Significant new taxonomic tool for Carabidae (Insecta: Coleoptera): endophallus inflation methods revised

Важный инструмент для таксономии Carabidae (Insecta: Coleoptera): пересмотр метода «надувания» эндофаллуса

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Abstract. A review of the use of the genital structures in taxonomic studies of Carabidae Latreille, 1802 is provided. The pros and cons of different existing methods are briefly discussed. A modified method of preparing and using the aedeagal endophallus is elaboratated. An evaluation of the utility of using this method for different groups of Carabidae is discussed. Most importantly, this method seems to be important for subfamilies Carabinae Latreille, 1802, Cicindelinae Latreille, 1802, Pterostichinae Bonelli, 1810, Harpalinae Bonelli, 1810.

Резюме. Дан обзор истории изучения эдеагуса в целях систематики Carabidae Latreille, 1802. Кратко обсуждаются преимущества и недостатки различных существующих методов. Описыватся улучшенная методика изготовления препаратов эндофаллуса. Дан прогноз полезности использования этого метода для различных групп Carabidae. Наиболее переспективно использование этого метода для видов из подсемейств Carabinae Latreille, 1802, Cicindelinae Latreille, 1802, Pterostichinae Bonelli, 1810, Harpalinae Bonelli, 1810.

Introduction

The structure of carabid beetle genitalia has been used for determination of species for more than one hundred years. The methods of this kind of research are continously improving. Karl Holdhaus [1924] was the first to our knowledge to begin the study of the aedeagal endophallus and thereafter many other researchers continued to develop these methods [Franz, 1929; Kurnakov, 1962; Lindroth, 1963–1969; Meurgues, Ledoux, 1966; Sturani, 1967, and many other carabidologists since then]. The initial approach was to investigate only the chitinized parts of the endophallus, using the method of clearing. The male genitalia was first placed in 10% potassium hydroxide (KOH) solution. Usually, a cold KOH solution is used, but hot KOH can be used to clear specimens within a short time of 30 minutes. Then it is dehydrated in absolute ethanol and after these manipulations, the permanent preparation is made in a drop of canadian balsam, or other modern analog of it. This method still is most widespread, nevertheless it has some limitations. The shape of soft and transparent tissue is not visible to the eye of a researcher, and it is the most important limitation. Moreover, many species have very similar shapes and number of sclerotized spines on the endopahallus, and only an inflated endophallus shows that the shape of these structures in these species is absolutely different and the spatial arrangement of spines can be distinquished.

Some authors are extracting the endophallus of large size species of the genus *Carabus* Linnaeus, 1758 (Coleoptera: Carabidae) using a hook, or by blowing it out with water. This method partly helps to recognise the specific shape of soft tissue, nevertheless the sac will be significantly deformed. The photos of the sac using this method appear to be useless for determination of species, as we can see in the case of a recent investigation on ground beetles of genus *Carabus* from the Caucasus [Retezár, 2008].

The structure of the fully inflated endophallus has been studied the most in Carabidae [Berlov, Berlov, 1996; Shilenkov, 1996; Berlov, 1997, 2000; Matalin, 1998, 1999a, b; Sota et al., 2005; Sasakawa, 2005a, b, 2006; Sasakawa et al., 2006; Sasakawa, Kubota, 2007a,]. A significant contribution to scientific studies of endophalli of members of the genus *Carabus* has been supplied by Ruslan Panin in recent years [Panin, 2013]. His photos are avalaible on the "Carabidae of the World" web-poject [URL: http://www.carabidae.org].

Investigation of the inflated endophallus in other families of Coleoptera has been started recently: Chrysomelidae Latreille, 1802 [Flowers, Eberhard, 2006], Cerambycidae Latreille, 1802 [Rubenyan, 2002; Anichtchenko, Verdugo, 2004; Kasatkin, 2006], Curculionidae Latreille, 1802 [Arzanov, 2006], Staphylinidae Latreille, 1802 [Khatschikov, 2005], Silphidae Latreille, 1807 [Khatchikov, Popov, 2006]. Even so, we still have insufficient information about the structure of the endophallus in most families of beetles. The structure of the endophallus in the families Carabidae, Silphidae, Chrysomelidae and Cerambycidae is very sophisticated (fig. 1–6), and studies of these structures can reveal interesting results. The endophallus of other families, for example, Tenebrionidae Latreille, 1802 and Scarabaeidae Latreille, 1802, have been less investigated and await more study.

Endophallus extraction and study is well-known and has proven to be a very reliable solution to difficult taxonomic issues. Nevertheless, even now not all specialists study the structure of a maximally inflated endophallus for description of new species, even of such a compelling genus as *Carabus*, where this method should be mandatory. The lack of use possibly can be explained by a bias about the apparent difficulty of making preparations. However, a method that is really simple could be suitable for avoiding a number of taxonomic mistakes and resulting synonyms.

Material and methods

The structure of the aedeagal endophallus was studied for male adults of 962 species of ground beetles in the following genera and the number of studied species for each genus is indicated in parentheses. *Cicindela* Linnaeus, 1758 (57), *Cephalota* Dokhtouroff, 1883 (8), *Cylindera* Westwood, 1831 (5), *Lophyra* Motschulsky, 1859 (1), *Myriochila* Motschulsky, 1862 (1), *Therates* Latreille, 1816 (3), *Megacephala* Latreille, 1802 (4), *Tetracha* Hope, 1838 (5), *Omus* Eschscholtz, 1829 (2), *Mantichroa* Fabricius, 1781 (1), *Carabus* Linnaeus, 1758 (377), *Calosoma* Linnaeus, 1758 (11), *Callisthenes* Fischer von Waldheim, 1820 (17), *Callistenia* Lapouge, 1929 (2), *Carabomimus* Kolbe, 1895 (2), *Carabophanus* Kolbe, 1895 (1), *Ceroglossus* Solier, 1848 (7), *Cychrus* Fabricius, 1794 (5), *Cychropsis* Boileau, 1901 (1), *Scaphinotus* Dejean, 1826 (2), *Leistus* Frölich, 1799 (5), *Nebria* Latreille, 1802 (11), *Broscus* Panzer, 1813 (2), *Cerotalis* Castelnau, 1867 (1), *Chaetobroscus* Semenov, 1900 (2), *Clivina* Latreille, 1802 (1), *Scarites* Fabricius, 1775 (5), *Penetretus* Motschulsky, 1865 (5), *Astigis* Rambur, 1838 (1), *Myas* Sturm, 1826 (1), *Abax* Bonelli, 1810 (3), *Percus* Bonelli, 1810 (4), *Pseudopercus* Motschoulsky, 1866 (1), *Ancholeus* Dejean, 1828 (1), *Cryobius* Chaudoir, 1838 (12), *Eutrichopus* Tschitscherin, 1897 (2), *Orthomus* Chaudoir, 1838, *Poecilus* Bonelli, 1810 (6), *Pterostichus* Bonelli, 1810 (31), *Steropus* Dejean, 1821 (4), *Sinosteropus* Sciaky, 1994 (1), *Aristochroa* Tschitscherin, 1898 (3), *Agonum* Bonelli, 1810 (5), *Andrewesius* Andrewes, 1939 (2), *Platyderus* Stephens, 1827 (25), *Laemostenus* Bonelli, 1810 (3), *Sphodrus* Clairville, 1806 (1), *Pristonychus* Dejean, 1828 (2), *Thermoscelis* Putzeys, 1873 (1), *Lindrothius* Kurnakov, 1961 (2), *Calathus* Bonelli, 1810 (11), *Pseudotaphoxenus* Schaufuss, 1865 (3), *Taphoxenus* Motschulsky, 1850 (2), *Amara* Bonelli, 1810 (25), *Curtonotus* Bonelli, 1810 (10), *Zabrus* Clairville, 1806 (47), *Anisodactylus* Dejean, 1829 (2), *Scybalicus* Schaum, 1862 (1), *Ophonus* Dejean, 1821 (13), *Pseudoophonus* Motschulsky, 1844 (2), *Parophonus* Ganglbauer, 1891 (2), *Acinopus* Dejean, 1821 (3), *Carterus* Dejean, 1830 (2), *Eocarterus* Stichel, 1923 (1), *Tschitscherinellus* Csiki, 1906 (1), *Harpalus* Latreille, 1802 (47), *Trichotichnus* A. Morawitz, 1863 (1), *Helluo* Bonelli, 1813 (1), *Licinus* Latreille, 1802 (3), *Dinodes* Bonelli, 1810 (3), *Haplochlaenius* Lutshnik, 1933 (1), *Chlaenius* Bonelli, 1810 (4), *Tefflus* Leach, 1819 (2), *Masoreus* Dejean, 1821 (2), *Graphipterus* Latreille, 1802 (2), *Trymosternus* Chaudoir, 1873 (3), *Cymindis* Latreille, 1806 (5), *Brachinus* Weber, 1801 (6), *Aptinus* Bonelli, 1810 (1), *Termophilum* Basilewsky, 1950 (1), and *Anthia* Weber, 1801 (2).

Studied specimens have been deposited in the collections:

cAA – private collection of Alexandr Anichtchenko (Spain);

DUBC – Daugavpils University Biological Collection (Latvia).

High-resolution endophallus images are available at “Carabidae of the World” web-project [URL: http://www.carabidae.org].

The first entomologists who suggested inflating an internal sac with the help of a syringe were Meurgues and Ledoux [1966]. For this proposed method, the aedeagus has to be boiled in 15% solution of KOH for 2–3 minutes, or 15–20 minutes in 20% acetic acid, and washed in distilled water afterwards. After this procedure, the endophallus is filled up with a mixture of glycerine and gelatine by a syringe with a fine needle with a straigth-cut terminal. then the endophallus is immersed in cold water. When the mixture is cooled, it maintains the shape. Subsequently, the endopahallus should be preserved in 4% solution of formalin. A much more convenient method was suggested by Oleg Berlov [1992]. The endophallus was filled with toothpaste, and then dried with a glow-lamp and the necessary time was dependent upon the size of the endophallus. All modern methods are variations of this last method. Now, however, a more commonly used method is of dry preparations of endophallus, as they are much more convenient for storage and study.

The recommend process herein comprises the following process.

Extraction of the endophallus can be obtained without any special preliminary preparation, if the beetle is fresh, or it is collected and kept in ethyl acetate. It is enough to merely immerse the aedeagus in hot water for 20–30 minutes. For old material that has been kept in formaline, it has to be boiled in 15% solution of KOH for 3–5 minutes. In order to facilitate the work with the aedeagus, the softened parameres are removed and water remnants from the tube with the aedeagus are dried with absorptive filter paper. Then with a strip of Parafilm “M” (approx. 15 mm to 3–4 mm), the aedeagus is fixed on the straigth-cut end of the syringe point. We recommended having needles of several diameters to be selected depending on the diameter of the aedeagus. If the opening of the basal bulb is large, a needle can be inserted into it; however, for a very small opening, it is best to insert the basal bulb inside a needle of an appropriate slightly larger size than the aedeagus.

The endophally filling material will result without shrinkage after the drying process. Ideally, the filling substance would be the light-curing dental composites, used in stomatology: however, the only limitation of them is the high price. Good results have been obtained using toothpaste, and we will describe this method further.

Some specialists inflate the sac with air, and then dry it by glow-lamp while maintaining the pressure. As we mentioned previously, the endophallus should be filled with toothpaste, making the inflation maximum level, if possible. The air or water bubbles that appear inside the endophallus often during the procedure must be remove and to do so, it is necessary to prick them with a very fine needle. Then, without removing the syringe, the endophallus has to be dried under glow-lamp.

The resulting preparation can be stored on a small rectangular card that can be attached on the pin under the beetle. In order to avoid a possible deformation of the preparation, we recommend making all necessary drawings and/or images of it immediately after the preparation.

Each of these methods has its drawbacks. Our recommended method is very suitable for fresh material, but it also has some limitations. Sometimes, especially when the preparation is old, the endophallus has perforations (small openings). In this case, it is much more difficult to inflate it by air, but it is very easy to fill it with thick toothpaste. Another limitation is that the endophallus inflated by air is very fragile, and can change it's shape during storage due to the impact of moisture. In the case of using the air inflation method, if one sees that endophallus is damaged, the best method is to fill it with toothpaste.

The main disadvantage of using toothpaste is that we don't know how it will react with air and moisture over time. Historically, some preparations made with some brands of toothpaste stored in laboratory are in excellent condition after 10 years, but in other cases after just months the wall of the endophallus is damaged by the growth of salt crystals. Male adults of some species have an endophallus with a very long sclerotized spiral, or flagellum that prevents inflation with a thick paste. In this case, it is recommended to unscrew the endophallus with water first, then gently remove the water with an absorbent filter paper and fill it again with a thick paste.

Discussion

Sclerotized structures of the endophallus, clearly visible in transmitted light, successfully have been used in the taxonomy for many groups of ground-beetles. Often, however, some sclerotized structures are not apparent using light, and in this case the endophallus looks like a set of difficult to differentiate folds, fields of scales, setae, or even hidden spines. In this case, a study of an inflated endophallus can reveal absolutely some unexpected results and additional arguments can be made to solve difficult taxonomic issues in many cases, for example, in the case of the genera *Platyderus* [Anichtchenko, 2005, 2009, 2011, 2012] and *Zabrus* [Anichtchenko, Ruiz-Tapiador, 2008; Anichtchenko, Gueorguiev, 2009] (Color plate 3: fig. 7–12). Some species of the genus *Carabus* are almost impossible to distinquish by visual examination of external features. The study of the endophallus in this genus (Color plate 4: fig. 13–16) has helped to solve many difficult issues [Brinev, 2002; Anichtchenko, 2004].

In general, the value of attributes of the endophallus for species diagnosis is now very well recognized. The use of structures of the endophallus for phylogenetic reconstruction requires the formulation of preliminary hypotheses about the patterns of evolution of these structures. Such hypotheses in turn, need to clarify the functions of the endophallus and subsequently an understanding of the interaction of the parts.

The theory about marked relationship between attributes of the endophallus and the bursa-copulatrix of females as a "lock-and-key" mechanism was first offered by Dufor [1844] and many subsequent authors [Ishikawa, 1973; Arnqvist, 1997; Sasabe et al., 2010; Okuzaki et al., 2012; for a summary of this hypothesis see Shapiro and Porter, 1989]. According to this theory, the genital structure that is less impacted by an external environment and only due to natural selection, has the role of stabilization of species (i.e., lack of hybridiazation). Nevertheless, the latest studies [Sota, Kubota, 1998; Takami, 2002, 2007] shows that a lock-and-key condition is not always in affect. Our studies of the structure of the female copulatory pouches in the genus *Zabrus* showed that there is no relationship between shape of bursa-copulatrix and shape of endophallus.

Therewith, the high uniformity of structure of the endophallus for some Coleoptera is evidence that a lock-and-key mechanism is not the universal method for isolation of species. Even within Carabidae, species from subfamilies Carabinae, Pterostichinae, Harpalinae and Cicindelinae have significantly complex and individual structure of the endophallus (Color plate 3–4: fig. 1, 10–12, 21–25), while within other subfamilies, for example, Platyninae Bonelli, 1810 (Color plate 4: fig. 19–20) or some Lebiini Bonelli, 1810 and Licinini Bonelli, 1810, the endophallus is quite simple and uniform.

There are still a number of unrevealed issues. One such questions is about the evolution of the structure of the endopallus – there is no explanation so far, as to why in case of the genus *Carabus*, the evolutionary changes developed in the very complex structure of endophallus, while that of the closely related genus *Calosoma* remained particularly primitive and uniform (Color plate 4: fig. 17–18). The most illustrative examples have been discovered in the genera *Amara* and *Zabrus*. In closely related species that occupy the same ecological niche, and representing the same biomorphs, the structure of endophallus can be extremely complex to very simple (Color plate 3: fig. 10–11). These examples lead us to believe that the sense of the endophallus shape is not necessarily used to create "lock-and-key", or an "anchor" mechanism. Likely, this means that a complex reproductive behavior, geographic, biotopic, or temporary disconnection often plays a leading role in providing of species crossbreeding prevention, even with a relatively simple and uniform endophallus. Thus, the study of an inflated endophallus significantly increases the number of attributes that can be used for diagnosis of species, and probably, for phylogenetic analysis, as well.

New studies are necessary to investigate and explain the aforementioned issues. Nevertheless, the authors certainly acknowledge that investigations of maximumlly inflated endophallus together with other methods of studies, especially with genetical analysis [Sasakawa, Kubota, 2007a], can substantially contribute to clarification of phylogenetic relationships and understanding of evolutionary processes.

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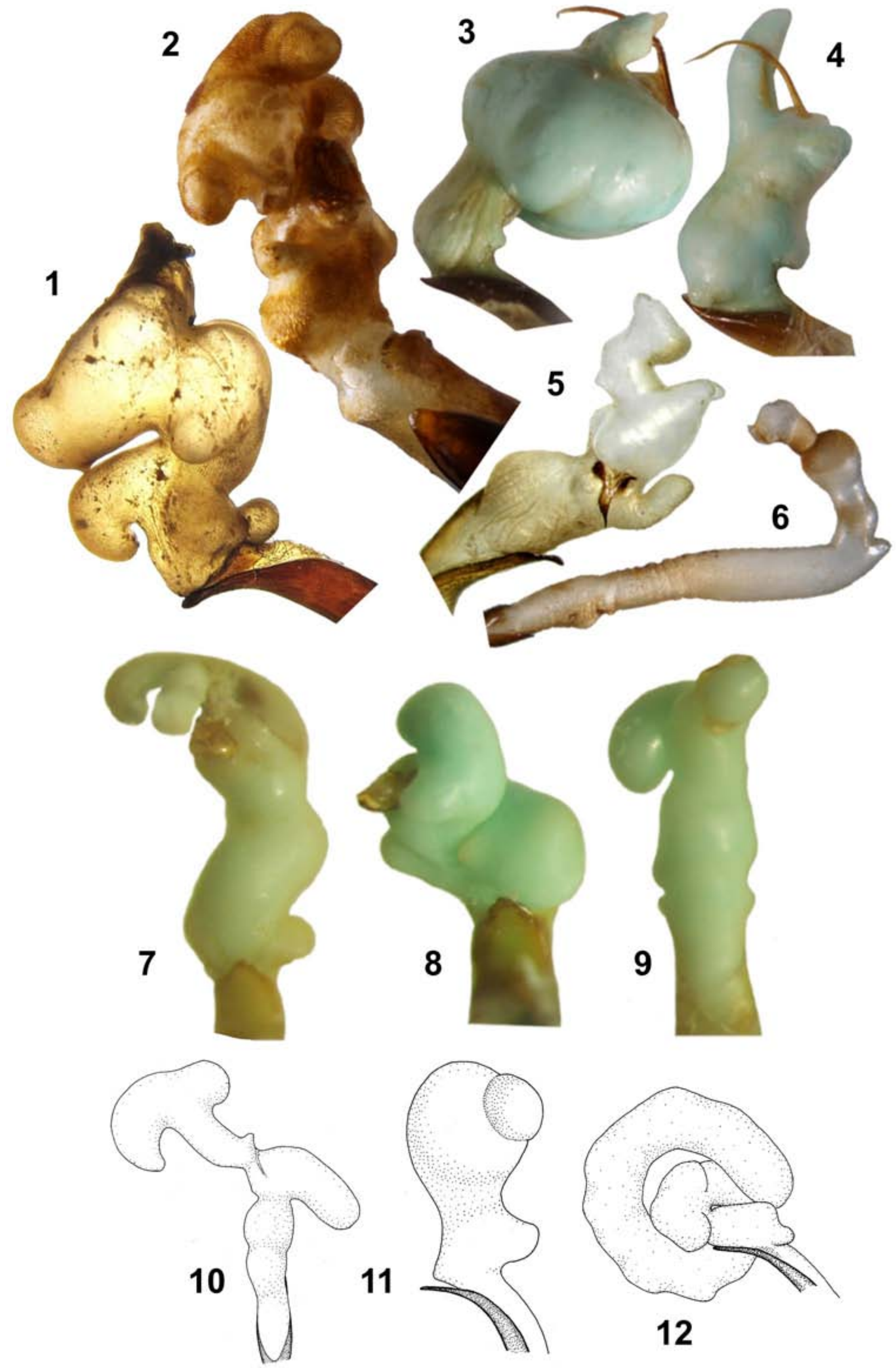

Fig. 1–12. Internal sack of aedeagus of Coleoptera, lateral view.

Рис. 1–12. Внутренний мешок эдеагуса Coleoptera, вид сбоку.

1 – *Carabus* (*Morphocarabus*) *spasskianus shoriensis* Obydov, 1999 (Carabidae); 2 – *Nicrophorus vespilloides* Herbst, 1783 (Silphidae); 3–4 – *Timarcha* sp. (Chrysomelidae); 5 – *Euryphagus lundii* (Fabricius, 1792) (Cerambycidae); 6 – *Dorcadion suturale* Chevrolat, 1862 (Cerambycidae); 7 – *Platyderus salmantinus* Jeanne, 1996 (Carabidae); 8 – *P. lusitanicus* (Dejean, 1828) (Carabidae); 9 – *P. speleus* Cobos, 1961 (Carabidae); 10 – *Zabrus* (*Iberozabrus*) *laurae* Toribio, 1989 (Carabidae); 11 – *Z.* (*I.*) *prietoi* Ruiz-Tapiador et Anichtchenko, 2008 (Carabidae); 12 – *Z.* (*I.*) *coiffaiti* Jeanne, 1970 (Carabidae).

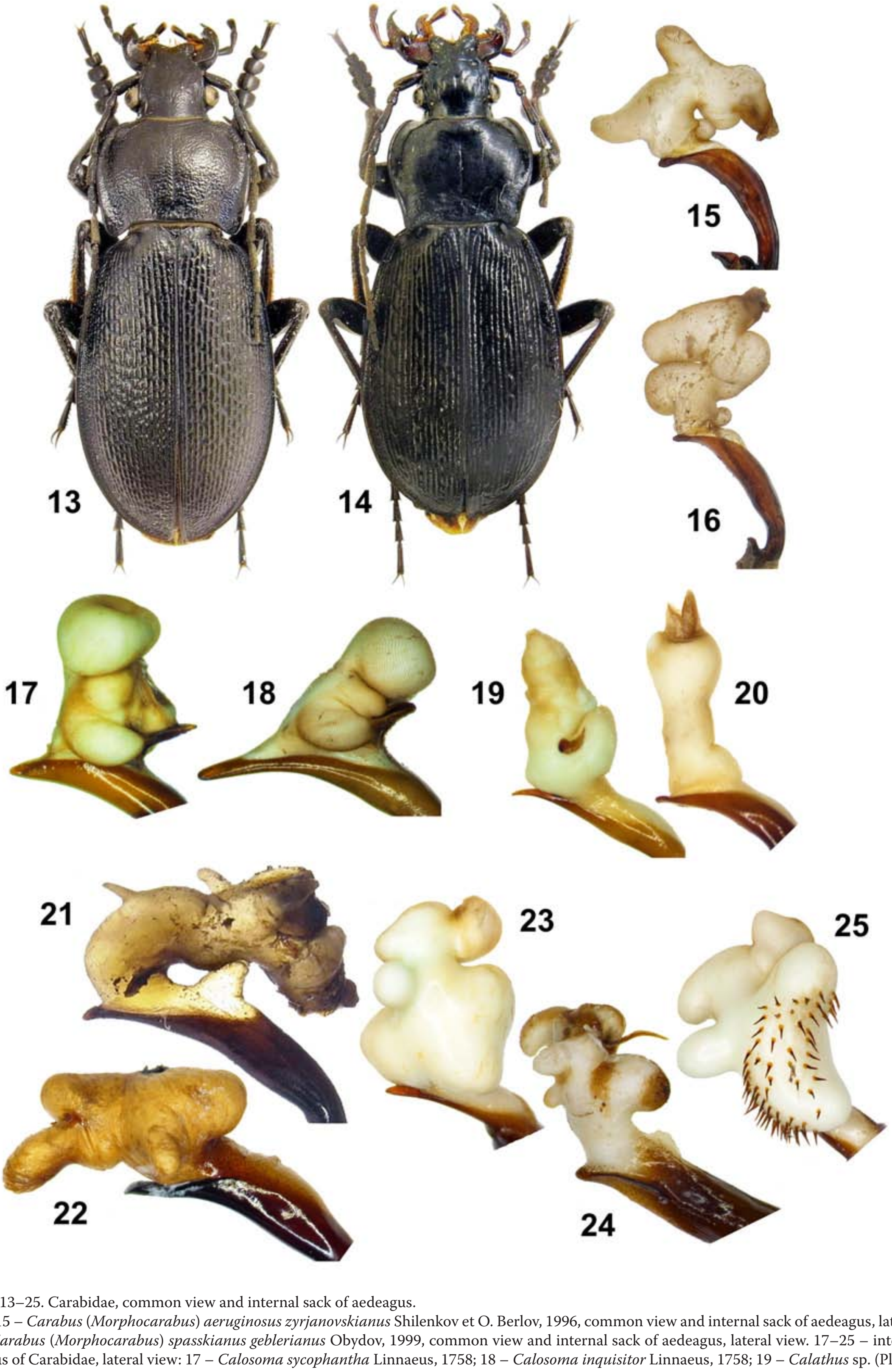

Fig. 13–25. Carabidae, common view and internal sack of aedeagus.

13, 15 – *Carabus* (*Morphocarabus*) *aeruginosus zyrjanovskianus* Shilenkov et O. Berlov, 1996, common view and internal sack of aedeagus, lateral view; 14, 16 – *Carabus* (*Morphocarabus*) *spasskianus geblerianus* Obydov, 1999, common view and internal sack of aedeagus, lateral view. 17–25 – internal sack of aedeagus of Carabidae, lateral view: 17 – *Calosoma sycophantha* Linnaeus, 1758; 18 – *Calosoma inquisitor* Linnaeus, 1758; 19 – *Calathus* sp. (Platyninae); 20 – *Laemostenus* (*Pristonychus*) *sericeus* Fischer von Waldheim, 1824; 21 – *Carabus* (*Hygrocarabus*) *variolosus nodulosus* Creutzer, 1799; 22 – *Abax* (*Abax*) *parallelepipedus subpunctatus* Dejean, 1828; 23 – *Acinopus* (*Acinopus*) *gigantopus* Dejean, 1831; 24 – *Cicindela restricta restricta* Fischer von Waldheim, 1828; 25 – *Acinopus* (*Acinopus*) *laevigatus* Ménétriès, 1832.

Рис. 13–25. Carabidae, общий вид и внутренний мешок эдеагуса.

13–15 – *Carabus* (*Morphocarabus*) *aeruginosus zyrjanovskianus* Shilenkov et O. Berlov, 1996, общий вид и внутренний мешок эдеагуса, вид сбоку; 14, 16 – *Carabus* (*Morphocarabus*) *spasskianus geblerianus* Obydov, 1999, общий вид и внутренний мешок эдеагуса, вид сбоку. 17–25 – внутренний мешок эдеагуса Carabidae, вид сбоку: 17 – *Calosoma sycophantha* Linnaeus, 1758; 18 – *Calosoma inquisitor* Linnaeus, 1758; 19 – *Calathus* sp. (Platyninae); 20 – *Laemostenus* (*Pristonychus*) *sericeus* Fischer von Waldheim, 1824 (Platyninae); 21 – *Carabus* (*Hygrocarabus*) *variolosus nodulosus* Creutzer, 1799; 22 – *Abax* (*Abax*) *parallelepipedus subpunctatus* Dejean, 1828; 23 – *Acinopus* (*Acinopus*) *gigantopus* Dejean, 1831; 24 – *Cicindela restricta restricta* Fischer von Waldheim, 1828; 25 – *Acinopus* (*Acinopus*) *laevigatus* Ménétriès, 1832.

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