

Histomorphology of the Male Reproductive System and Spermatogenesis of *Phyllobius (Ectomogaster) fulvago* Gyllenhal, 1834 (Coleoptera, Curculionidae): A Light and Scanning Electron Microscope Study

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Abstract—The morphology and histology of the male reproductive system of *Phyllobius fulvago* were described and discussed in relation to other Coleoptera species. The results show that *Ph. fulvago* has two-lobed flower-shaped arranged testes, each lobe with fourteen follicles. There are two types of glands in the male reproductive system in *Ph. fulvago*: tubular accessory glands and lobed prostate glands.

Keywords: testis, differentiation zones, light microscope, SEM

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Curculionidae is known as one of the most speciose insect families with approximately 60000 species in the World and 13000 in the Palaearctic Region (Varlı, 1998). Many weevils of the genus *Phyllobius* Germar live on cultivated shrubs and trees, most often of the families Fagaceae, Betulaceae, Salicaceae, and Rosaceae (Pesarini, 1980). Larvae of many *Phyllobius* feed on the roots, while the adults feed mostly on the shoots and leaves (Dieckmann, 1980). Therefore, they can cause significant economic losses to agricultural crops and forest trees (Pesarini, 1980). *Phyllobius (Ectomogaster) fulvago* Gyllenhal, 1834 is widespread on oaks (*Quercus* spp.) across the Middle East (Avcın and Colonnelli, 2011). As this species causes economic damage feeding in numbers on many plants important for agriculture and abundant in natural landscapes, research focusing on the male reproductive system of *Ph. fulvago* may contribute to agricultural pest control studies.

The internal morphology of beetles is commonly used for taxonomic characterization of insect species in the same genus or different genera in the same family (Russo, 1926; Williams, 1945). The histological and

morphological knowledge of the male reproductive system of Coleoptera is poor (Cerezke, 1964; Barker, 1989; Salazar et al., 2016; Özyurt Koçakoğlu et al., 2019) although several investigations of the spermatogenesis and reproductive system have been previously reported (Goldson and Emberson, 1981; Yahiro, 1996; Diefenbach et al., 1998; Sasakawa, 2007; Opitz, 2014; Senarat et al., 2019). The male reproductive system in the Coleoptera including Curculionidae consists of a pair of testes, *vasa deferentia*, seminal vesicles, accessory glands, prostate glands, and an ejaculatory duct (Aslam, 1961; Barker, 1989; Hoffman and Raffa, 1992; Özyurt Koçakoğlu et al., 2019). Each testicular follicle contains germ cells at different stages of development. The germarium, which is the upper part of testis, contains spermatogonia and spermatocytes. In the next zones, spermatocytes develop into spermatids and finally into mature spermatozoa (Barker, 1989; Özyurt Koçakoğlu et al., 2019). In the center of each testis, there is a seminal vesicle. Extending from the ends of the seminal vesicles, there are paired *vasa deferentia*. The paired accessory glands arising from the *vasa deferentia* are long blind-ended tubules. In the posterior

part of the accessory glands there is a prostate gland, which opens into the *vas deferens* (Barker, 1989). The prostate glands have 7 or 8 lobes (Goldson and Emberson, 1981). During mating, stored sperm in the seminal vesicles is mixed with secretions of the glands before transfer to the female in the form of a spermatophore (Chapman, 1971; Barker, 1989). The *vas deferens* unites with the ejaculatory duct which enters the aedeagus (Barker, 1989).

Here, we describe the morphology and histology of the male reproductive system of adult *Ph. fulvago* for the first time. The purpose of this contribution is to offer new information about the reproductive system of Curculionidae.

MATERIALS AND METHODS

Stereomicroscopy (SM). Twenty adult males of *Ph. fulvago* were collected with a sweeping net from meadows in different localities of Yozgat Province of Turkey in May 2018. The samples were placed in a plastic container with good air ventilation along with food sources and were brought to the laboratory. Next, ten insects were anesthetized with ethyl acetate fumes. The dissection was done in phosphate buffer by cutting open the dorsal part of the abdomen from posterior to the anterior in order to expose the male reproductive organs which were photographed using a stereomicroscope Olympus SZX7.

Light microscopy (LM). For histological preparations, ten samples were fixed in formalin fixative for 24 hours. Next, the tissues were underwent tissue processing for eight hours. First, the 10% neutral formalin was removed by washing in tap water for a day. Next, the samples were dehydrated in subsequently graded from 50% to 100% ethanol for an hour in each alcohol series. The tissues were left in the mixture of xylene and paraffin for fifteen minutes. Then the tissues were twice infiltrated with paraffin wax. Last, tissues were embedded in histological paraffin, and 5–6 μm thick sections were taken from the tissues using microtome (Micom HM 310). Finally, the serial sections were stained with Harris's hematoxylin and eosin (H & E) staining. The stained slides were observed and photographed under a light microscope with a digital camera Olympus BX51.

Scanning electron microscopy (SEM). For scanning electron microscopy (SEM), ten adult samples were fixed in 2.5% glutaraldehyde (pH 7.2, phosphate buffered), rinsed with phosphate buffer three times, and the tissues were dehydrated in ethanol series from 50% to 100% at room temperature. The tissues were dried with hexamethyldisilazane, mounted on aluminum stubs, and finally coated with gold in a Polaron SC 502 sputter coater. The samples were examined with a JEOL JSM 6060 LV SEM at 10 kV, and digital photos were taken.

RESULTS

The male reproductive system of adult *Phyllobius fulvago* comprises a pair of testes, a pair of seminal vesicles, a pair of *vasa deferentia*, a pair of prostate glands, a pair of accessory glands, an ejaculatory duct, and an aedeagus (Fig. 1, *a, b*). The testes are located dorso-laterally in the abdominal cavity. Each testis has two separate flower-shaped lobes (Fig. 1, Fig. 2). The testis is enclosed by epithelial sheath with a flat surface, and the testis surface is covered with trachea and tracheole networks (Fig. 2, *a*). Histological examinations have shown that each testis lobe is composed of 14 testicular follicles (Fig. 2, *b*). In each of these follicles, different stages of sperm development (spermatogenesis) are seen. Sperm development is observed from the periphery to the center of each testis lobe (Fig. 2, *b*; Fig. 3, *a*). There is a seminal vesicle in the center of each testicular lobe (Fig. 3, *b*). At larger magnifications of LM, different stages of the germ cells which are spermatogonia, spermatocytes, spermatids, and spermatozoa are observed in each follicle (see Fig. 3, *a*). In the growth zone, towards the periphery of each follicle, spermatogonia and spermatocyte cysts, which are known as spherical sac, are found. At this stage (spermatogonia and spermatocyte cysts) part of the sperm's tail is located at the back of the head and is quite short (Fig. 4, *a, b*).

In the maturation zone, spermatocytes undergo two meiotic divisions and turn into spermatids (Fig. 5, *a, b*). At this stage, the sperm head is round and its tail is quite long and filiform (Fig. 6, *a, b*). Spermatids are seen in bundles together. In the differentiation zone, the spermatids turn into spermatozoa, and the sperm heads turn from oval to spindle-shaped structures (Fig. 7, *a, b*). In the spermatozoon head, the middle part of the head is

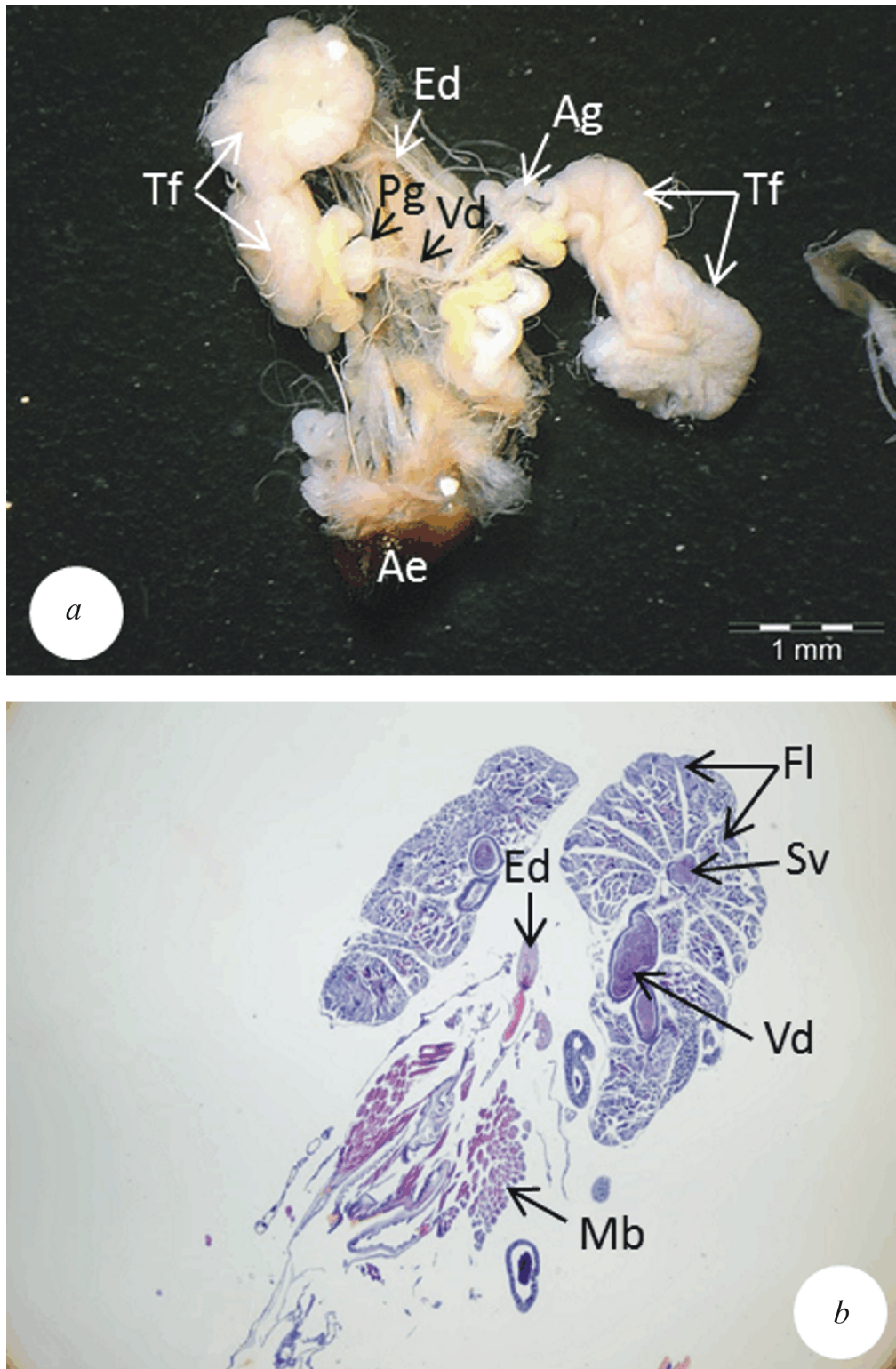


Fig. 1. *Phyllobius fulvago* Gyll., general morphology (*a*; SM) and histological structure (*b*; LM) of male reproductive system and testes. (Ae) aedeagus, (Ag) accessory gland, (Ed) ejaculatory duct, (Fl) testis follicle, (Mb) muscle bundles, (Pg) prostate gland, (Sv) seminal vesicle, (Tf) testis lobe, (Vd) *vas deferens*, (LM) light microscope, (SM) stereomicroscope.

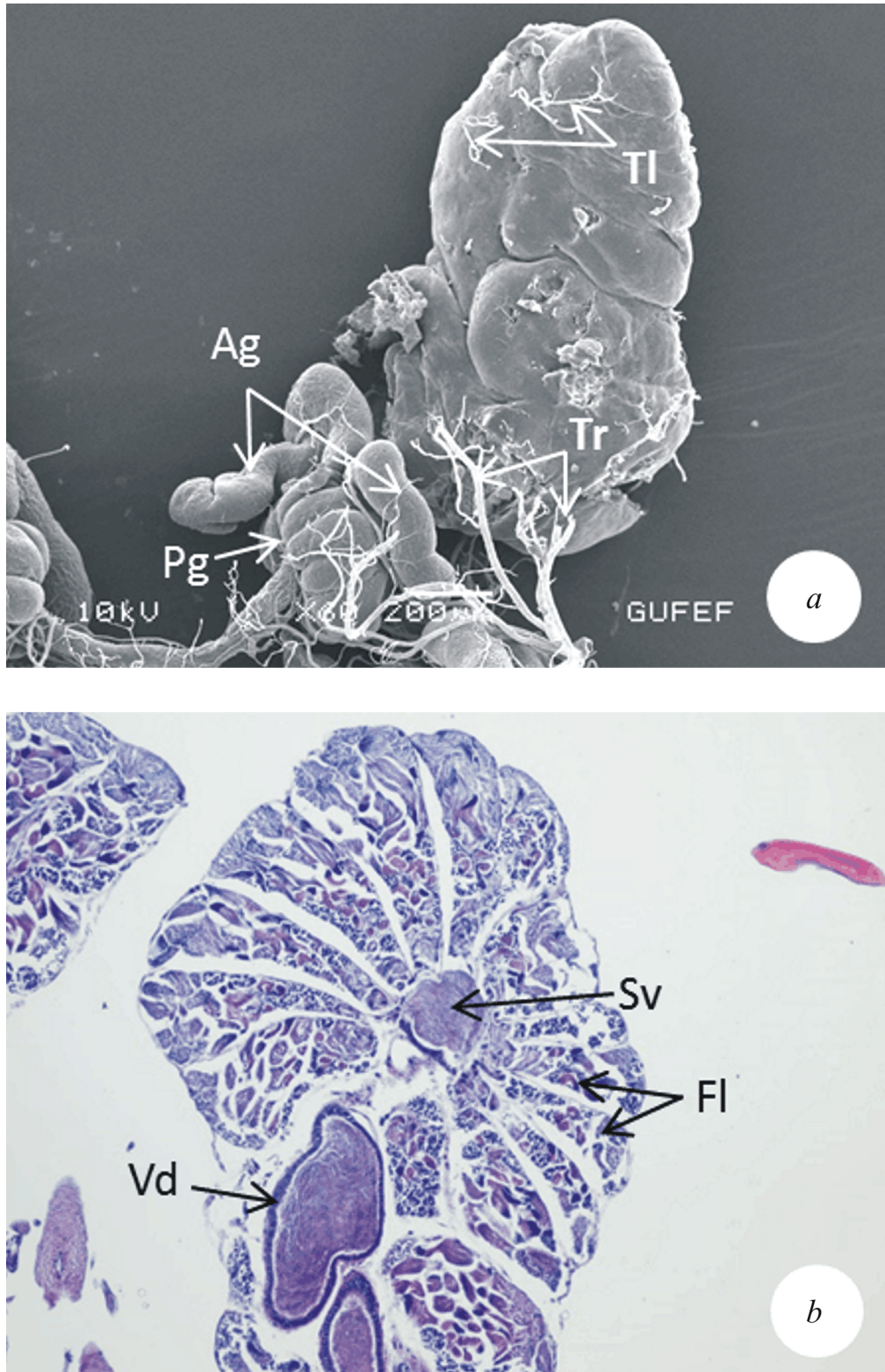


Fig. 2. *Phyllobius fulvago* Gyll.: (a) trachea (Tr) and tracheoles (TI) on the surface of the testis sheath, SEM; (b) cross section of a testis lobe and seminal vesicle (LM; $\times 100$). (SEM) scanning electron microscopy. For other designations see Fig. 1.

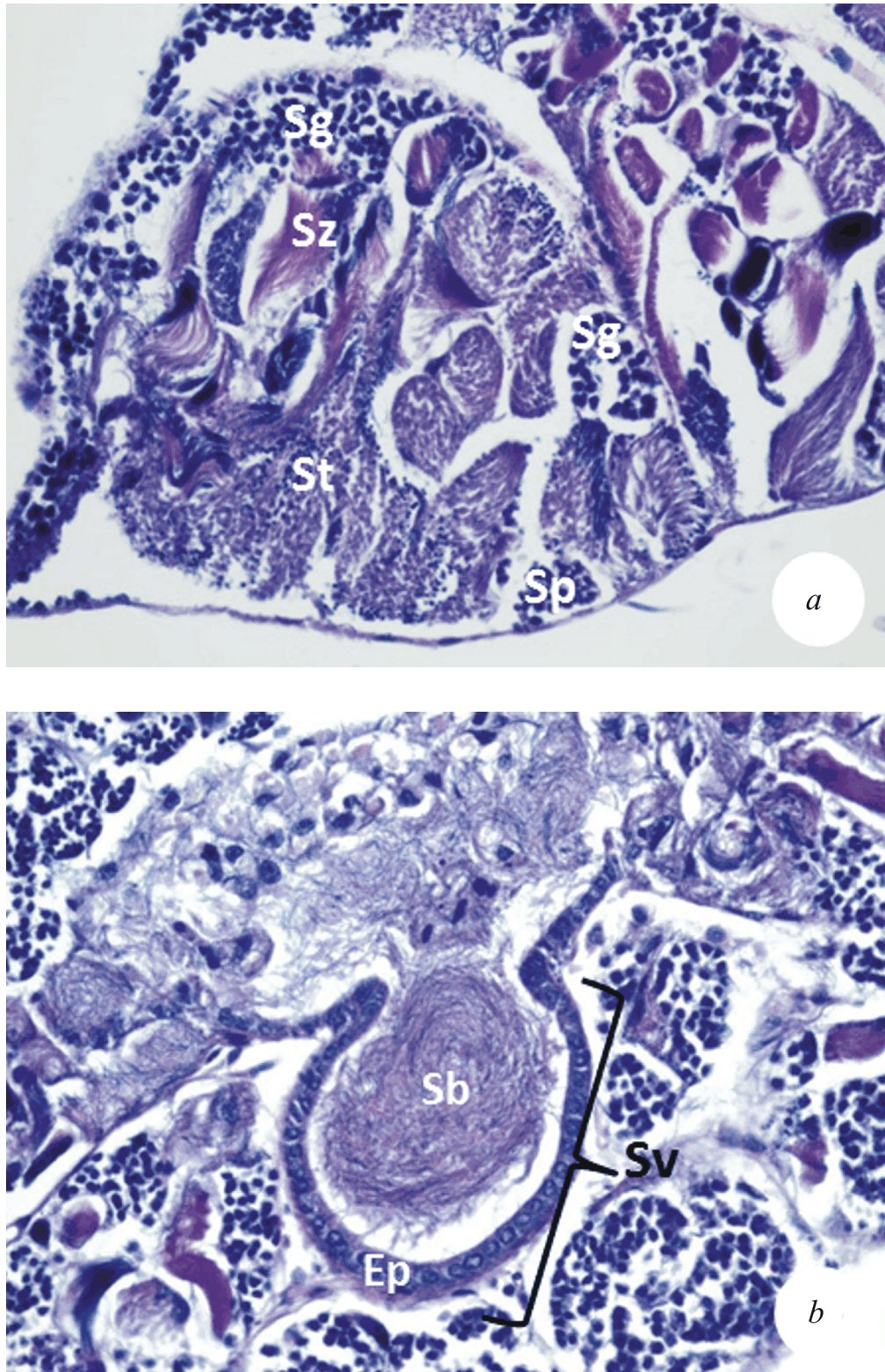


Fig. 3. *Phyllobius fulvago* Gyll., LM: (a) differentiation zones in a testis follicle ($\times 200$), (b) connection of the seminal vesicle to the testis ($\times 400$). (Ep) epithelial cell, (Sb) sperm bundles, (Sg) spermatogonia, (Sp) spermatocytes, (St) spermatids, (Sz) spermatozoa. For other designations see Fig. 1.

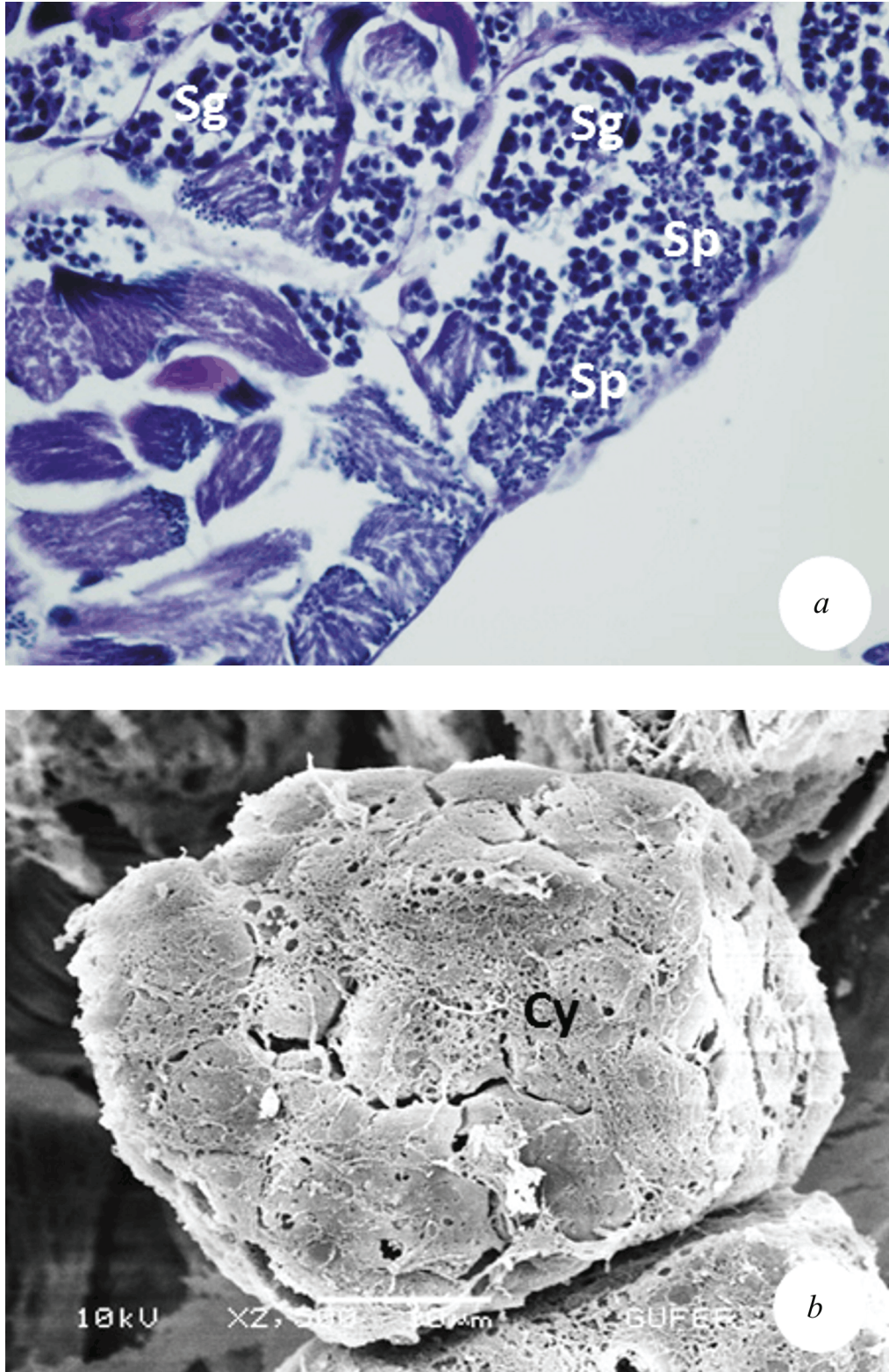


Fig. 4. *Phyllobius fulvago* Gyll.: (a) spermatogonia and spermatocytes located at the periphery of the testicular lobe (LM; $\times 400$); (b) the appearance of spermatocytes in cyst, SEM. (Cy) cyst. For other designations see Figs. 1–3.

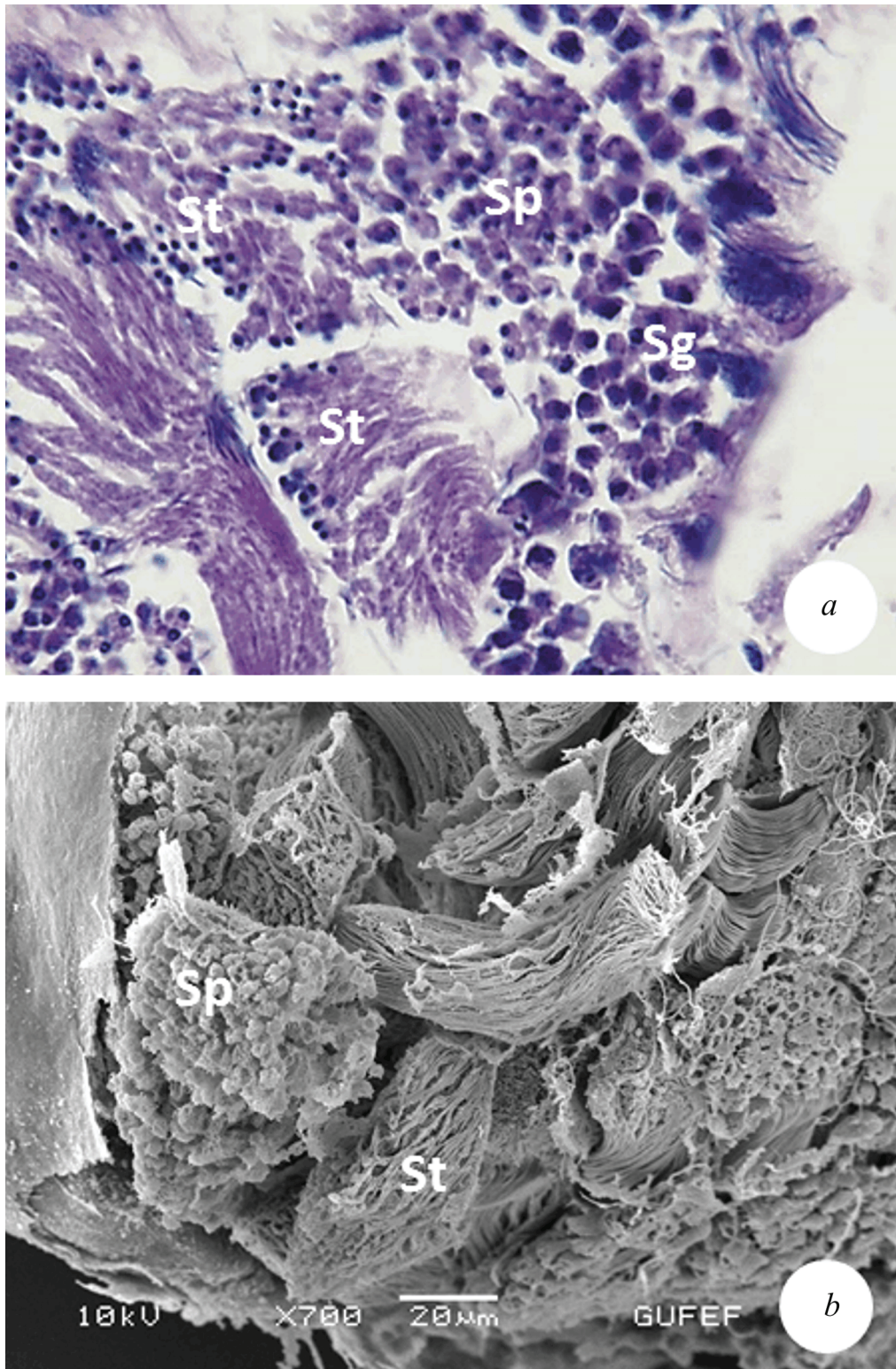


Fig. 5. *Phyllobius fulvago* Gyll., conversion of spermatocytes into spermatids in testicular follicle: (a) LM ($\times 1000$), (b) SEM. For designations see Figs. 1–3.

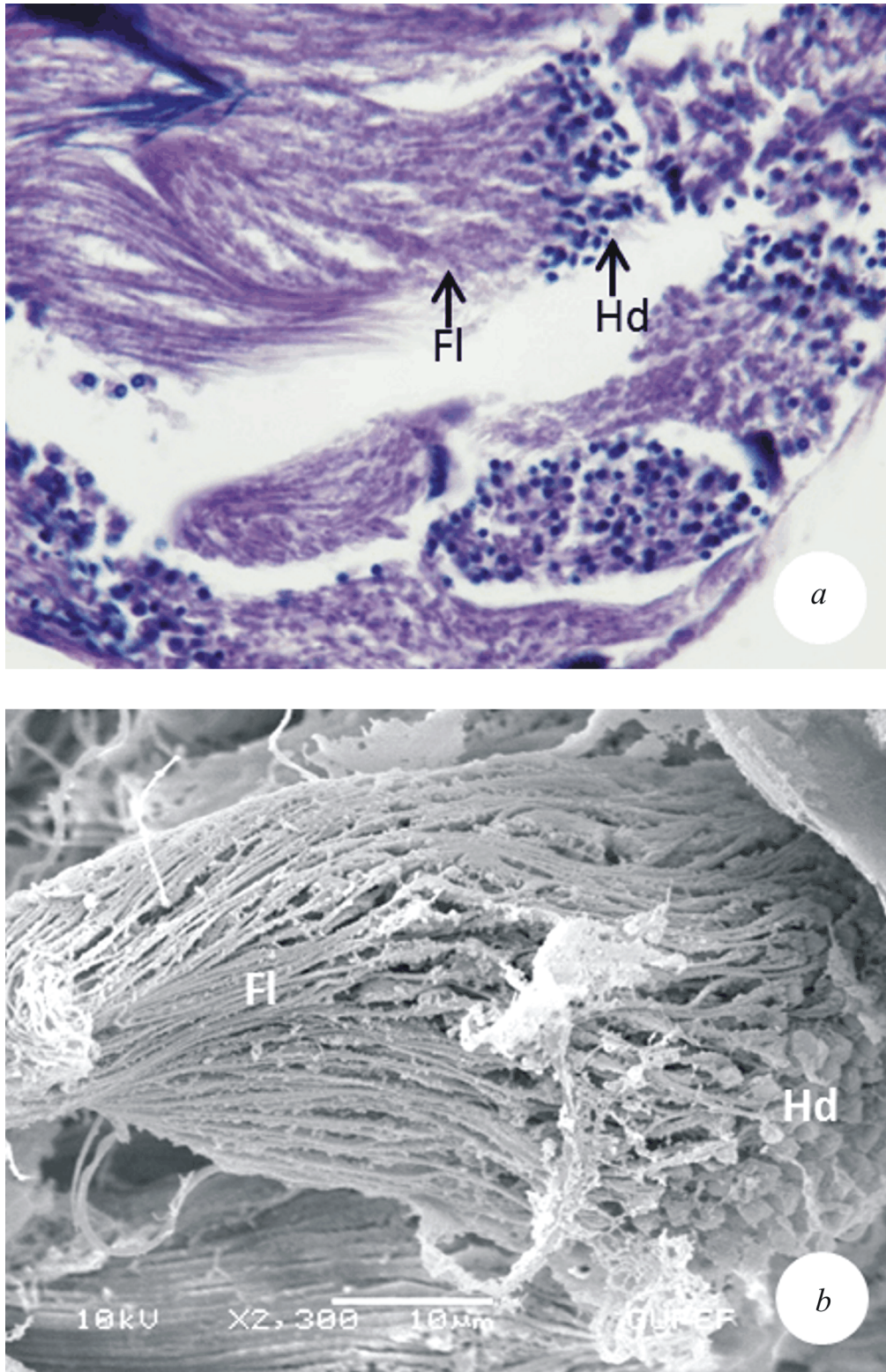


Fig. 6. *Phyllobius fulvago* Gyll., detailed view of the head and flagellum parts in spermatids (H & E): (a) LM ($\times 1000$), (b) SEM. (Fl) flagellum, (Hd) head. For other designations see Figs. 1–5.

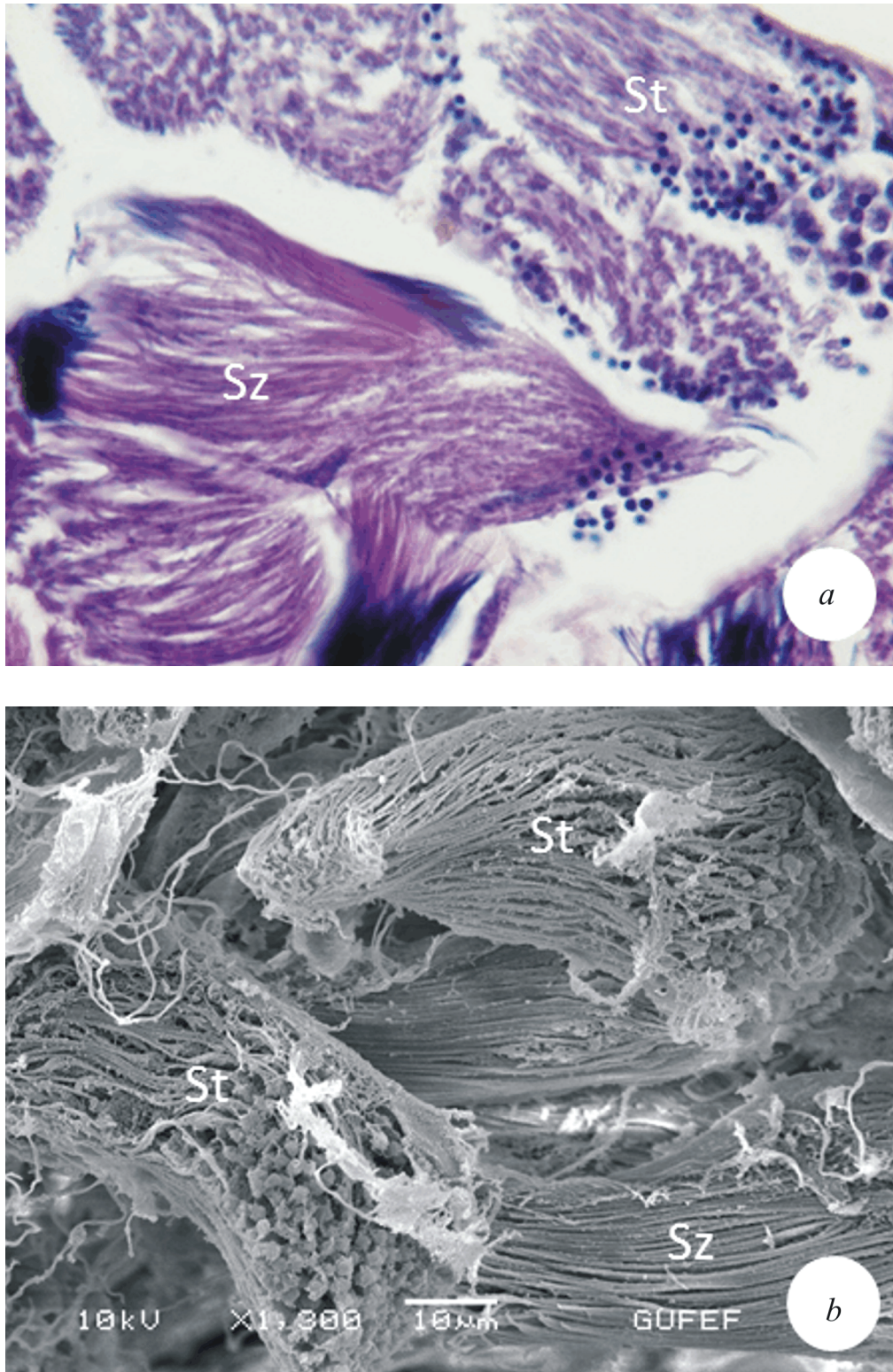


Fig. 7. *Phyllobius fulvago* Gyll., differentiation of spermatids into spermatozoa in testicular follicle: (a) LM ($\times 1000$), (b) SEM. For designations see Figs. 1–6.

wide, and the anterior and posterior parts are narrow (Fig. 9, *b*). The tail parts are long, strand-shaped, and form regular bundles (Fig. 8, *a, b*; Fig. 9, *a*). In the middle of each testicular lobe, there is a seminal vesicle (Fig. 10, *a, b*). The wall of the seminal vesicle is surrounded with a monolayer epithelium, and there are sperm bundles in the lumen (Fig. 10, *a*). The sperm stored in the seminal vesicles is mixed with accessory and prostate glands secretions before transfer to the female insect. The sperm bundle travels from the seminal vesicle to the *vas deferens*. The *vas deferens* wall is surrounded with monolayer cubic epithelium and muscle layer from the inside (Fig. 11, *a, b*). Toward the middle of the *vas deferens*, there are pairs of long, curved, blind-ended accessory glands. Also, trachea and tracheole networks are found on the surface (Fig. 12, *b*). The accessory glands are surrounded with monolayer cylindrical epithelium with large spherical nuclei, and secretory material is found in the lumen (Fig. 12, *a*; Fig. 13, *a, b*). Below the accessory glands, there are multi-lobed prostate glands (Fig. 14, *a, b*). The epithelium of the prostate glands consists of cuboidal cells with round nuclei (Fig. 14, *a*). A lower concentration of secretory material is found in the lumen as compared to the accessory glands (see Fig. 12, *a*; Fig. 14, *a*). The prostate glands are connected to the ejaculator duct by a thin *vas deferens* duct (see Fig. 14, *a, b*). The thickness of the muscle layer in the ejaculator duct wall is considerably larger than that of other ducts (Fig. 15, *a, b*). The nuclei and streaks in the muscle layer are clearly distinguished (see Fig. 15, *a*). The ejaculator duct is connected to the aedeagus via dense muscle bundles just below the duct (Fig. 16, *a, b*). The muscles are involved in the retraction and protraction of the aedeagus which is a sclerotized tube.

DISCUSSION

The results of this study show that the general morphology of the male reproductive system of *Phyllobius fulvago* is typical of that in the beetles of the suborder Polyphaga (Aslam, 1961; Barker, 1989; Hoffman and Raffa, 1992; Werner et al., 2002; Wu et al., 2017; Özyurt Koçakoğlu et al., 2019). The general appearance of *Phyllobius fulvago* testes is similar to that of other curculionids (Aslam, 1961; Barker, 1989; Hoffman and Raffa, 1992; Özyurt Koçakoğlu et al., 2019). However, the morphology of the testis varies between species. The testes in *Ph. fulvago* are flower-shaped, while *Dendro-*

ctonus monticolae (Curculionidae: Scolytinae) has the testes bean-shaped (Cerezke, 1964). *Rhysodes comes* (Lewis, 1888) of the Adephaga (Rhysodidae) has ovoid testes (Yahiro, 1996). *Spasalus silvarum* (Polyphaga: Passalidae) has fusiform testes (Salazar et al., 2016). The testes of *Tentyria cypria* (Polyphaga: Tenebrionidae) look like four-leaf clover (İzzetoğlu and Gülmez, 2018).

Each testis consists of a number of follicles which contain the male germ cells; the number of follicles in each testis and the number of male accessory glands in the examined species of Coleoptera are given in Table 1.

The follicles of the sexually mature males are full of sperm cysts, within which spermatogenesis occurs (Phillips, 1970; Wu et al., 2017). Sperm development in *Ph. fulvago* testis proceeds as described by Cerezke (1964), Salazar et al. (2016), Senarat et al. (2019) and Özyurt et al. (2019) for other beetles.

Each testicular lobe of *Ph. fulvago* is connected to the *vas deferens* via the seminal vesicle. The same structures were found in other Curculionidae: *Listronotus bonariensis*, *Hypothenemus hampei* and *Tanymecus dilaticollis* (Goldson and Emberson, 1981; Barker, 1989; Rubio et al., 2008; Özyurt Koçakoğlu et al., 2019)

The morphology of the male accessory glands is known to vary between species (Barker, 1989). *Phyllobius fulvago* has a pair of long, tubular accessory glands and a pair of multilobed prostate glands. In *Dendroctonus monticolae*, some of the accessory glands are tubular, and others have four to six lobes (Cerezke, 1964). *Listronotus bonariensis* has large accessory glands and prostate glands with seven or eight lobes (Barker, 1989). The accessory glands in *Phyllobaenus pallipennis* are trilobed (Opitz, 2014). *Isohydnocera aegra* and *Wolcottia sobrina* have accessory glands swollen at base and particularly long (Opitz, 2014). *Axina bifasciata* has biramous accessory glands of which the medial pair is much shorter than the lateral one (Opitz, 2014). In *Cardiostychus gabonicus*, the medial gland is biramous and lateral gland is uniramous (Opitz, 2014). *Clerus mutilarius* has medial glands paired and not branched, but its lateral glands are paired biramously (Opitz, 2014). In *Epiclines basalis*, the medial gland is not branched and highly diverticulated at base, and the lateral gland is biramous (Opitz, 2014). *Neoscribiger patricius* has spheroid lobe at the base of the medial gland, and its

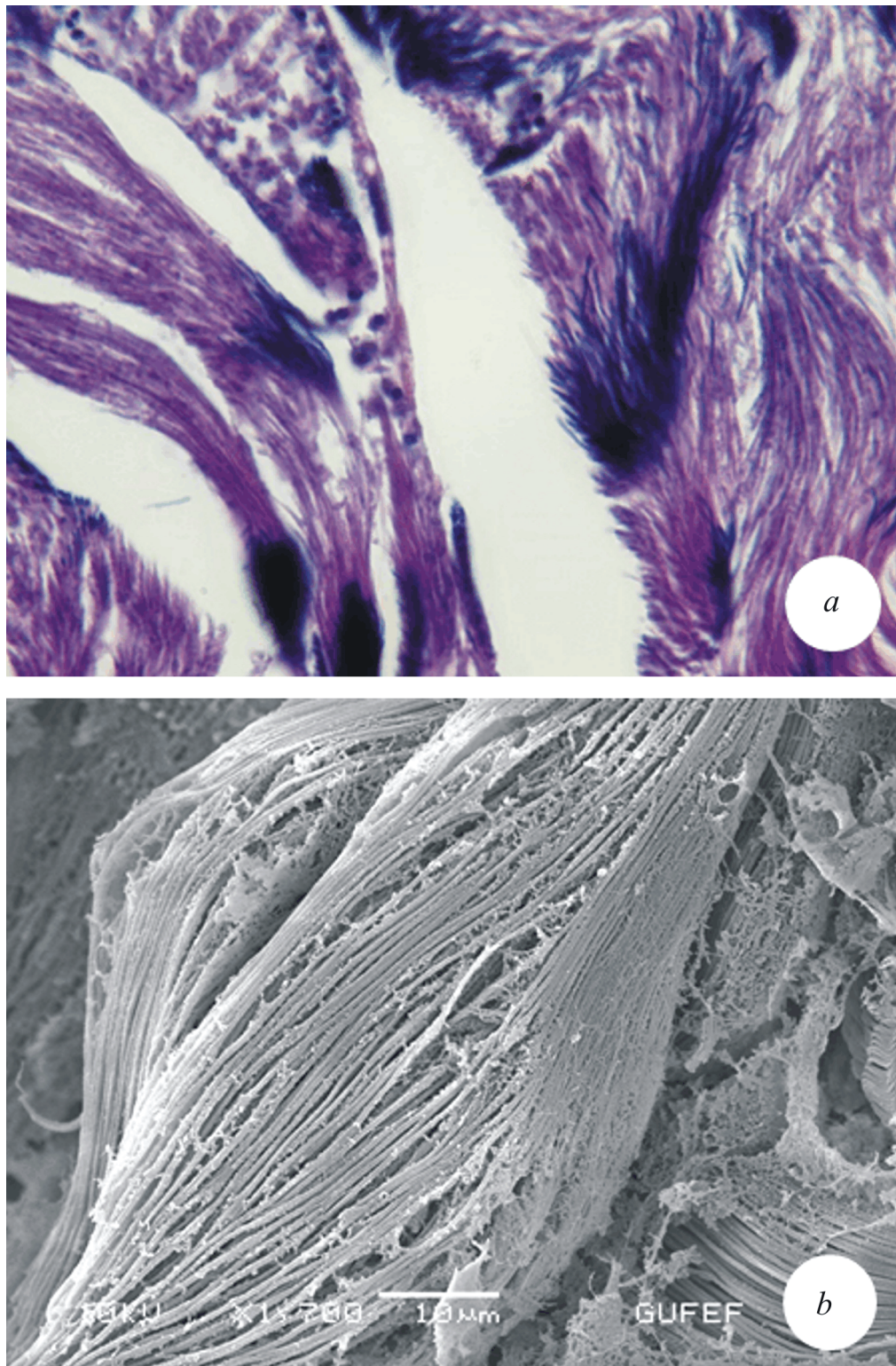


Fig. 8. *Phyllobius fulvago* Gyll., detailed view of spermatozoon bundles: (a) LM ($\times 1000$), (b) SEM. For designations see Figs. 1–7.

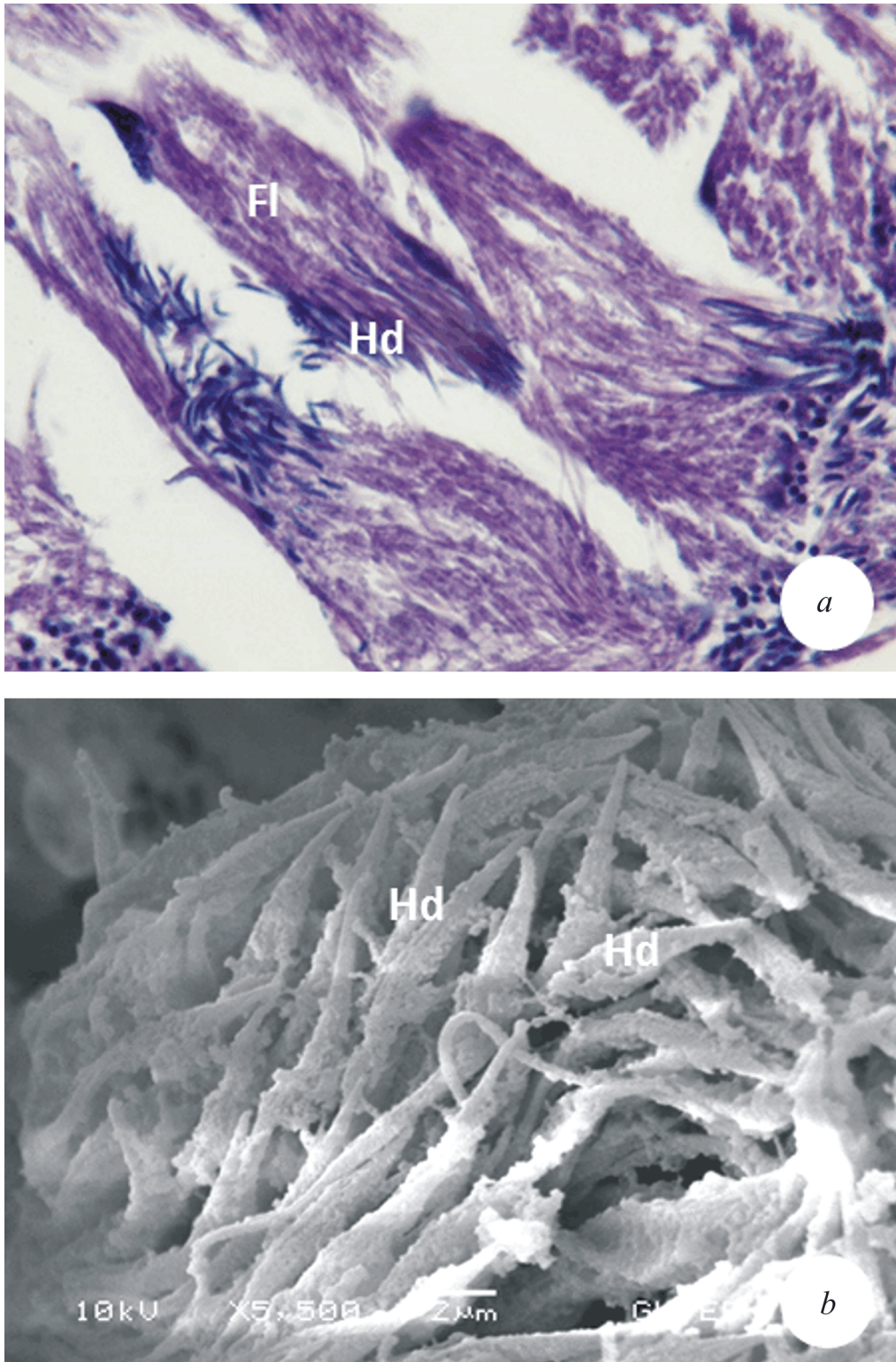


Fig. 9. *Phyllobius fulvago* Gyll.: (a) appearance of the spermatozoon head and flagellum parts, LM ($\times 1000$); (b) spermatozoon head parts (SEM). For designations see Figs. 1–8.

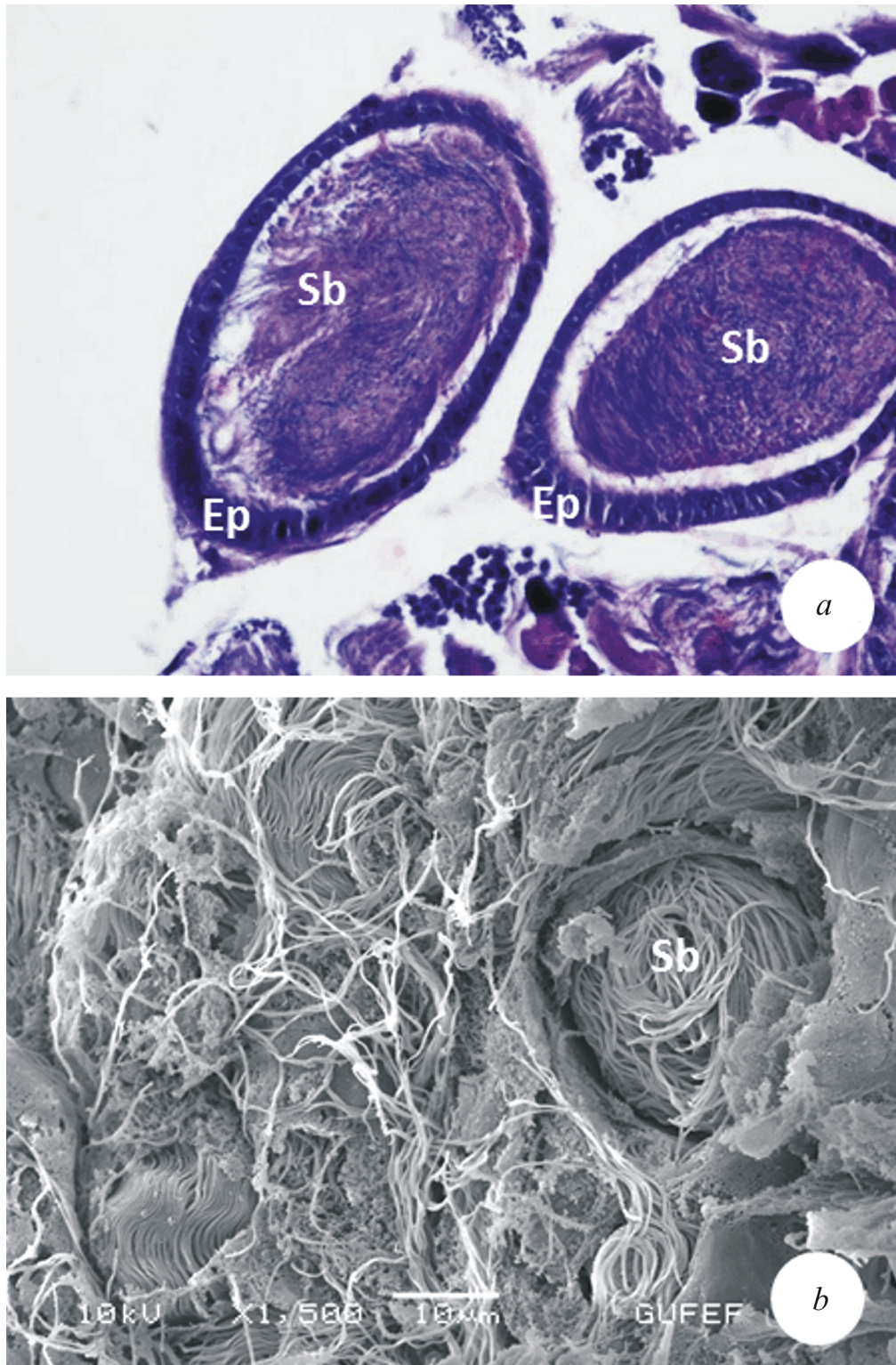


Fig. 10. *Phyllobius fulvago* Gyll.: (a) histological structure of the seminal vesicle, LM ($\times 1000$); (b) mature sperm bundles in the lumen of the seminal vesicle (SEM). For designations see Figs. 1–3.

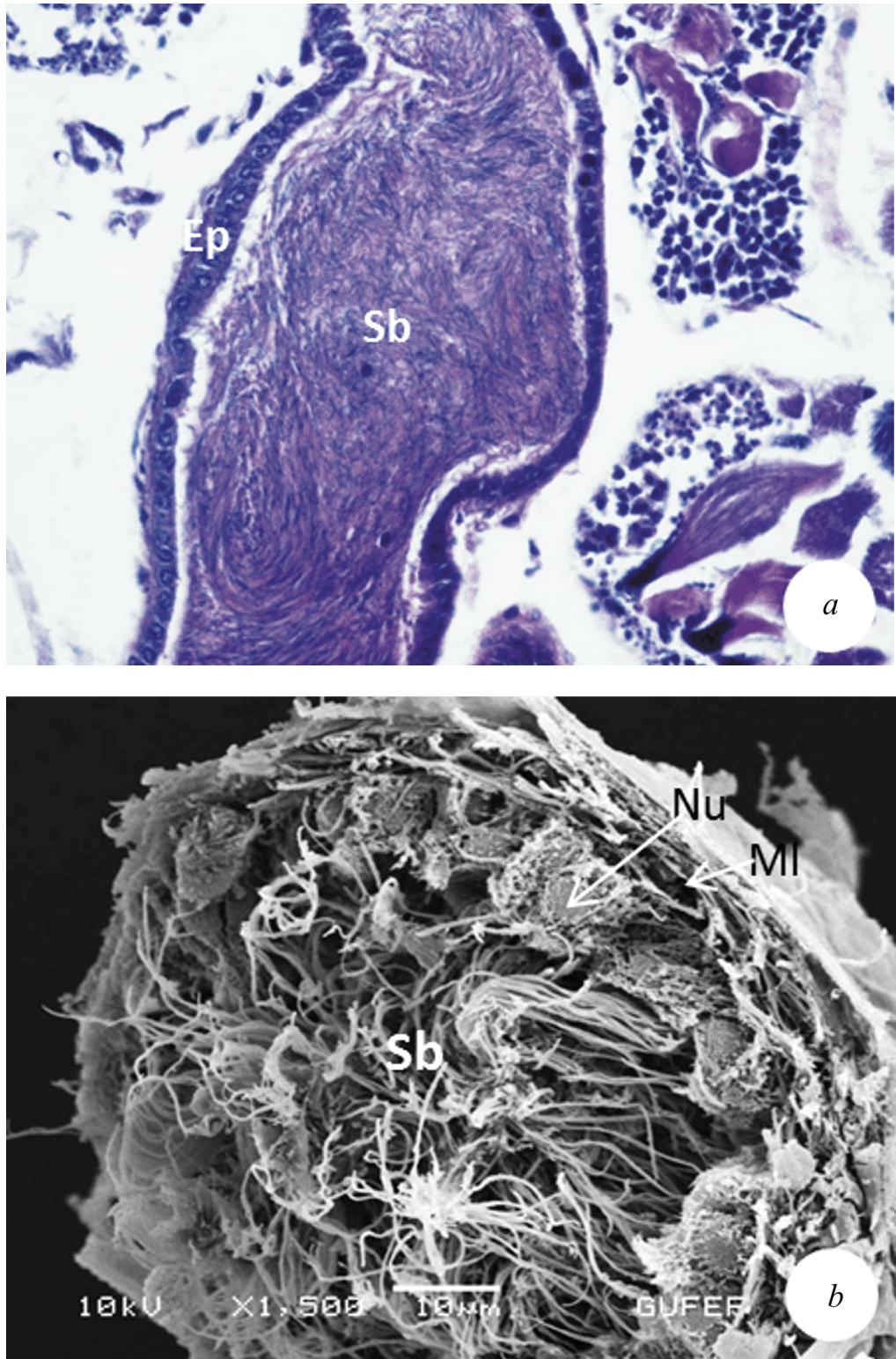


Fig. 11. *Phyllobius fulvago* Gyll., *vas deferens*: (a) histological structure (LM; $\times 1000$), (b) fine structure (SEM). (MI) muscle layer, (Nu) nucleus. For other designations see Figs. 1, 3.

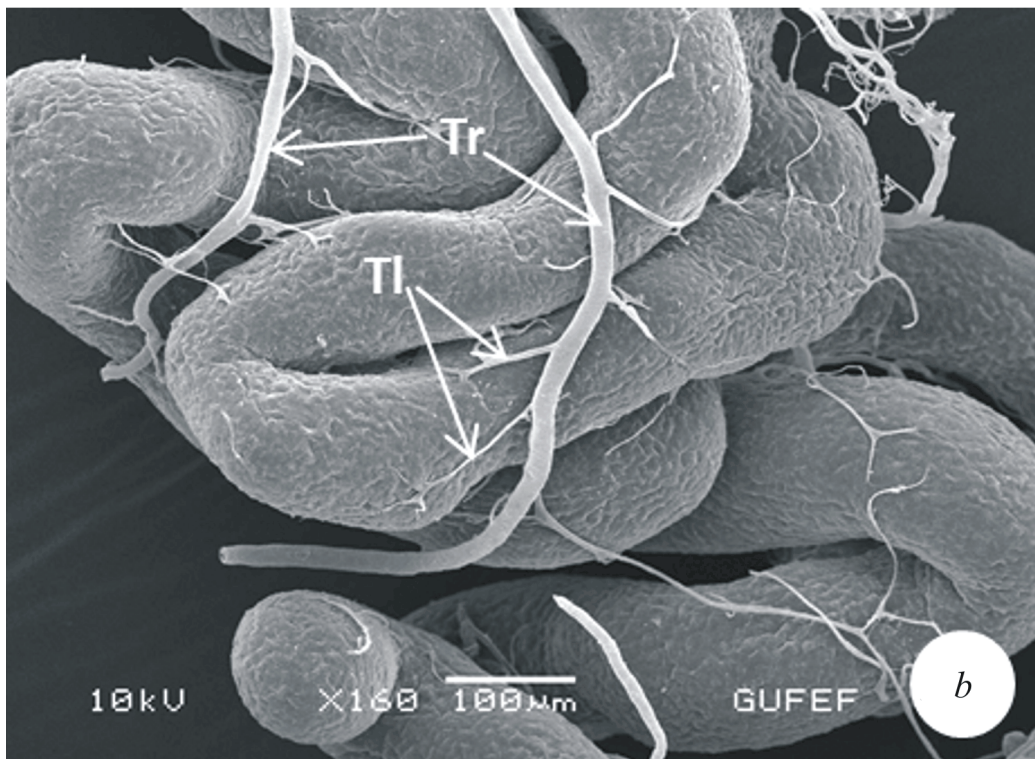
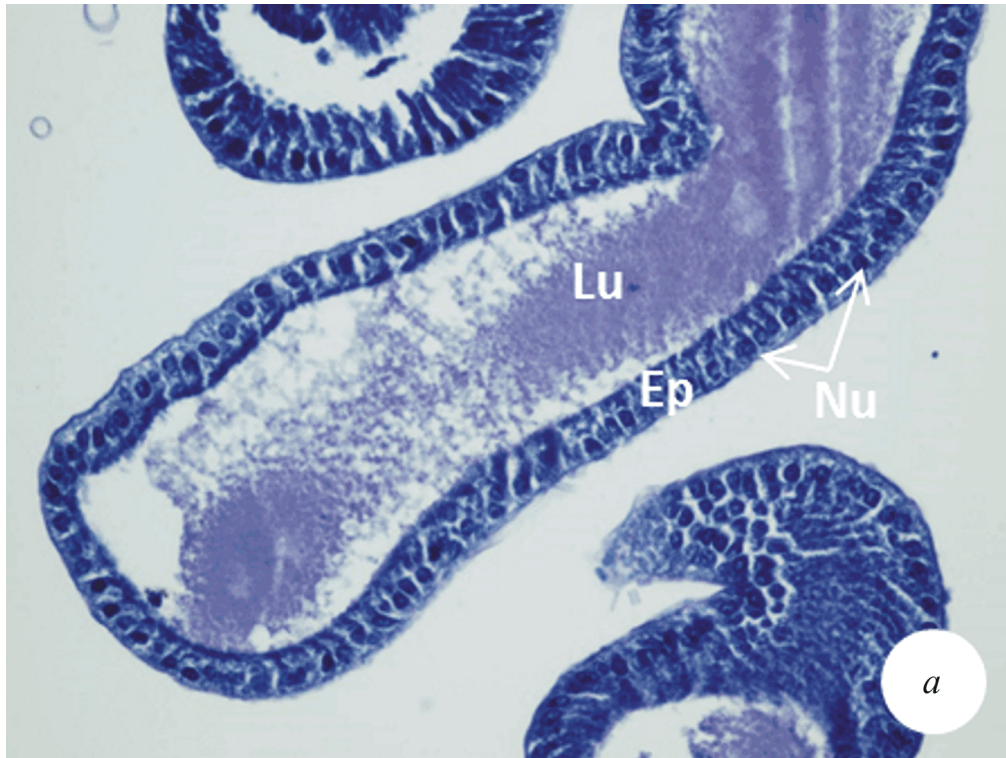


Fig. 12. *Phyllobius fulvago* Gyll.: (a) histological structure of the accessory glands (LM; $\times 400$), (b) fine structure of trachea and tracheoles on the surface of the accessory glands (SEM). (Lu) lumen. For other designations see Figs. 1–11.

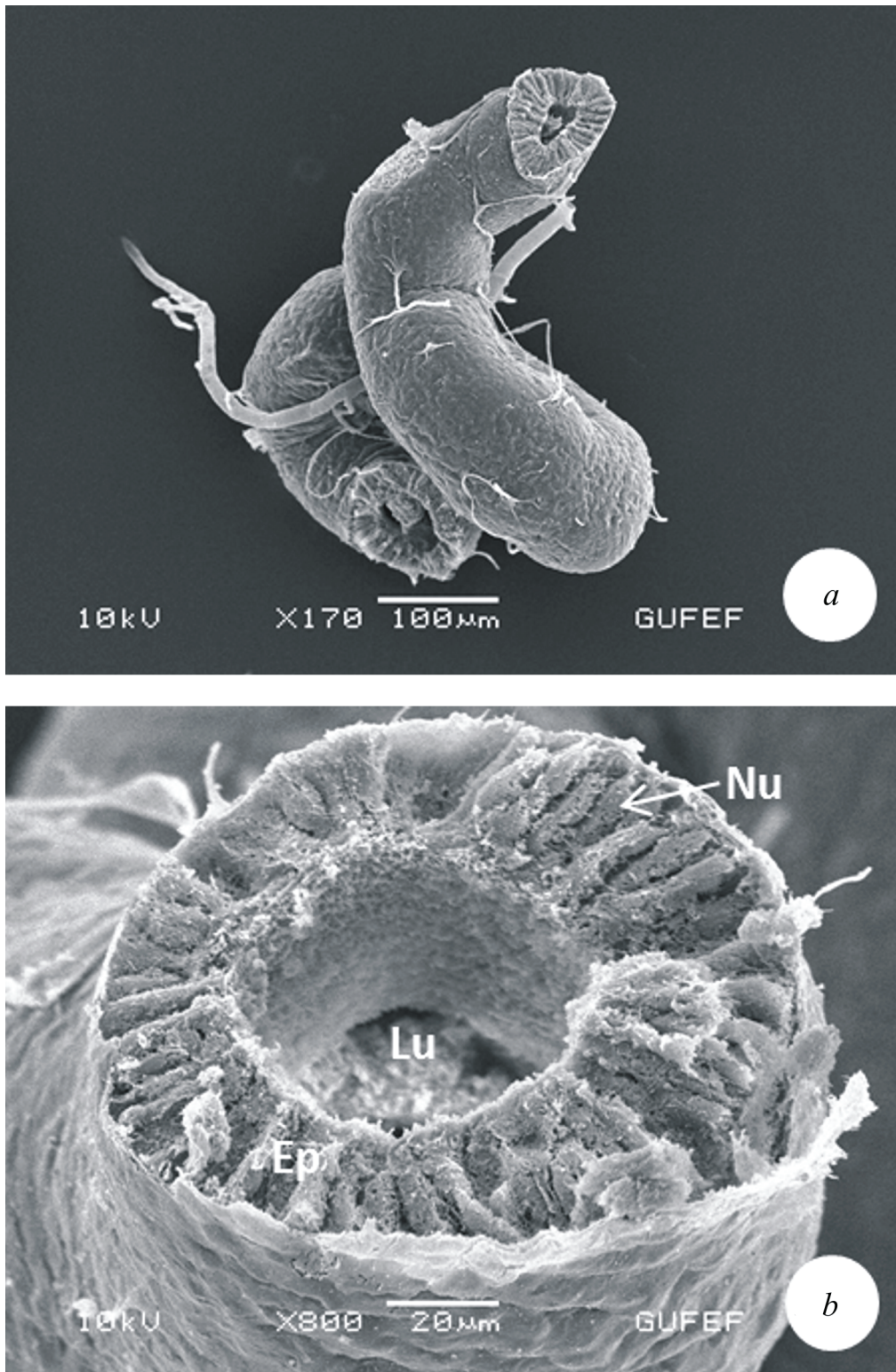


Fig. 13. *Phyllobius fulvago* Gyll., fine structure of the accessory gland (*a*) and its lumen (*b*), SEM. For designations see Figs. 1–12.

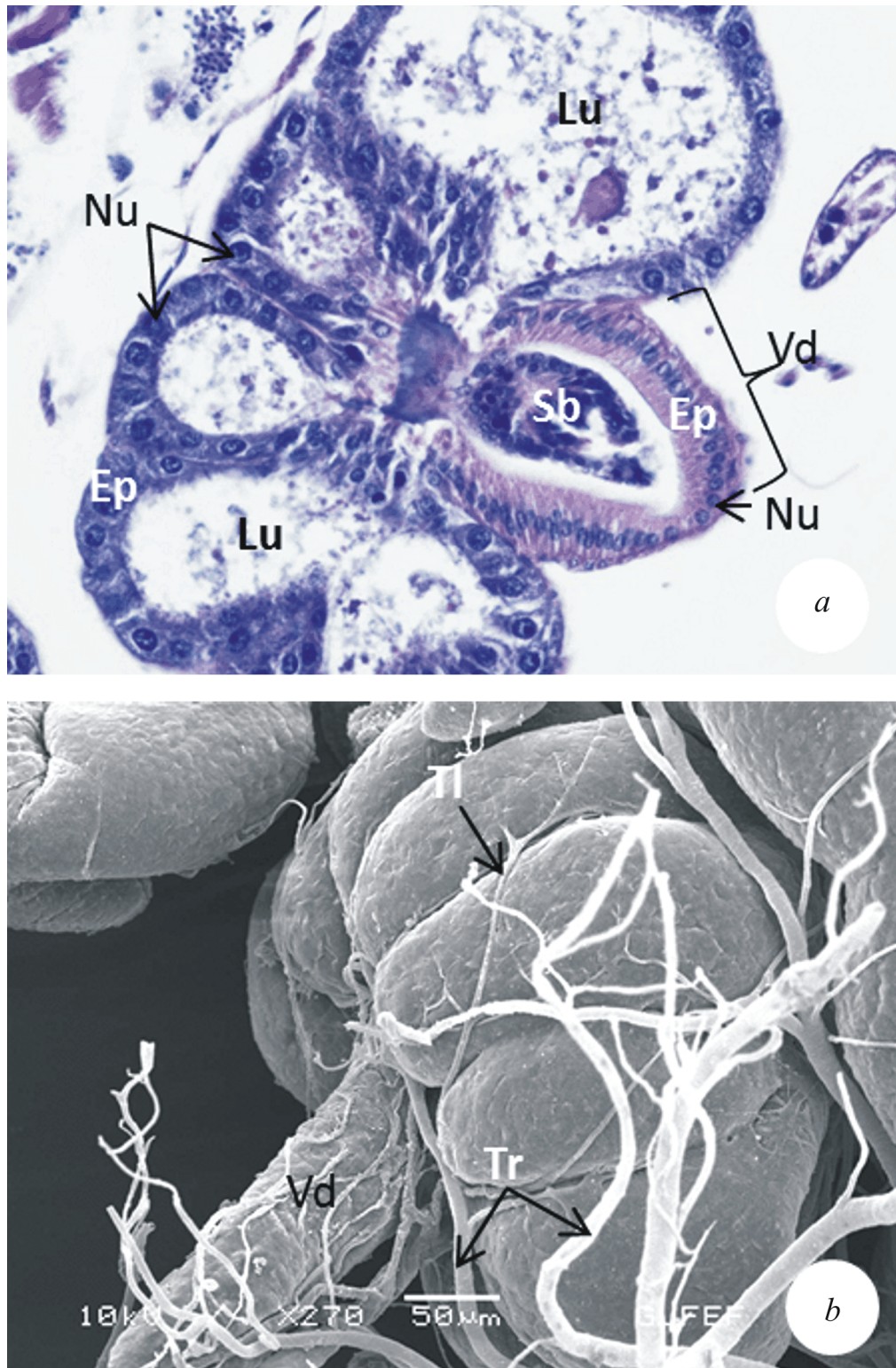


Fig. 14. *Phyllobius fulvago* Gyll., connection of prostate gland with *vas deferens*: (a) histological structure (LM; $\times 400$), (b) morphology (SEM). For designations see Figs. 1–13.

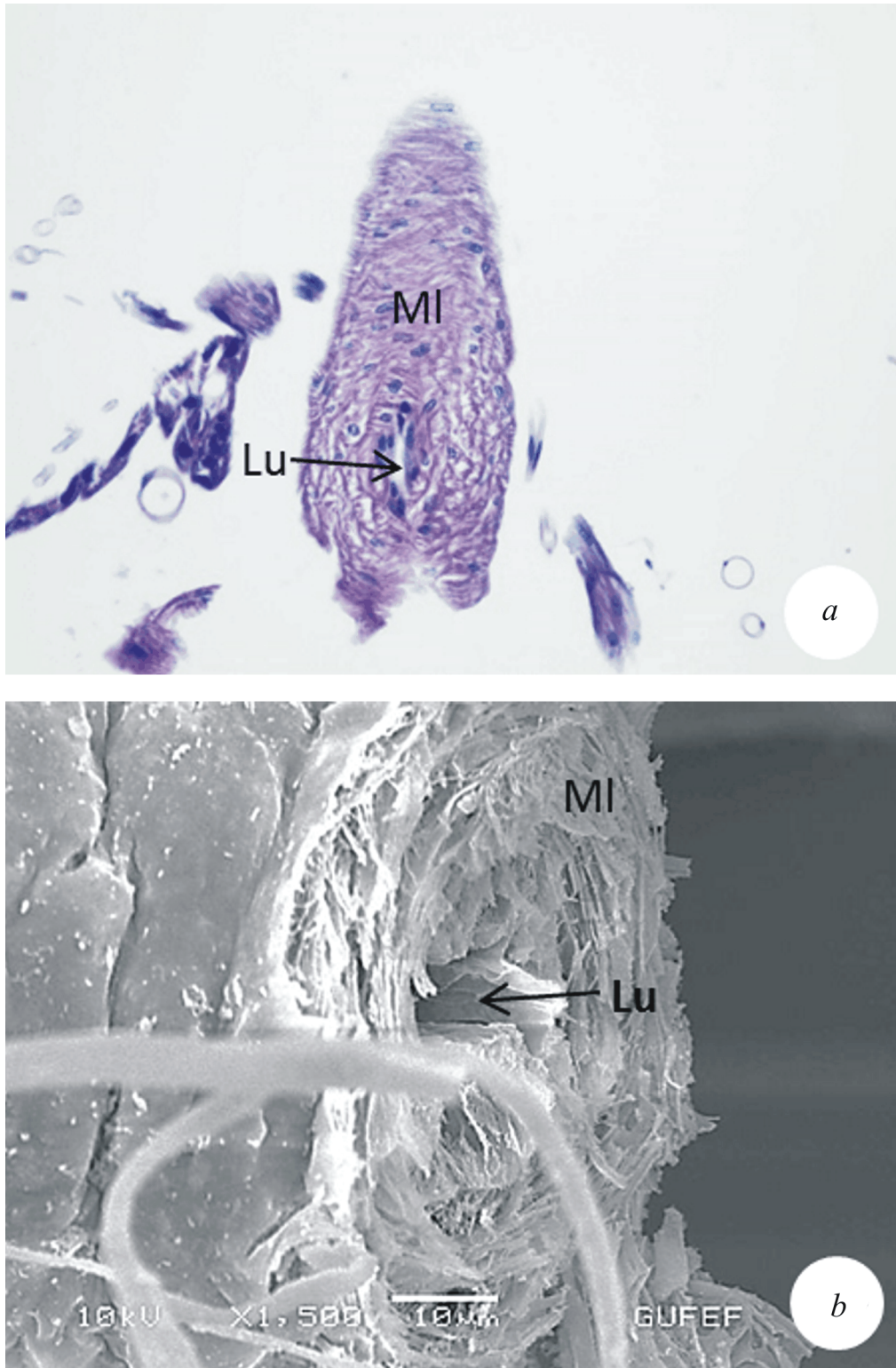


Fig. 15. *Phyllobius fulvago* Gyll., ejaculator duct wall, histological structure (*a*; LM, ×400) and fine structure (*b*; SEM). For designations see Figs. 1–14.

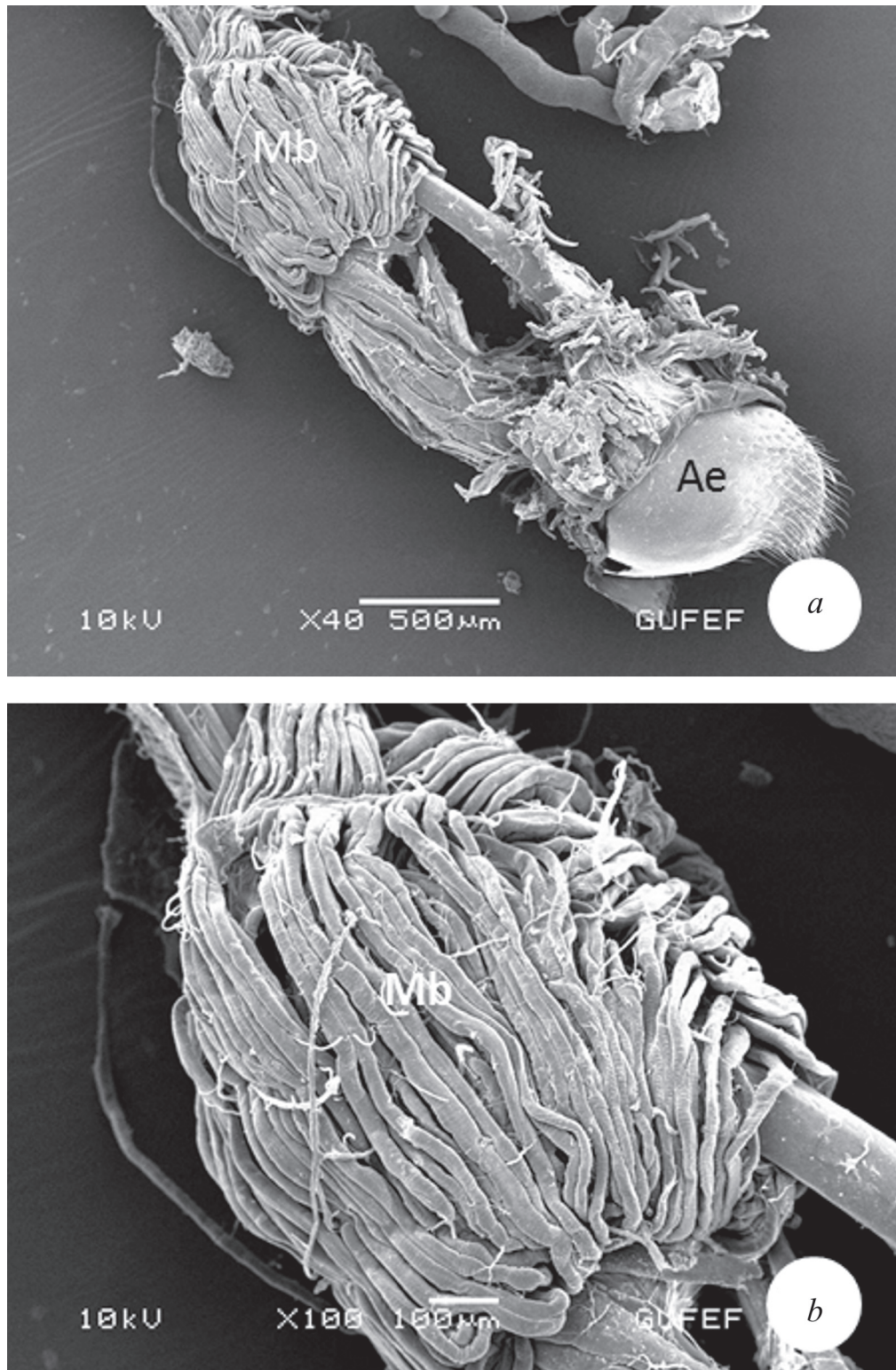


Fig. 16. *Phyllobius fulvago* Gyll., the attachment of muscles to the aedeagus (*a*) and a detailed view of the muscles (*b*). For designations see Figs. 1–15.

Table 1. Number of testicular follicles and accessory glands in Coleoptera

Species	Family	The number of follicles in each testis	The number of accessory glands	Reference
<i>Spasalus silvarum</i> Kuwert	Passalidae	2	4	Salazar et al., 2016
<i>Phytalus sanctipauli</i> Blanchard	Scarabaeidae	6	2	Diefenbach et al., 1998
<i>Axina bifasciata</i> (Chevrolat)	Cleridae	44	4	Opitz, 2014
<i>A. plagiata</i> Schenkling	Cleridae	49	4	Opitz, 2014
<i>Caestron concinnus</i> (Gorham)	Cleridae	11	4	Opitz, 2014
<i>Cardiostychus gabonicus</i> (Thomson)	Cleridae	20	4	Opitz, 2014
<i>Chiloclerus mimus</i> Solervicens	Cleridae	over 70	4	Opitz, 2014
<i>Cladiscus obeliscus</i> Lewis	Cleridae	6	4	Opitz, 2014
<i>Clerus mutillarius</i> (Fabricius)	Cleridae	26	4	Opitz, 2014
<i>Cylidrus abdominalis</i> Klug	Cleridae	6	2	Opitz, 2014
<i>Cymatodera antennata</i> Schaeffer	Cleridae	1	4	Opitz, 2014
<i>C. dietrichi</i> Barr	Cleridae	1	4	Opitz, 2014
<i>C. horni</i> Wolcott	Cleridae	1	4	Opitz, 2014
<i>C. inornata</i> (Say)	Cleridae	1	4	Opitz, 2014
<i>C. tutoides</i> Barr	Cleridae	1	4	Opitz, 2014
<i>C. undata</i> Spinola	Cleridae	1	4	Opitz, 2014
<i>Cymatoderella collaris</i> (Spinola)	Cleridae	1	4	Opitz, 2014
<i>Epiclines basalis</i> Blanchard	Cleridae	multifollicular	4	Opitz, 2014
<i>Eurymetopum maculatum</i> Blanchard	Cleridae	12	2	Opitz, 2014
<i>Isohydnocera aegra</i> (Newman)	Cleridae	6	2	Opitz, 2014
<i>I. tabida</i> (Leconte)	Cleridae	12		Opitz, 2014
<i>Lecontella cancellata</i> (Leconte)	Cleridae	1	4	Opitz, 2014
<i>Lemidia nitens</i> (Newman)	Cleridae	12	2	Opitz, 2014
<i>Monophylla californica</i> (Fall)	Cleridae	1	4	Opitz, 2014
<i>Neoscrobiger patricius</i> (Klug)	Cleridae	multifollicular	4	Opitz, 2014
<i>Omadius semicarinatus</i> Chevrolat	Cleridae	multifollicular	4	Opitz, 2014
<i>Opilo taeniatus</i> (Klug)	Cleridae	11	4	Opitz, 2014
<i>Orthocladiscus dispar</i> (Gorham)	Cleridae	6	4	Opitz, 2014
<i>Phyllobaenus antillae</i> (Wolcott)	Cleridae	6		Opitz, 2014
<i>Ph. pallipennis</i> (Say)	Cleridae	6	2	Opitz, 2014
<i>Priocera hypocrita</i> Chevrolat	Cleridae	25		Opitz, 2014
<i>Thanasimus ceylonicus</i> Kraatz	Cleridae	12	4	Opitz, 2014

Table 1. (Contd.)

Species	Family	The number of follicles in each testis	The number of accessory glands	Reference
<i>Thanasimus dubius</i> (Fabricius)	Cleridae	12	4	Opitz, 2014
<i>Th. formicarius</i> (Linnaeus)	Cleridae	12	4	Opitz, 2014
<i>Tilloidea unifasciata</i> (Fabricius)	Cleridae	1	4	Opitz, 2014
<i>Trichodes alvearius</i> (Fabricius)	Cleridae	multifollicular	4	Opitz, 2014
<i>Trogodendron fasciculatum</i> (Schreibers)	Cleridae	multifollicular	4	Opitz, 2014
<i>Wolcottia sobrina</i> (Fall)	Cleridae	multifollicular		Opitz, 2014
<i>Listronotus bonariensis</i> (Kuschel)	Curculionidae	8–10		Goldson and Emberson, 1981 (as <i>Hyperodes bonariensis</i>); Barker, 1989
<i>Tanymecus dilaticollis</i> Gyllenhal	Curculionidae	2	4	Özyurt Koçakoğlu et al., 2019
<i>Phyllobius fulvago</i> Gyllenhal	Curculionidae	28	6	Authors' data
<i>Dendroctonus armandi</i> Tsai et Li	Curculionidae: Scolytinae	20		Wu et al., 2017
<i>D. monticolae</i> Hopkins	Curculionidae: Scolytinae	6	6	Cerezke, 1964

lateral pair is not branched (Opitz, 2014). In *Omadius semicarinatus*, the medial pair is not branched, and the lateral pair has a basal vesicle (Opitz, 2014). In *Thanasimus dubius* and *Th. formicarius*, medial pair is biramous and the lateral pair is not branched, but in *Th. ceylonicus* both glands are biramous (Opitz, 2014). *Trichodes alvearius* has a short medial pair but the lateral pair is very long (Opitz, 2014). In *Trogodendron fasciculatum*, the medial glands are much longer than the lateral ones (Opitz, 2014). In *Cymatoderella collaris*, the medial pair is not branched but the lateral one is biramous (Opitz, 2014). In *C. inornata* the medial pair is not branched but lateral gland is bilobed and very long (Opitz, 2014). In *Tanymecus dilaticollis*, the accessory glands have large and blind-ended tubules, and the prostate glands have multilobed lobes (Özyurt Koçakoğlu et al., 2019). These glands have two functions in general: formation of sperm fluid and formation of the spermatophore (Sehna, 1985; Gillott, 2005; Klowden, 2008; İzzetoğlu and Gülmez, 2018).

In *Phyllobius fulvago*, the ejaculatory duct is a thick-walled muscular tube with inner epithelium. This duct opens to the aedeagus. No distinct intima was observed

in our study. Similar structures were found in *Dendroctonus monticolae*, *Listronotus bonariensis* and *Tanymecus dilaticollis* (Cerezke, 1964; Barker, 1989; Özyurt Koçakoğlu et al., 2019).

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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