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Morphology of *Pholeoixodes* species associated with carnivores in the western Palearctic: Pictorial key based on molecularly identified *Ixodes* (*Ph.*) *canisuga*, *I.* (*Ph.*) *hexagonus* and *I.* (*Ph.*) *kaiseri* males, nymphs and larvae

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ABSTRACT

Three Palearctic members of the subgenus *Pholeoixodes*, i.e., *Ixodes canisuga*, *Ixodes hexagonus* and *Ixodes kaiseri* are frequently collected from dogs, cats, red foxes, badgers and other carnivorous/insectivorous hosts in Europe. While a pictorial identification key has been reported for female *Pholeoixodes* ticks, a similar work has not been done on their male, nymphal and larval specimens. This study was initiated in order to clarify and re-examine those morphological characters of these three tick species, which can be used relatively easily to identify/distinguish them. In the case of larvae the aims included finding alternatives to chaetotaxy, which is difficult to observe and its usefulness is also affected by uncertainties in literature data.

For this, 609 *Pholeoixodes* ticks (males, nymphs and larvae) were collected from carnivores, hedgehogs and their environment in six European countries (representing Western, Central and Southeastern Europe), followed by detailed morphological examination and/or molecular analyses to confirm the identity of their species. Based on the morphology of 84 molecularly analyzed specimens and a new identification key compiled accordingly, altogether 116 *I. canisuga*, 277 *I. hexagonus* and 216 *I. kaiseri* males, nymphs and larvae were identified. *Ixodes kaiseri* was not found in Western Europe, where *I. canisuga* predominated. In Central Europe, all three *Pholeoixodes* species were collected, the largest number of specimens represented by *I. hexagonus*. On the other hand, in Southeastern Europe *I. kaiseri* had the highest abundance.

In conclusion, the morphology of internal spur on the first coxae (as the traditionally used character to distinguish *I. hexagonus* from other *Pholeoixodes* species) is trustworthy to recognize males but is less informative in the case of nymphs and larvae. The latter can be identified more properly by observing the morphology of basis capituli. In particular, nymphs and larvae of *I. canisuga* have anteriorly flattened basis capituli, forming a plateau that surrounds the base of the hypostome. On the other hand, nymphs and larvae of *I. hexagonus* and *I. kaiseri* lack a similar plateau, but (unlike *I. canisuga*) have cornuae, which are either posterolaterally or caudally directed, respectively.

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1. Introduction

Among hard ticks (Acari: Ixodidae), the genus *Ixodes* contains the highest number of species (Guglielmone et al., 2014). These are grouped into subgenera, within which species share morphologic and ecologic characters (Estrada-Peña et al., 2017a). Members of the subgenus *Pholeoixodes* (Schulze, 1942) typically have short palps, reduced number/-length of coxal spurs, absence of auriculae and a well-developed tarsal hump (Nosek and Sixl, 1972; Clifford et al., 1973). These species are usually associated with burrow-dwelling mammals, as well as terrestrial birds that nest in cavities (tree holes or burrows). The most common species of this subgenus associated with carnivores (mainly Canidae, Felidae, Mustelidae) and hedgehogs (Erinaceidae) in the western Palearctic are *Ixodes canisuga*, *Ixodes hexagonus* and *Ixodes kaiseri* (Estrada-Peña et al., 2017a). Some authors consider *I. canisuga* as a junior synonym of *Ixodes crenulatus* (Černý, 1969; Guglielmone et al., 2014), and the latter is also often reported from Poland, Eastern Europe and Asia (Estrada-Peña et al., 2017b). A fifth species, *Ixodes rugicollis* occurs sporadically and is found rarely in Central and Western Europe (Estrada-Peña et al., 2017b).

As indicated by the name of their subgenus, *I. canisuga*, *I. hexagonus* and *I. kaiseri* are endophilic (pholeophilous). This implies that for scientific and diagnostic purposes they are most accessible from their host, where they frequently cause mixed infestation, necessitating accurate species identification. This becomes especially important in the context of pathogen transmission because the vector role of these three species is thought to be different (Estrada-Peña et al., 2017b; Hornok et al., 2020). However, even their females (i.e., the largest stage, with the broadest spectrum of structures allowing species identification) bear similarities and can be relatively easily confused with each other (Hornok et al., 2017). Accordingly, it has been stated that it is urgent and necessary to prepare a comparative overview of the morphologic details of *Pholeoixodes* ticks of carnivores occurring in Europe (Estrada-Peña et al., 2017b). While a simple, pictorial identification key has been reported for female *Pholeoixodes* ticks, including their molecular phylogenetic analyses and comparison with type specimens (Hornok et al., 2017), a similar work has not been done on their male, nymphal and larval specimens.

In this context nymphs, and particularly larvae may pose the biggest difficulty during identification of their species. Chaetotaxy, in particular the number of marginal dorsal and marginal ventral setae is important in distinguishing larvae within *Ixodes* subgenera, as reported for both Nearctic (Clifford and Anastos, 1960) and Palearctic species (Marquez et al., 1992). However, descriptions of chaetotaxy among larvae of Palearctic *Pholeoixodes* species are not exempt of contradictions (as elaborated in the Discussion). In addition, setae may not be easily observed during species identification.

On the other hand, conflicting data also exist in other aspects of morphology, as it can be seen from the following examples: the anal groove is discontinuous in front in the case of *I. canisuga* nymph according to Nosek and Sixl (1972), unlike shown in Cordas et al. (1993); cornuae on the basis capituli of larvae are lacking (Cordas et al., 1993) or are present (Estrada-Peña et al., 2017b) in *I. canisuga* larvae, and lacking (Arthur, 1965) or rounded (Manilla, 1998) on *I. hexagonus* larvae. In light of these uncertainties, traditional morphology-based identification of *Pholeoixodes* larvae and nymphs will benefit from molecular comparison of representative specimens (Hornok et al., 2017).

This study was initiated in order to clarify and re-examine those morphological characters of the three most common Palearctic *Pholeoixodes* species, which can be used relatively easily in a dichotomous

Table 1
Data of samples used in this study, and results of *cox1* and 16S rRNA gene sequence analyses. Localities are shown in separate rows only if corresponding tick host species were different. Upper index of a GenBank accession number indicates that the sample originated from the locality with the same upper index.

Region	Country	Locality or region of collection	Sample origin (n = number of hosts per tick sp.)	*Ixodes* species (number of M = males, N = nymphs, L = larvae)	GenBank accession numbers of *cox1* [or 16S rRNA] sequences detected in this study and already reported (n = number of sequences)	GenBank accession numbers of *cox1* [or 16S rRNA] sequences detected in this study and new in the country (n = number of sequences)
Western Europe	Ireland	Wicklow	badger (8)	*I. canisuga* (10 N, 2 L)	–	MT659136 (3), MT659137 (2), [MT658762 (5)]
			badger (1)	*I. hexagonus* (1 N)	–	MT659138 (1), [MT658763 (1)]
	France	Bernay	fox burrow	*I. canisuga* (12 M, 1 N)[1]	KY962049 (6), [KY962074 (2)]	–
		Nancy, Nantes, Carquefou	badger (5)	*I. canisuga* (15 N, 1 L)	KY962049 (1), [KY962074 (2)]	–
			red fox (7)	*I. canisuga* (8 N, 38 L)	KY962044 (1), [KY962068 (2)], [KY962069 (1)]	–
	Germany	Thuringia	red fox (22)	*I. hexagonus* (1 M, 24 N, 110 L)	KY962046 (3), [KY962070 (14)]	MT659139 (1), [MT658767 (3)]
			red fox (12)	*I. kaiseri* (1 M, 14 N, 42 L)	KY962042 (1), [KY962067 (4)]	[MT658769 (1)], [MT658770 (1)], [MT658771 (1)]
Central Europe	Hungary	Aggtelek	badger (2)	*I. canisuga* (5 N)	KY962013 (2), [KY962053 (2)]	–
		Veszprém, Kapolcs	cave entrance	*I. canisuga* (2 M)[1]	[KY962053 (2)]	–
		Porrogszentkirály	badger (1)	*I. canisuga* (1 N)	[KY962053 (1)]	MT659130 (1)
			badger (1)	*I. kaiseri* (2 N)	KY962015 (2)	[MT658758 (2)]
		Iharos	dog (1)	*I. hexagonus* (1 N)	–	MT659131 (1), [MT658759 (1)]
		Budapest	hedgehog (1), cat (1), indoors	*I. hexagonus* (1 M[1], 73 N, 62 L)	–	MT659132 (4), [MT658760 (6)]
	Croatia	Zaprešić, Petrinja, Vrbovec[2]	red fox (3)	*I. hexagonus* (4 M)	[KY962077 (1)]	[MT658768[2] (1)]
South-eastern Europe	Romania	Harman, Cusuiuș, Cefa, Popesti, Targu Secuiesc[5], Turia, Coșeni[6,8], Sudrigiu[3], Crânceşti[9], Sâmpetru, Ilia, Tăbărăști[4,7]	red fox (4)	*I. canisuga* (21 L)	KY962021 (1), KY962022 (3), KY962023 (1), KY962025 (1), [KY962060 (4)], [KY962061 (1)]	[MT658764[3] (1)]
			red fox (10)	*I. kaiseri* (1 M, 38 N, 118 L)	KY962020 (11), [KY962059 (13)], [KY962062 (1)]	MT659133[4] (1), MT659134[5] (1), MT659135[6] (1), [MT658761[7] (1)], [MT658765[8] (1)], [MT658766[9] (1)]

[1]These ticks were unengorged.

Table 2
Literature review of important differential diagnostic characters of males, nymphs and larvae of the three most common *Pholeoixodes* species associated with carnivores and hedgehogs in the Western Palearctic. Reference number is shown in black circle next to each statement.

(A) Male:

View	Body part	Structure	Character according to species		
			Ixodes canisuga	*Ixodes kaiseri*	*Ixodes hexagonus*
dorsal	gnathosoma	palp	laterally 6–7 short hair ❼ medially 3–5 hair ❷	laterally 8–9 hair ❸ medially 10–12 hair ❷	laterally 6–7 short hair ❸ medially 3–5 hair ❶
		basis	W ≈ 2 × L ❽	–	W ≈ 1.5 × L ❽
	idiosoma	AG anteriorly	–	straight (broadly rounded) ❸	pointed ❶ ❸
		median plate	narrow ❺	narrow, as long as anal plate ❷	very broad (W = L) ❷❺
		adanal plate	L ≈ 2 × W ❶ ❽	L > 3 × W ❶ ❷	L ≈ 3 × W ❽
ventral	gnathosoma	palp	no lateral "bump" on 2nd palpal segment ❶❼	no lateral "bump" on II. palpal segment ❷	lateral "bump" on 2nd palpal segment ❶❽❼
		basis	–	auricular ridges inapparent ❷	auriculae as long ridges ❷
	legs	coxa I.	chitinized inner margin ❶ ❽ or internal spur ❼	broad tapered internal spur ❷ (medial denticle: ❶)	short, broad ❷ or pointed ❽, sharp ❶ internal spur
		coxae II-IV.	slightly elevated external crests ❶ ❼	weak internal projections; no external spurs, perhaps external projections on III-IV. ❷	slightly elevated external spurs ❷ ❽ or crests ❽

(B) Nymph:

View	Body part	Structure	Character according to species		
			Ixodes canisuga	*Ixodes kaiseri*	*Ixodes hexagonus*
dorsal	scutum	margin/ shape	L > W, broadest anteriorly, posterolateral margin with two concavities ❶ ❾	L > W, broadest anteriorly, posterolateral margin with two concavities ❻	L ≈ W, broadest close to midlength, posterolateral margin with one concavity ❷
		CG	absent ❾	converging, then diverging ❷	nearly parallel ❷
	gnathosoma	palp	lateral hairs on segment II. longer than those on III. ❼	lateral hairs on segment II. longer than those on III. ❽	lateral hairs on segment II. longer than on III. ❷
		basis	basis with divergent sides, cornuae small, pointed ❽, posterolaterally directed ❶	basis with parallel sides, cornuae large, pointed ❷ ❻	cornuae blunt, posterolaterally directed ❼
ventral	gnathosoma	palp	–	–	ventral spur on segment I. ❷
		basis	auriculae absent ❽	short auricular ridges present ❷	auriculae as ridge-like thickenings ❷
	legs	coxa I.	no internal spur ❺ ❼; no external spur ❺	internal spur as a marginal projection ❷; external spur ❷	short, pointed internal spur ❶ ❷ ❺ ❽; small, blunt external spur ❶ ❺
		coxae II-IV.	no spurs ❺	external spur ❷	small, blunt external spur ❶ ❺

(C) Larva:

View	Body part	Structure	Character according to species		
			Ixodes canisuga	*Ixodes kaiseri*	*Ixodes hexagonus*
dorsal	gnathosoma	palp	no dermal sensilla at mid-length ❸	dermal sensilla at mid-length ❸ ❻	no dermal sensilla at mid-length ❸
		basis	cornuae lacking ❼	cornuae present, pointed ❻	cornuae lacking ❷ or rounded ❽
	idiosoma	surface	–	MD 8× ❸ ❻, MD ≈ MS ❶ ❹ or L of MD ≈ 2 × L of MS ❻	MD 8× ❸, L of MD ≈ 2 × L of MS ❶ ❹
ventral	gnathosoma	basis	auriculae absent ❽	auriculae as short ridges ❻	auriculae as thickened ridges ❷
	legs	coxa I.	no internal spur ❺ ❼ ❽; no external spur ❺ ❽	broad, blunt internal spur ❻	internal spur ❺ small, blunt ❽ and broad ❷; no external spur ❺
		coxae II-III.	no spurs ❺ ❽	broad, blunt "median" spur on coxa II. ❻	no internal spur ❷ or on coxa II. blunt internal spur ❶; no external spur ❺, only external crests ❽
	idiosoma	surface	MV 4× ❶ ❹	–	MV 4× ❸

Abbreviations: AG - anal groove; CG - cervical groove; MG - marginal groove; W - width; L - length; MD - marginal dorsal setae; MS - marginal scutal setae; MV - marginal ventral setae.
References (in an arbitrary, decreasing order of comprehensiveness, accessibility and quality of illustrations):
❶ Estrada-Peña et al. (2017b), ❷ Arthur (1965), ❸ Filippova (1977), ❹ Marquez et al. (1992), ❺ Nosek and Sixl (1972), ❻ Filippova and Uspenskaya (1973), ❼ Cordas et al. (1993), ❽ Manilla (1998).

key to identify them. For this, male ticks, nymphs and larvae were collected from carnivores, hedgehogs and their environment in six European countries (representing Western, Central and Southeastern Europe), followed by detailed morphological examination and subsequent molecular analyses to confirm the identity of their species (Hornok et al., 2017). In this work digital stereomicroscopic pictures are used. These are the most informative when put (similarly to a voucher specimen) next to the sample under evaluation, and therefore the authors consider them superior for everyday usage in comparison with scanning electron microscopic pictures (for which instruments are not available in most laboratories and clinics) and drawings (which may easily miss important structures and only "mimic" microscopic views).

It should be taken into account that there are adequate descriptions of Palearctic *Pholeoixodes* species in various sources (Filippova, 1977; Manilla, 1998; Pérez-Eid, 2007) as stated by Estrada-Peña et al. (2017a). Thus, it was not among the aims of this study to redescribe any of these species. At the same time, some of these sources are hard to access or are not in English, therefore the most important characters mentioned in these sources are also reviewed here.

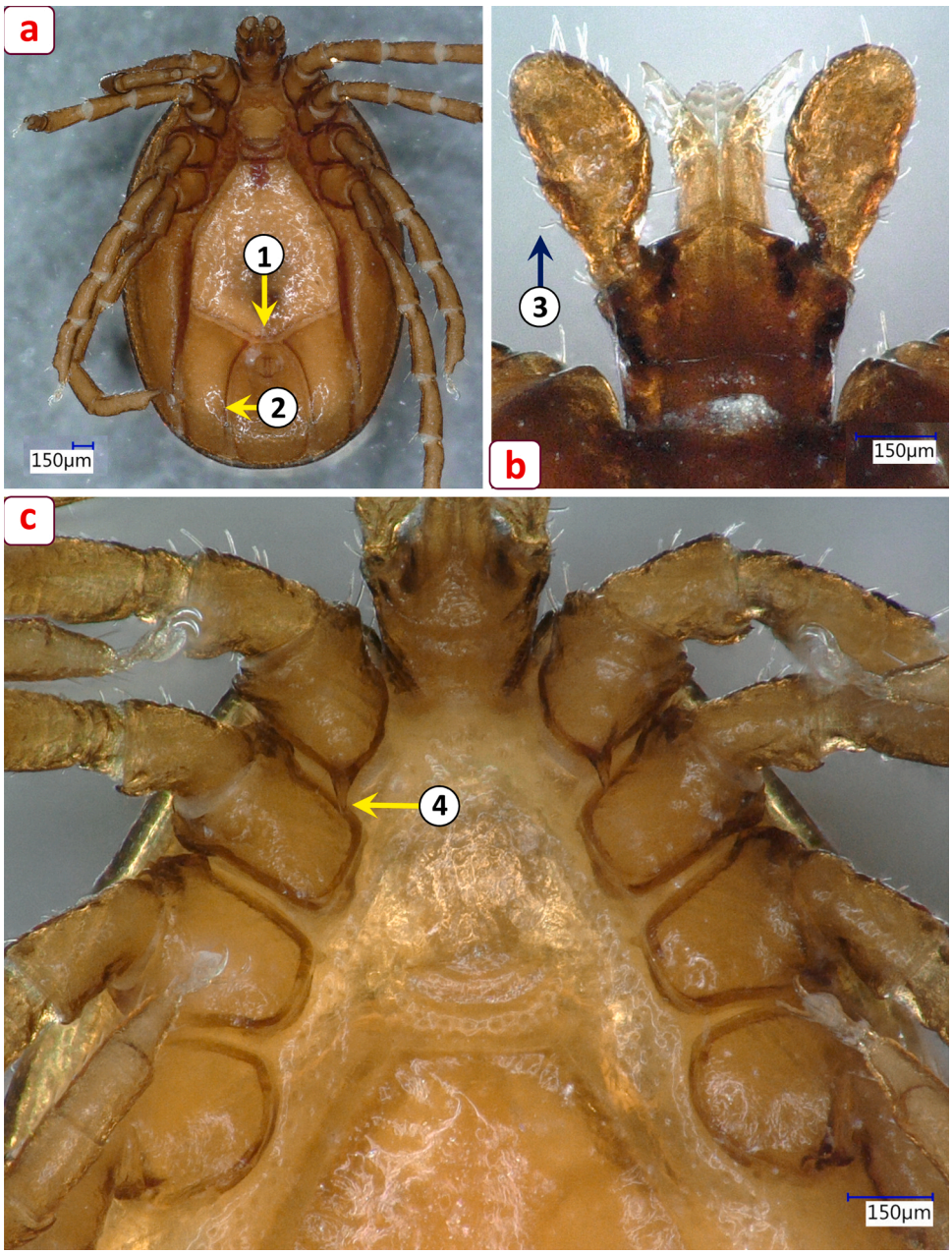

Fig. 1. Key features of *Ixodes hexagonus* males. (a) Habitus, ventral surface; (b) gnathosoma, dorsal surface; (c) coxae I-IV. Numbered arrows indicate: (1) preanal groove straight anterior to and (2) its branches curved behind the anus; (3) short setae on palpal article II; (4) long, narrow and pointed internal spur on coxa I. Specimens on the pictures were collected in Croatia.

2. Materials and methods

2.1. Tick collection and morphological identification

For this study, 609 *Pholeoixodes* males, nymphs and larvae were selected from specimens collected from four species of carnivores (red foxes, dog, cat, badgers), a hedgehog and the environment between 2008 and 2019 (Table 1). Specimens from Germany were kept frozen until evaluation, while others were stored in 96 % ethanol. Preliminary identification of these stages was performed according to monographs and papers (Table 2). Eighty-four ticks (*I. canisuga*: 8 males, 15 nymphs, 8 larvae; *I. hexagonus*: 4 males, 4 nymphs, 19 larvae; *I. kaiseri*: 2 males, 6 nymphs and 18 larvae) were studied morphologically in detail, implying pictures of diagnostically important structures with a VHX-5000 digital microscope (Keyence Co., Osaka, Japan). This subset (i.e., >10 % of all ticks) was then molecularly and phylogenetically analyzed in comparison with sequences of females identified based on type specimens as reported previously (Hornok et al., 2017). The remaining 525 ticks were identified based on the pictures of molecularly identified specimens.

2.2. Mounting of tick larvae

In order to observe setae and key characters noted during digital microscopic analyses, 13 larvae (four *I. canisuga*, three *I. kaiseri* and six *I. hexagonus*) were mounted on slides for light microscopic examination. For this, larvae were cleared in 50 % lactic acid for two days, then incubated for approximately one week (until evaporation is completed) in a solution containing 50 % glycerine, 10 % ethanol and 40 % distilled water. Finally, they were mounted on slides in glycerine under a coverslip surrounded by Diamount (Diapath, Martinengo, Italy).

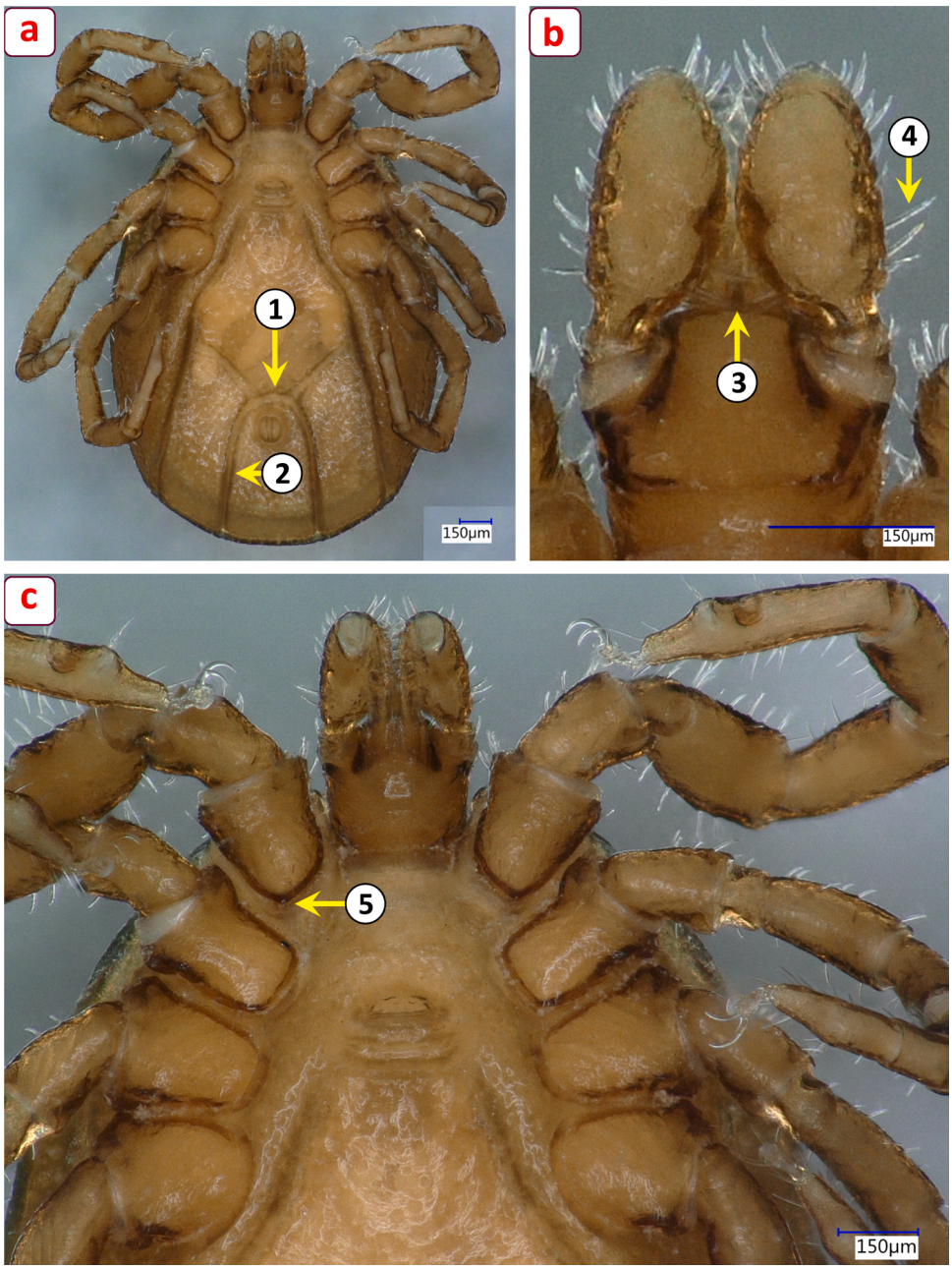

Fig. 2. Key features of *Ixodes canisuga* males. (a) Habitus, ventral surface; (b) gnathosoma, dorsal surface; (c) coxae I-IV. Numbered arrows indicate: (1) preanal groove curved anterior to and (2) its branches nearly parallel behind the anus; (3) basis capituli anteriorly flattened; (4) moderately long setae on palpal article II; (4) inconspicuous (short, blunt) internal spur on coxa I. Specimens on the pictures were collected in France.

2.3. Molecular and phylogenetic analyses

DNA was extracted from 84 ticks individually with the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction, including an overnight digestion in tissue lysis buffer and 6.6 % Proteinase-K at 56 °C. Part of the cytochrome *c* oxidase subunit I (*cox*1) gene and/or the 16S rRNA gene were amplified with the primer pairs HCO2198 (5'-TAA ACT TCA GGG TGA CCA AAA AAT CA-3') and LCO1490 (5'-GGT CAA CAA ATC ATA AAG ATA TTG G-3'), or 16S + 1 (5'-CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3') and 16S-1 (5'-CCG GTC TGA ACT CAG ATC AAG T-3'), respectively, as reported (Black and Piesman, 1994; Folmer et al., 1994; Hornok et al., 2017).

Purification and sequencing of the PCR products were done by Biomi Ltd. (Gödöllő, Hungary). One representative sequence (for each tick species according to each country) were submitted to GenBank (accession numbers for the *cox*1 gene: MT659130-MT659139; for the 16S rRNA gene: MT658758-MT658771). Sequences of Palearctic tick species from other studies (retrieved from GenBank) were included in the phylogenetic analyses only if they had nearly 100 % coverage with sequences from this study. In addition, *I. cookei* was also included in the phylogenetic analysis, on account of its morphological similarity to *I. hexagonus*. This dataset was resampled 1,000 times to generate bootstrap values. Phylogenetic analyses were conducted with the Maximum Likelihood method (Tamura-3 and HKY models) by using MEGA version 7.0.

2.4. Ethical approval

In Ireland, ethical approval for examination of badgers was granted by Trinity College Dublin's Animal Research Ethics Committee (Project

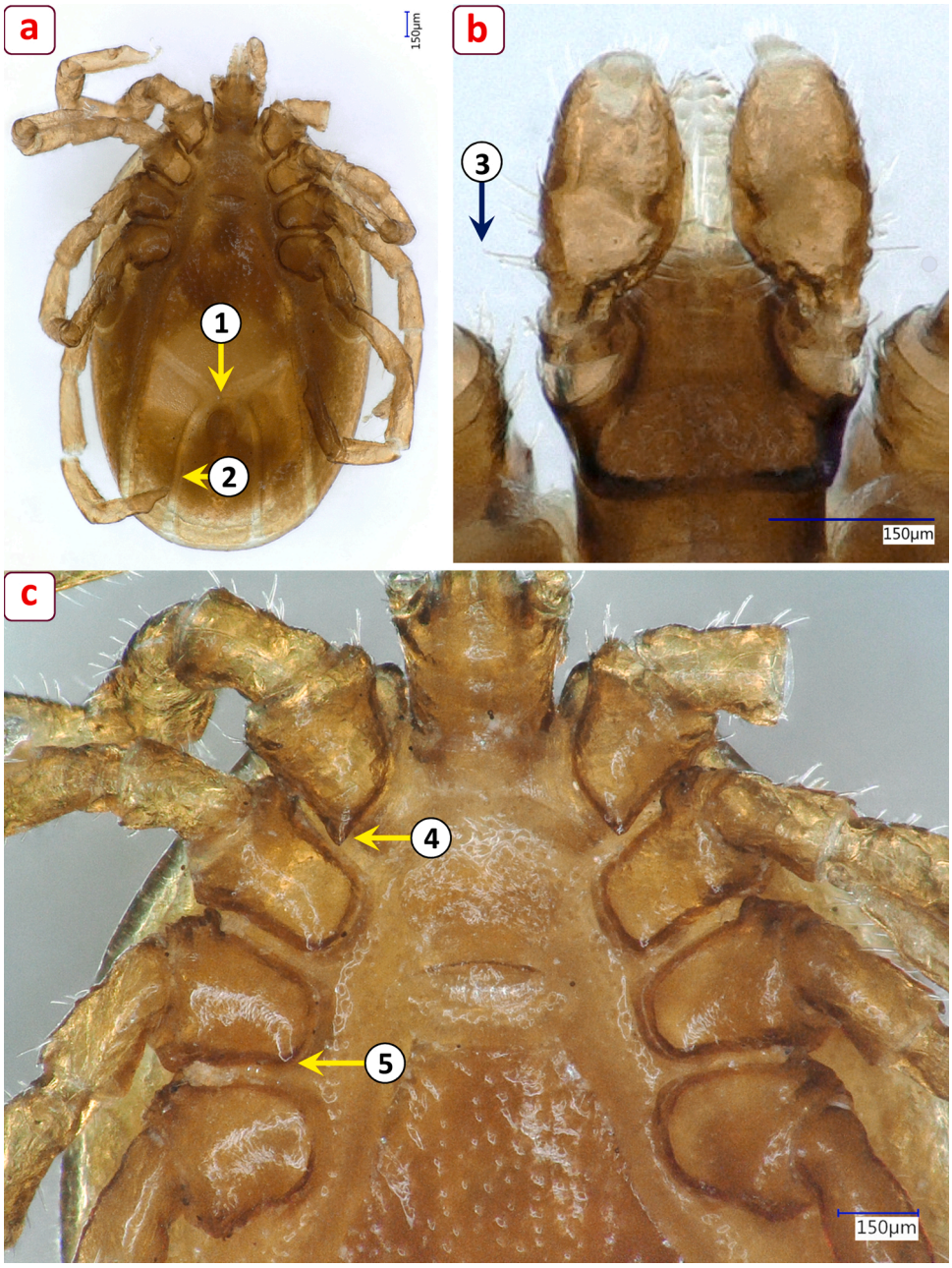

Fig. 3. Key features of *Ixodes kaiseri* males. (a) Habitus, ventral surface; (b) gnathosoma, dorsal surface; (c) coxae I-IV. Numbered arrows indicate: (1) preanal groove curved anterior to and (2) its branches nearly parallel behind the anus; (3) moderately long setae on palpal article II; (4) medium length, broad internal spur on coxa I and (5) short, blunt internal spur on coxa III. Specimens on the pictures were collected in Romania (a, c) and Germany (b).

No. 290516) and the Health Products Regulatory Authority (Project No. 7024754). Badgers were captured under the following licences: NPWS Nos. 101/2009, 04/2010, 13/2010, C123/2010, 03/2011, C040/2011, C03/2013, C005/2013 and C001/2015. In France, Hungary, Croatia and Romania ticks were collected from corpses of foxes, badgers, a hedgehog and a cat, which were either provided by official hunters or were road-killed. In Germany, sampled red foxes were provided by the Veterinary and Food Inspection Office (VLÜÄ) and hunters during a campaign against rabies and tapeworms (January 1, 2009 to December 31, 2009; TLV, Bad Langensalza).

3. Results

3.1. Identification of species and characteristics of tick infestations

Based on the morphology of molecularly analyzed specimens, altogether 116 *I. canisuga*, 277 *I. hexagonus* and 216 *I. kaiseri* males, nymphs and larvae were identified. *Ixodes kaiseri* was not found in Western Europe, where *I. canisuga* predominated (Table 1). In Central Europe, all three *Pholeoixodes* species were collected, the largest number of specimens represented by *I. hexagonus*. On the other hand, in Southeastern

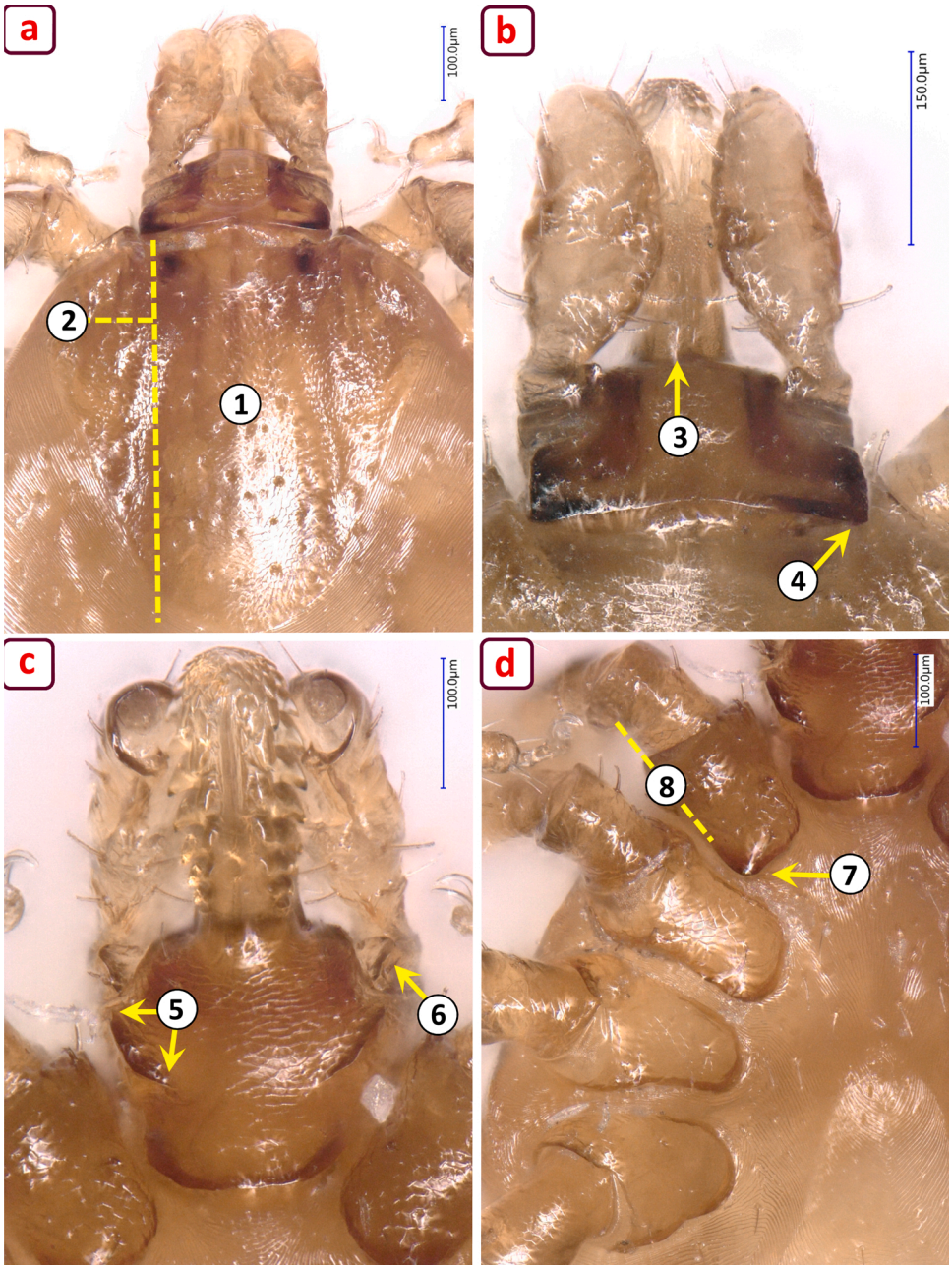

Fig. 4. Key features of *Ixodes canisuga* nymphs. (a) Gnathosoma and scutum; (b) gnathosoma, dorsal surface; (c) gnathosoma, ventral surface; (d) coxae I-IV. Numbered arrows indicate: (1) elongated scutum (viewed perpendicular to its surface) and (2) its maximum breadth, relative to its length (dashed line); (3) anteriorly flattened basis capituli; (4) absence of cornua; (5) long, moderately thick auricular ridge and (6) protuberance on palpal segment I; (7) broad internal spur on coxa I, (8) its lateral edge aligning with the lateral edge of trochanter I. Specimens on the pictures were collected in Germany (a, c, d) and France (b).

Europe *I. kaiseri* had the highest abundance (Table 1). Phylogenetic analyses of both the *cox*1 and 16S rRNA genes showed that all molecularly identified specimens clustered with conspecific ticks, confirming their identity (Supplementary Figs. 1–2).

Most male specimens were collected off-host (Table 1). Co-infestations with nymphs and/or larvae of different species were also recorded, involving four foxes in Germany (*I. canisuga* and *I. kaiseri* nymphs in two cases, *I. hexagonus* nymphs with *I. canisuga* larvae and *I. kaiseri* nymphs with *I. hexagonus* larvae), one badger in Hungary (*I. canisuga* and *I. kaiseri* nymphs) and two foxes in Romania (coinfestations with *I. canisuga* and *I. kaiseri* larvae). However, the exact number of co-infestations could not be evaluated, because intact specimens were selected from a larger number of ticks collected from the same host individuals. The maximum number of ticks on the same host individual was 73 for nymphs (*I. hexagonus*, from hedgehog, Hungary), and 62 for larvae (*I. kaiseri*, from red fox, Romania). During this study on-host copulating ticks were collected in Germany, Hungary and Croatia: in such cases always an *I. ricinus* male mated with an *I. hexagonus* female (Supplementary Fig. 3).

3.2. Key to the males

1a. Lateral setae on palpal segment II are short (*ca* 30 µm, i.e., half of the diameter of the stalk of palpal segment II); preanal groove straight anterior to the anus, its branches curved behind the anus; there is a long, narrow and pointed internal spur on coxa I (Fig. 1)…*Ixodes hexagonus*

1.b. Lateral setae on palpal segment II are moderately long (*ca* 50 µm, i.e., as the diameter of the stalk of palpal segment II); preanal groove curved anterior to the anus, its branches nearly parallel behind the anus ……2

2.a. Basis capituli anteriorly flattened, forming a plateau that dorsally surrounds the base (and is perpendicular to the axis) of hypostome; internal spur on coxa I is usually inconspicuous (short, blunt) (Fig. 2) ……*Ixodes canisuga*

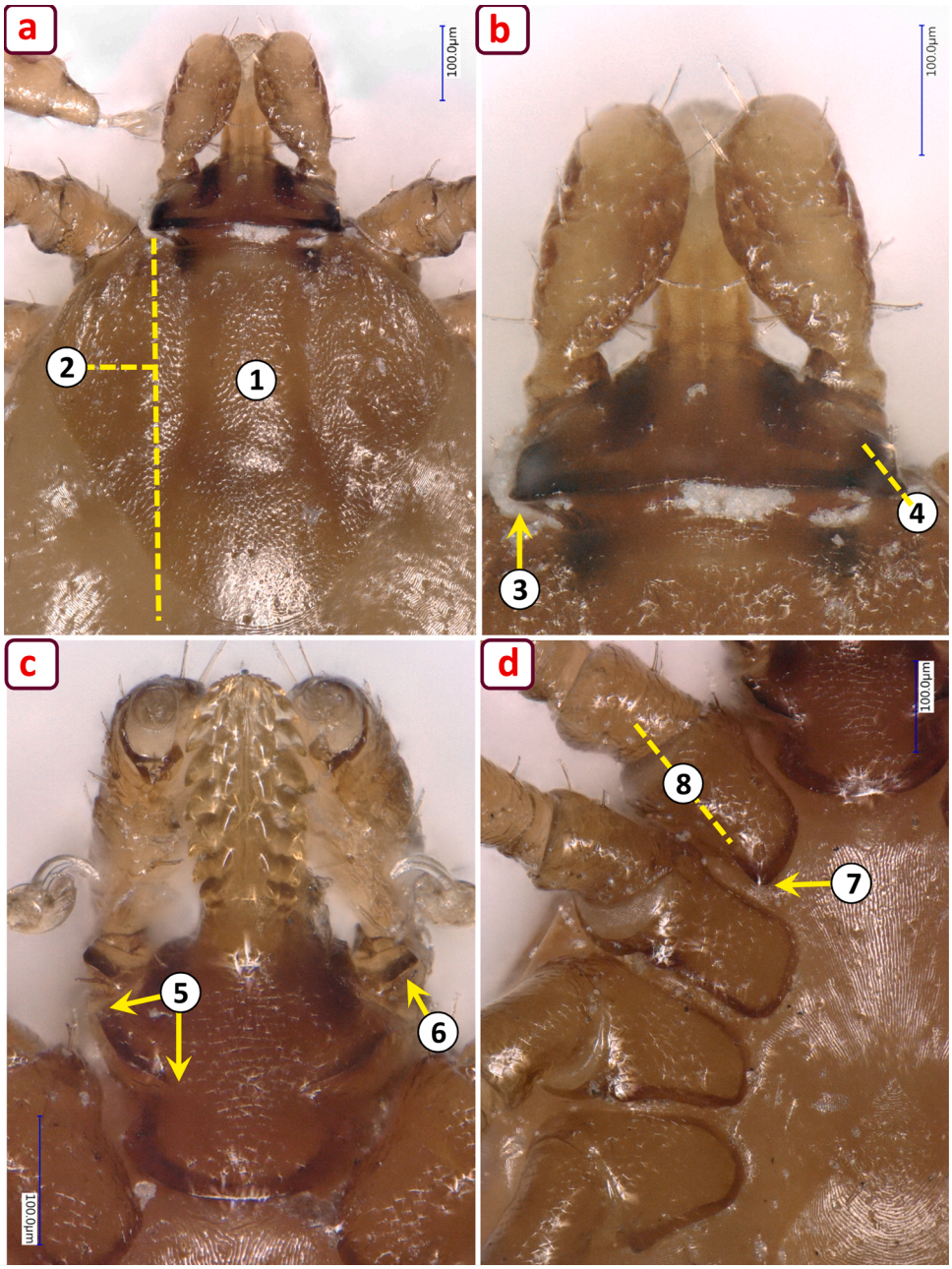

Fig. 5. Key features of *Ixodes hexagonus* nymphs. (a) Gnathosoma and scutum; (b) gnathosoma, dorsal surface; (c) gnathosoma, ventral surface; (d) coxae I-IV. Numbered arrows indicate: (1) broad scutum (viewed perpendicular to its surface) and (2) its maximum breadth, relative to its length (dashed line); (3) cornua and (4) direction of its axis (dashed line); (5) long, thick auricular ridge and (6) protuberance on palpal segment I; (7) broad internal spur on coxa I, (8) its lateral edge aligning with the axis of trochanter I. Specimens on the pictures were collected in Hungary (a, b, d) and Germany (c).

2.b. Basis capituli without plateau; there is a medium length, broad internal spur on coxa I, and short, blunt internal spur on coxae II-III (Fig. 3) .....*Ixodes kaiseri*

3.3. Key to the nymphs

1.a. Basis capituli anteriorly flattened, forming a plateau that dorsally surrounds the base (and is perpendicular to the axis) of hypostome; cornuae are absent; scutum elongated, reaching its maximum breadth at one fourth of its length; basis capituli ventrally with long, moderately thick (less sclerotized) auricular ridges and protuberance on palpal segment I; broad internal spur on coxa I, its lateral edge aligning with the lateral edge of trochanter I (Fig. 4).....*Ixodes canisuga*

1.b. Cornuae present, all coxae with external spur.....2

2.a. Cornuae posterolaterally directed; scutum broad, reaching its maximum breadth at one third of its length; basis capituli ventrally with long, thick (well sclerotized) auricular ridges and protuberance on palpal segment I; broad internal spur on coxa I, its lateral edge aligning with the axis of trochanter I (Fig. 5).....*Ixodes hexagonus*

2.b. Cornuae caudally directed; scutum elongated, reaching its maximum breadth at one fourth of its length; basis capituli ventrally with short, thin (usually inconspicuous) auricular ridges but anteriorly rounded and well sclerotized protuberance on palpal segment I; broad internal spur on coxa I (Fig. 6).....*Ixodes kaiseri*

3.4. Key to the larvae

1.a. Basis capituli semicircular in shape, anteriorly flattened, forming a plateau that dorsally surrounds the base (and is perpendicular to the axis) of hypostome; posterolateral corners of basis without cornuae, but dark and sclerotized; dorsal posterior edge of basis with central concavity; scutum slightly elongated, reaching its maximum breadth behind one fourth of its length; basis capituli ventrally with short auricular ridges, separated by a distance twice of their length; broad internal spur on coxa II, can be well-developed on coxa I (Fig. 7).....*Ixodes canisuga*

1.b. Basis capituli triangular in shape; cornuae present.....2

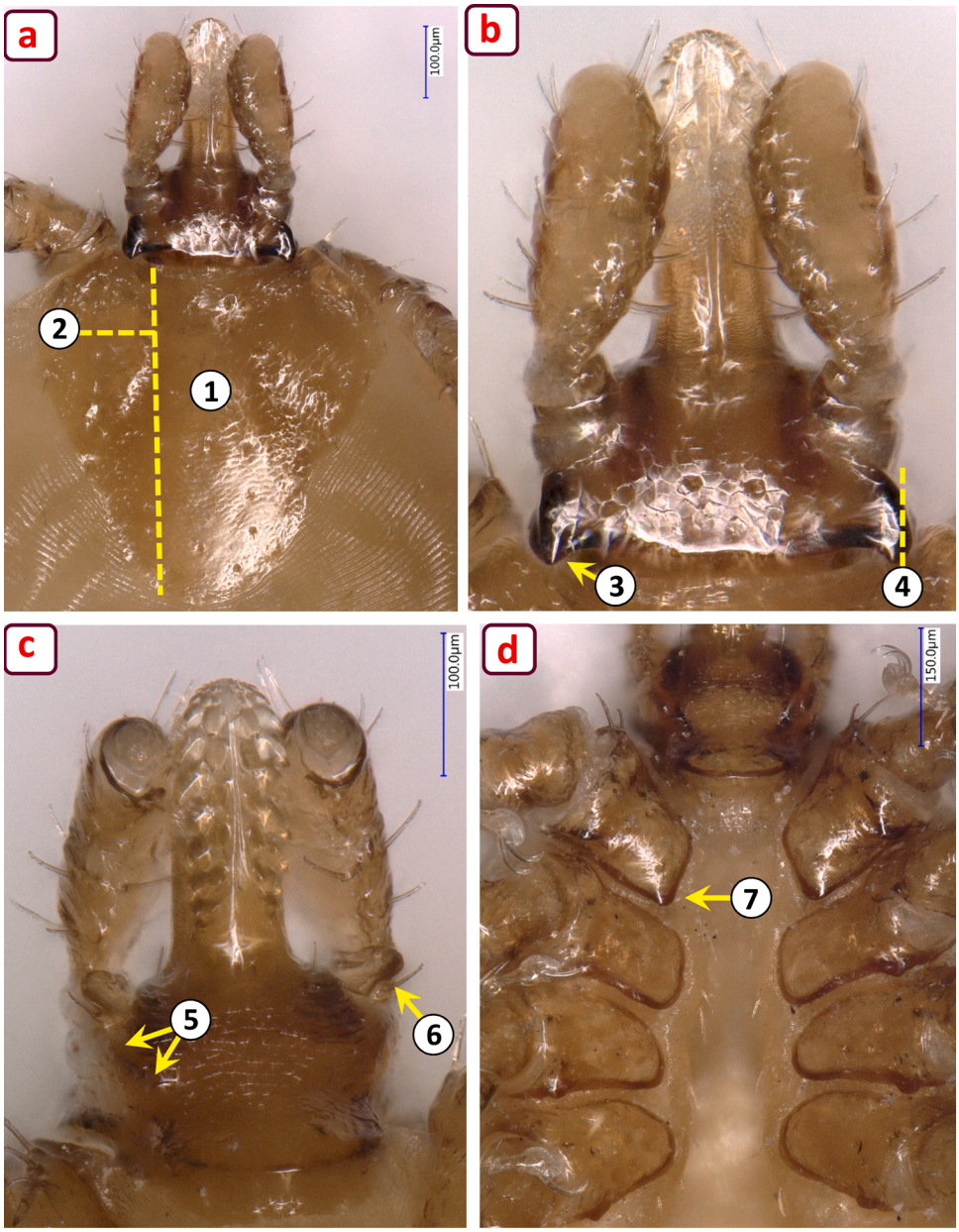

Fig. 6. Key features of *Ixodes kaiseri* nymphs. (a) Gnathosoma and scutum; (b) gnathosoma, dorsal surface; (c) gnathosoma, ventral surface; (d) coxae I-IV. Numbered arrows indicate: (1) elongated scutum (viewed perpendicular to its surface) and (2) its maximum breadth, relative to its length (dashed line); (3) cornua and (4) direction of its axis (dashed line); (5) short, thin auricular ridge and (6) anteriorly rounded and well sclerotized protuberance on palpal segment I; (7) broad internal spur on coxa I. Specimens on the pictures were collected in Hungary (a, b, c) and Romania (d).

2.a. Cornuae posterolaterally directed, their dark (sclerotized) color with short medial extension; dorsal posterior edge of basis with three concavities; scutum broad, reaching its maximum breadth at one third of its length; basis capituli ventrally with long, thick (well sclerotized) auricular ridges, separated by a distance of their length; internal spur broad on coxa II, well-developed on coxa I (Fig. 8)....*Ixodes hexagonus*

2.b. Cornuae more caudally directed, their dark color has sharp edge and long medial extension; usually an anteriodorsal ridge is also visible on the basis; scutum laterally rounded, reaching its maximum breadth behind one fourth of its length; basis capituli ventrally with short, thin (frequently inconspicuous) auricular ridges; broad, short internal spur on coxae I-II (Fig. 9).....*Ixodes kaiseri*

Note (1): *Ixodes canisuga* males, nymphs and larvae were observed both with well-developed and with inconspicuous internal spur on coxa I, when the angle of view was adjusted as perpendicular to the ventral surface of idiosoma (Fig. 7c. vs d, Supplementary Fig. 4). For instance, males of this species from France had inconspicuous internal spur on coxa I, whereas both males from Hungary had well-developed one.

Note (2): Larvae of all three *Pholeoixodes* species were also checked after mounting on slides, and (apart from chaetotaxy) the shape of the dorsal posterior edge of basis and cornuae confirmed their identity (Supplementary Fig. 5).

4. Discussion

The present study was conducted in an attempt to solve uncertainties in the morphological identification of *Pholeoixodes* species as known from previous literature data (see below), as well as to compile and to provide a more practical and "user friendly" identification key for future studies. With this in mind, it was essential to focus on easily discernible structures, most of which are observable under "regular" stereomicroscopes. This is a continuation of our previous work on *Pholeoixodes* females examined in a similar context (Hornok et al., 2017), now completing the differentiation of all stages/sexes of these widespread Eurasian tick species.

While chaetotaxy (the number, arrangement and size of setae) is regarded as important in distinguishing larvae between and within

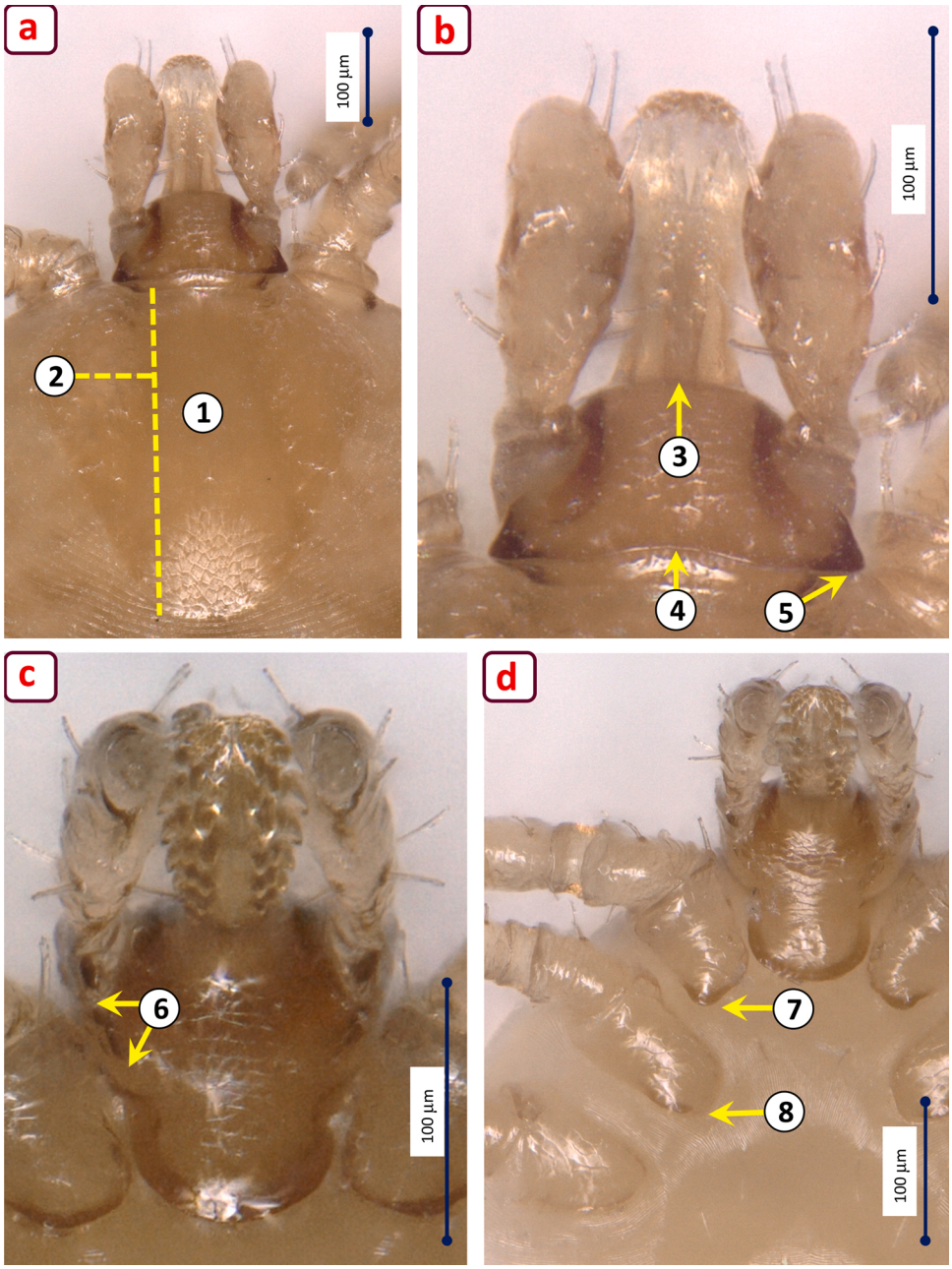

Fig. 7. Key features of *Ixodes canisuga* larvae. (a) Gnathosoma and scutum; (b) gnathosoma, dorsal surface; (c) gnathosoma, ventral surface; (d) coxae I-III. Numbered arrows indicate: (1) elongated scutum (viewed perpendicular to its surface) and (2) its maximum breadth, relative to its length (dashed line); (3) anteriorly flattened basis capituli; (4) central concavity in the dorsal posterior edge of basis; (5) absence of cornua, dark and sclerotized posterolateral corner of basis; (6) short auricular ridge; (7) well-developed broad internal spur on coxa I, (8) less protruding on coxa II. Specimens on the pictures were collected in Romania (a, b), France (c) and Germany (d).

Ixodes subgenera (Clifford and Anastos, 1960; Marquez et al., 1992), relevant data available on the larvae of Palearctic *Pholeoixodes* species contain numerous contradictions. For instance, marginal dorsal setae (critical in species differentiation) on drawings of *I. hexagonus* larvae number eight in Manilla (1998), whereas seven in Estrada-Peña et al. (2017b); these setae are longer than marginal scutal setae on larvae of *I. kaiseri* (Filippova and Uspenskaya, 1973), unlike written and shown elsewhere (Marquez et al., 1992) where this character is used to delineate larvae of *I. kaiseri* and *I. hexagonus*. The number of marginal ventral setae is three pairs on *I. crenulatus* larvae and this character may serve to distinguish these from larvae of *I. canisuga* (Marquez et al., 1992), unlike indicated by Feider (1965). Nymphs of *I. kaiseri* have equally long hair on palpal segments II and III (Arthur, 1965) or the latter are considerable shorter (Filippova, 1977). These contradictions reflect inherent difficulties of species delineation based on chaetotaxy alone. Consequently, in the same context other characters were sought for during the present study.

The morphologic characters illustrated and written in the keys provided here allow species identification, but different morphotypes (Filippova and Uspenskaya, 1973; Hornok et al., 2017) and unusual specimens might also exist (Supplementary Fig. 4). Therefore, in doubtful cases it is strongly suggested that morphologic identification of *Pholeoixodes* males, nymphs and larvae should be confirmed molecularly.

It was also demonstrated here that *I. hexagonus* females may mate with *I. ricinus* males (Supplementary Fig. 3). Thus, copulation is not enough to suppose that in mating pairs both ticks belong to the same species. In addition, this finding suggests that hybridization may occur between species within the subgenus *Pholeoixodes*, as reported within the subgenus *Ixodes* (Kovalev et al., 2015), as well as between members of subgenera *Pholeoixodes* and *Ixodes* (Patterson et al., 2017).

Results of phylogenetic analyses in this study concurred with morphologic characters, as *I. hexagonus* and *I. kaiseri* shared more key features with each other (e.g., well visible spur on coxa I, shape and

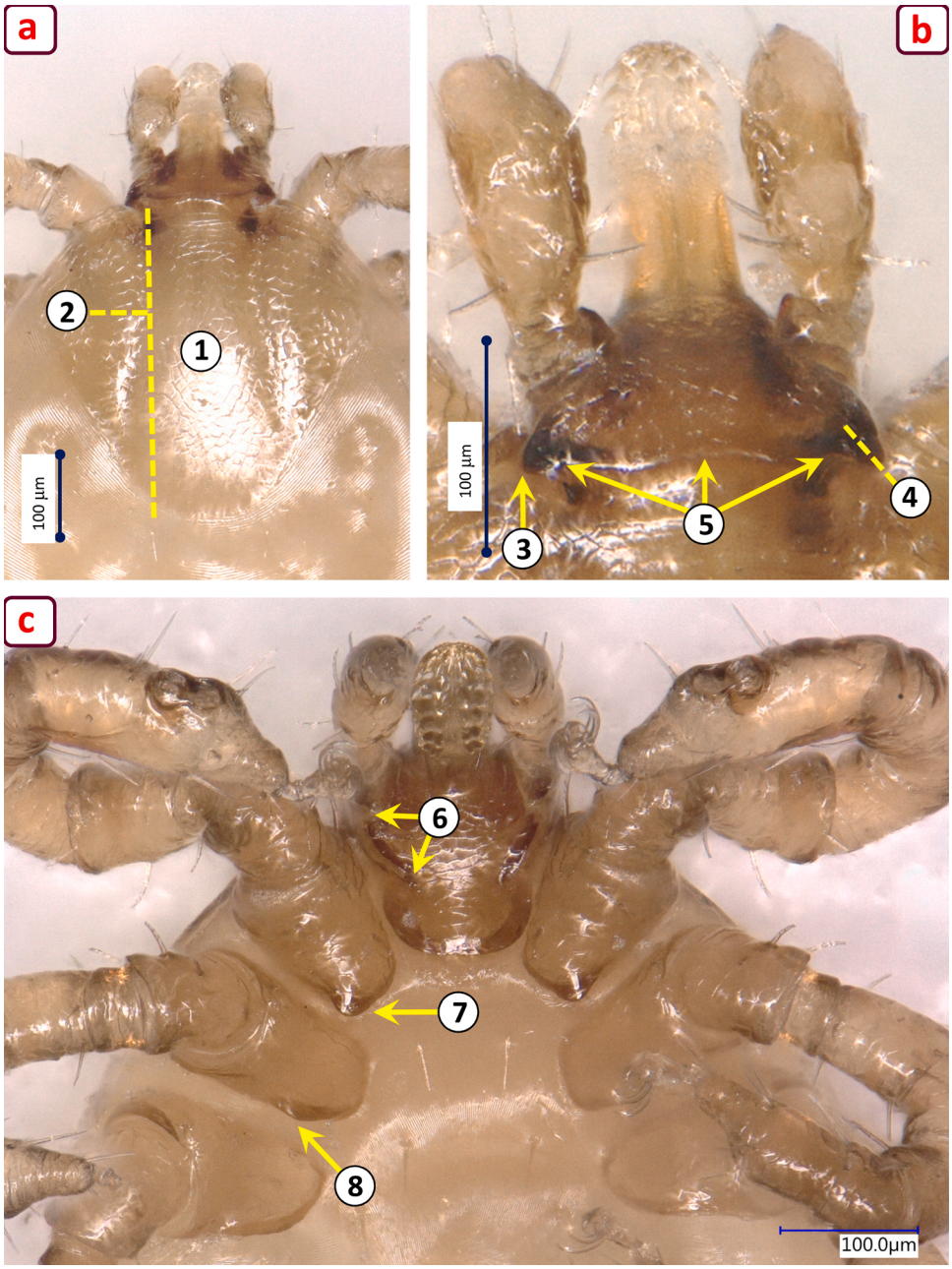

Fig. 8. Key features of *Ixodes hexagonus* larvae. (a) Gnathosoma and scutum; (b) gnathosoma, dorsal surface; (c) gnathosoma, ventral surface and coxae I-III. Numbered arrows indicate: (1) broad scutum (viewed perpendicular to its surface) and (2) its maximum breadth, relative to its length (dashed line); (3) cornua and (4) its axis (dashed line); (5) three concavities along the dorsal posterior edge of basis; (6) long, thick auricular ridge, separated from the other by a distance of its length; (7) well-developed internal spur on coxa I, (8) broad on coxa II. Specimens on the pictures were collected in Germany.

cornuae of basis capituli) than with *I. canisuga*, and this was reflected by the separation of their phylogenetic groups. It was also confirmed here that the subgenus *Pholeoixodes* is not monophyletic, unless including bat-associated ixodid ticks (subgenus *Eschatocephalus*), as reported (Hornok et al., 2017; Charrier et al., 2019). This observation highlights the relevance of ecological characters to the taxonomy of *Ixodes* subgenera (see Estrada-Peña et al., 2017a), as members of both *Pholeoixodes* and *Eschatocephalus* are adapted to the cavity- (burrow- or cave-) dwelling habit (i.e., pholeophily) of their hosts.

Data of this study reflect that *Pholeoixodes* males are very rarely found on-host, and it is more likely to collect them from burrows of carnivores. According to the present results not only larvae, but nymphs may also cause high intensity monospecific infestation of their host. This is most likely a consequence of the endophilic nature of *Pholeoixodes* species, i.e., the aggregated occurrence of immature stages in the burrows of their hosts, unlike in the case of exophilic ticks (such as *I. ricinus*) when only larvae show aggregated host questing on the vegetation (Nilsson, 1988).

It was reported that Nearctic *Pholeoixodes* larvae have "spur(s)" on the first palpal article, whereas these are absent from Palearctic species (Kleinjan and Lane, 2008). According to our observation, however, a ventral protrusion can be seen on the first palpal article of larvae in the case of all three *Pholeoixodes* species examined here. Interestingly, striking morphological similarities were found between Palearctic *I. hexagonus* larvae analyzed here, and Nearctic *I. cookei* larvae as reported (Coley, 2015). These included the triangular shape of the basis capituli, the posterolaterally directed triangular cornuae, the dorsal posterior edge of basis with three concavities and the internal spur on coxa I. Interestingly, both sequences of *I. cookei* (retrieved from GenBank) clustered as a sister group to the clade containing European isolates of *I. hexagonus* (Supplementary Figs. 1–2). Thus, these two tick species may exemplify parallel evolution and suggest that "species pairs" might exist in the categories of Palearctic and Nearctic *Pholeoixodes* species. However, confirmation of this would require further, large scale

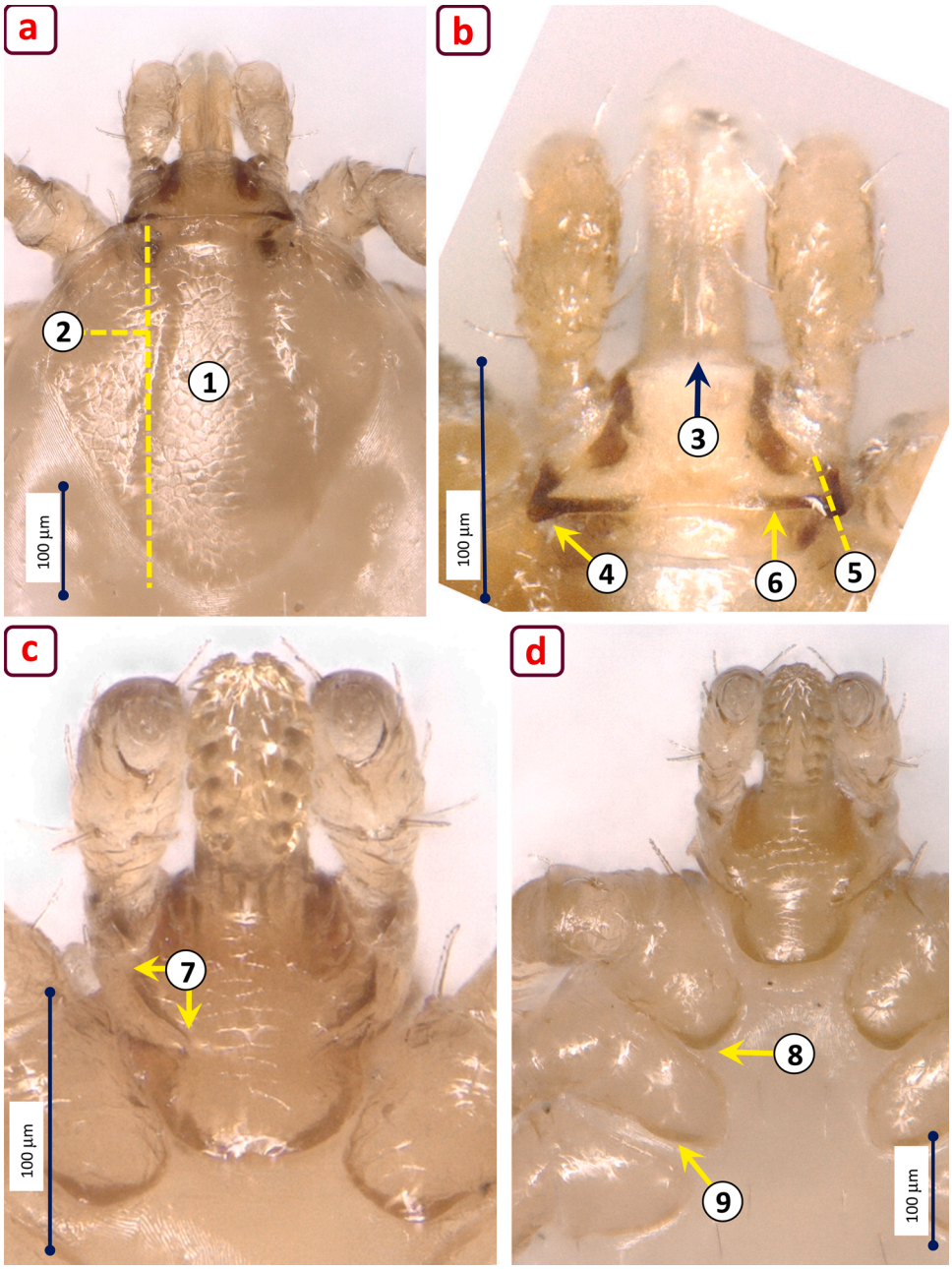

Fig. 9. Key features of *Ixodes kaiseri* larvae. (a) Gnathosoma and scutum; (b) gnathosoma, dorsal surface; (c) gnathosoma, ventral surface; (d) coxae I-III. Numbered arrows indicate: (1) elongated scutum (viewed perpendicular to its surface) and (2) its maximum breadth, relative to its length (dashed line); (3) anteriodorsal ridge of basis; (4) cornua and (5) direction of its axis (dashed line); (6) dark color of cornua with sharp edge and long medial extension; (7) short, thin auricular ridge; broad, short internal spur (8) on coxa I and (9) II. Specimens on the pictures were collected in Germany (a, c) and Romania (b, d).

(multi-species) morphologic and phylogenetic comparison of relevant specimens from both geographic regions.

Author statement

Sándor Hornok: conceptualization, methodology, writing, editing. **Jenő Kontschán**, **Nóra Takács**: methodology, visualization, investigation. **Elisabeth Meyer-Kayser, Olivier Plantard, Siobhán Cullen, Aoibheann Gaughran, Relja Beck, Sándor A. Boldogh, Gábor Horváth, Csaba Kutasi**: data curation, investigation. **Sándor Szekeres, Gábor Majoros**: methodology, software. **Attila D. Sándor**: conceptualization, supervision, editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2021.101715.

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