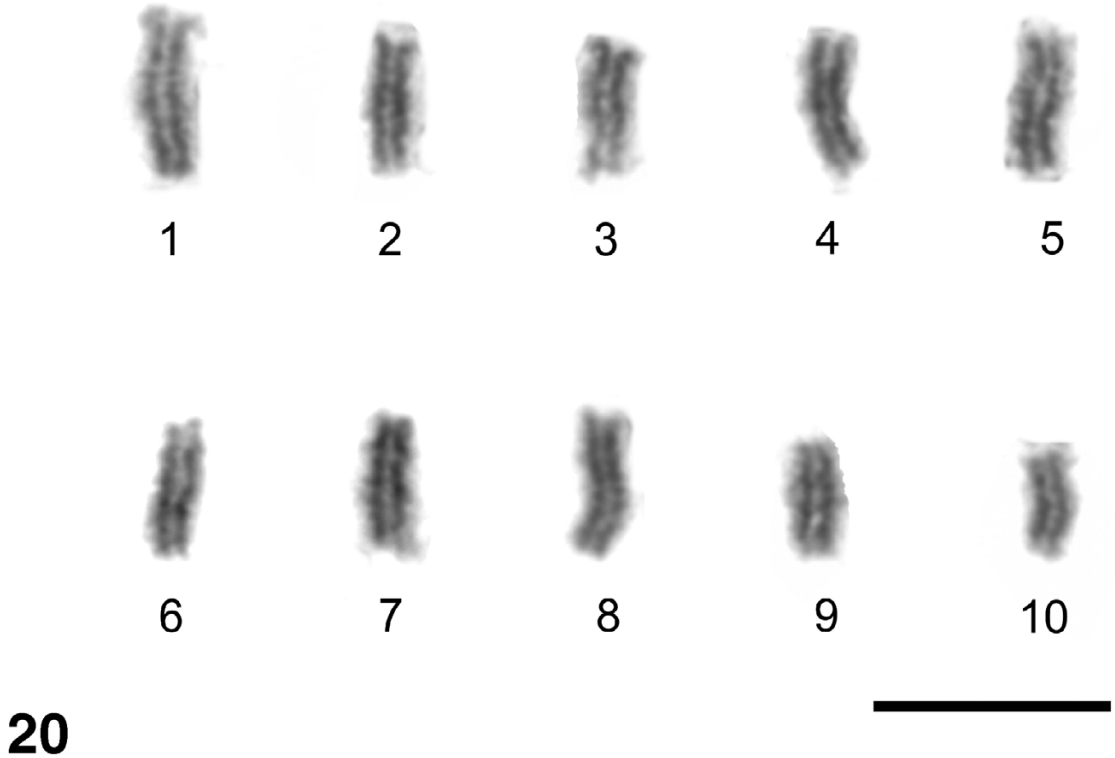
Discussion

The taxonomy of *T. magnimanus* was discussed by Lourenço (1987: 568) and Lourenço & Bruehmueller (2004: 286), who concluded that its type locality is not Brazil but Venezuela, Falcón Province, and that *T. falconensis* González-Sponga, 1974 (type locality: Cueva de Hueque, 5 km NE of Cabure, Sierra de San Luis, Falcón Province, Venezuela) is a synonym of *T. magnimanus.*

Tityus ythieri was described by Lourenço (2007a: 377) from specimens collected in Ecuador (Morona-Santiago Province, south of Yaupi) by local Indians (coll. J. Castro, October, 1993) and subsequently by E. Ythier (March 2005). In another paper, Lourenço (2007b: 477) also stated without further comment that *T. ythieri* occurs in Peru.

Lourenço (2007a: 381) compared *T. ythieri* with *T. elizabethae* Lourenço & Bruehmueller Ramos, 2004 from Brazil and with *T. magnimanus* from Venezuela, and distinguished *T. ythieri* from *T. magnimanus* by having (1) a paler reddish yellow coloration almost overall, (2) the basal middle lamella of the pectines more strongly dilated than in *T. magnimanus*, and (3) dorsal carinae of metasomal segments II to IV with one to three strong spinoid granules (whereas in *T. magnimanus* these granules are weakly developed).

Eric Ythier bred the holotype female and paratype male of *Tityus ythieri* (see Lourenço, 2007a: 381) and passed the young on to other breeders. Specimens labeled in this article as *T. ythieri* are direct descendants of the types, which Ythier at first identified as "*Tityus* cf. *ecuadorensis*" and sent early in 2004 to the fifth author (Tom van der Ende). In December 2006, Ythier wrote online"...I just want to inform people keeping those "*T.* cf. *ecuadorensis*" that they were misidentified by me. Actually it is a new species which has just been identified [described] by Lourenço as *Tityus ythieri*. The paper will be published soon in the *Ent. Mit. Zool. Museum Hamburg*." („Arachnoboards" forum: http://



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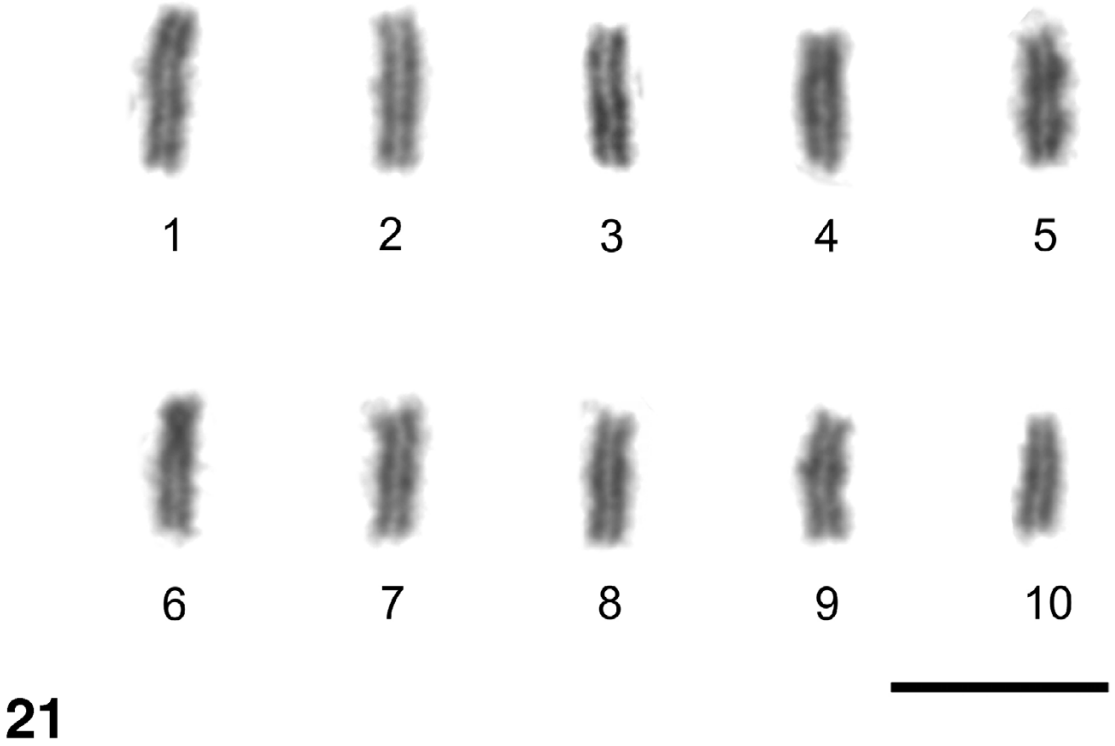

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Figures 20–21: Karyotypes of *T. magnimanus* (**20**) and *T. ythieri* (**21**). Chromosome complement of metaphase I cells consists of ten bivalents. Bar = 10 µm.

	1	2	3	4	5	6	7
1 ***Zabius fuscus***	-	0.214	0.202	0.222	0.222	0.222	0.222
2 ***Tityus simonsi***	0.185	-	0.198	0.213	0.213	0.213	0.213
3 ***Tityus nematochirus***	0.177	0.174	-	0.185	0.185	0.185	0.185
4 ***Tityus ythieri***	0.190	0.183	0.164	-	0.000	0.000	0.000
5 ***Tityus magnimanus* 1**	0.190	0.183	0.164	0.000	-	0.000	0.000
6 ***Tityus magnimanus* 2**	0.190	0.183	0.164	0.000	0.000	-	0.000
7 ***Tityus magnimanus* 3**	0.190	0.183	0.164	0.000	0.000	0.000	-

Table 3: A matrix of genetic distances: *p*-distance (below the diagonal); Kimura 2-parameter distance (above the diagonal).

www.arachnoboards.com/ab/archive/index.php/t-39142.html). Tom van der Ende brought the juveniles to maturity and passed the next generation on to the first author (František Kovařík), who continued the breeding process (see Table 1).

Specimens labeled in this paper as *T. magnimanus* originate from the Falcón Province of Venezuela (without a closer localization). Study of many captive-born specimens belonging to these two populations (*T. ythieri* and *T. magnimanus*) shows that (1) coloration changes with age and becomes darker (dark reddish) in older specimens; (2) the basal middle lamella of the pectines is somewhat variable but its dilation is nevertheless very similar in both populations and their hybrids; and (3) the spinoid granules on dorsal carinae of metasomal segments II to IV are developed to variable extents in both populations, strong in some specimens and weak in others.

Thus, diagnostic morphological differences between *T. ythieri* and *T. magnimanus* do not exist; moreover, since the two easily hybridize and their offspring remains fertile, we suggest that *T. ythieri* is a synonym of *T. magnimanus.* This idea is further supported by very similar karyotype of both species, regular pairing of homologous chromosomes during meiosis of their hybrids, as well as by identical DNA marker (COI) sequences.

Chromosomes of *T. magnimanus* and *T. ythieri* exhibit holocentric structure and achiasmate male meiosis, like other buthid scorpions studied so far (Shanahan 1989). From cytogenetic point of view, the genus *Tityus* belongs to best studied genera of scorpions due to its medical significance. We have found data on karyotypes of six species at literature (Toledo Piza, 1947, 1950b). This genus exhibits extensive inter- and intraspecific diversity of chromosome numbers. Karyotype of *T. magnimanus* and *T. ythieri* consists of 20 chromosomes. The same number of chromosomes was found also in other species of the genus *Tityus*, namely in *T. bahiensis* (Perty, 1834) and *T. mattogrossensis* Borelli, 1901 (see Toledo Piza, 1947). *T. bahiensis* showed a great variability of chromosome numbers, namely from 6 to 20 (Toledo Piza, 1949, 1950a, 1950b). Remaining examined species of the genus *Tityus* showed the following diploid chromosome numbers: *T. serrulatus* Lutz & Mello, 1922: 2n = 12 (Toledo Piza, 1947), *T. stigmurus* (Thorell, 1876): 2n = 16 (Toledo Piza, 1950b), *T. trivittatus* Kraepelin, 1898: 2n = 14 (Toledo Piza, 1948); and *T. neglectus* Mello-Leitão, 1932: 2n = 26, 27, 28 (Toledo Piza, 1950b). Variability of chromosome numbers found in *T. bahiensis* and *T. neglectus* reflects operation of chromosome fusions and fissions in their populations. Fusion/fission heterozygotes are common in these species (Toledo Piza, 1950b; White, 1973). We found no anomalies caused by fusion/fission heterozygosity or other pairing anomalies of homologous chromosomes in meiosis of studied species as well as in their hybrids. Similar distribution of relative sizes of chromosome bivalents in *T. magnimanus* and *T. ythieri* as well as regular pairing of homologous chromosomes in their hybrids support close relationship or even identity of *T. magnimanus* and *T. ythieri*.

The comparative analysis of selected gene sequences has been successfully used in scorpion taxonomy and phylogeography during the last decade. The most commonly used DNA markers in studies at intrageneric or intraspecific levels in scorpions appear to be mitochondrial 16S (e. g. Gantenbein et al., 1999, 2000, 2002; Fet, 2003) and COI rDNA sequences (Gantenbein & Largiadèr, 2003). Owing to the adequate divergence within these genes, it was possible to solve a number of problems in taxonomy of scorpion genus *Euscorpius* Thorell, 1876 (Gantenbein et al., 2000, 2002) or to study phylogeography of *Buthus occitanus* (Amoreux, 1789) populations across the Strait of Gibraltar (Gantenbein & Largiadèr 2003). In this context, zero variability of COI rDNA sequences between *T. magnimanus* and *T. ythieri* specimens seems to support the synonymy of these two taxa quite well. In principle, identical sequences from three juveniles of *T. magnimanus* coming from three different females may be explained by a lineal consanguinity because females come from the same locality, or it may be an effect of low genetic diversity within the population. Due to absence of molecular systematic studies within the genus

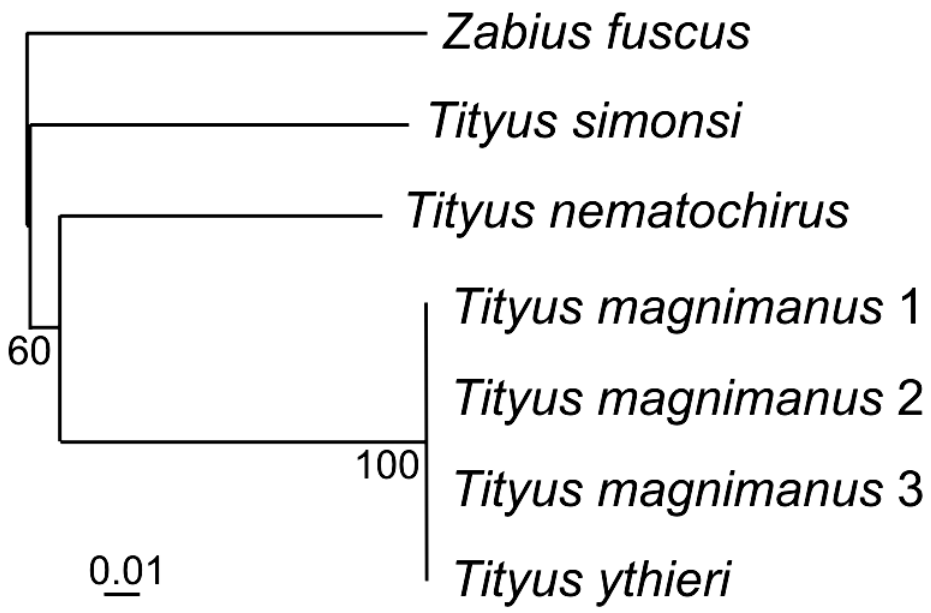

Figure 22: Neighbor-Joining (NJ) tree of studied species based on a 605 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene.

Tityus, and due to only a limited sample in this study, we cannot exclude also a possibility that variability of COI marker is not able to detect intraspecific diversity in this genus. On the other hand, the absolute identity of COI sequences between the studied specimens of *T. magnimanus* and *T. ythieri* supports their synonymy because there was no evidence of genetic divergence. Note also that COI marker is one of the mitochondrial sequences used in so-called "DNA barcoding" projects, which are supposed to discriminate among taxa at species level (see e.g. Ratnasingham & Hebert 2007).

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