New incipient species under reinforcement in the *Drusus discolor* new species complex (Limnephilidae, Trichoptera)

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Abstract: In a comprehensive survey high genetic differentiation with haplotype endemism was detected between mountain range populations of the montane caddisfly species *Drusus discolor* especially in the Pyrenees, Massif Central, and Western Alps. However no morphological divergences were recognised by traditional morphology. In the present study, covering the entire area of the species we have discovered stable paraproctal divergences in the same mountain ranges by applying the speciation trait approach together with fine structure analysis and demonstrated empirically by diverged trait matrices. Unlike other limnephilid taxa the parameres in *Drusus* genus vary stochastically with mutation, genetic drift, and recombination as well as modified by standing genetic variation and by fluctuating asymmetry of developmental instability and environmental plasticity. Morphological divergences of the speciation trait evolved from the ancestral species in peripatry during sexual selection processes. We theoretized the possibility that the reproductive barriers were reinforced or are under reinforcement in secondary contacts. Subtle and stable divergences resulted in the formation of phylogenetic incipient sibling species: *Drusus ferdes* sp. n., *D. kupos* sp. n., *D. leker* sp. n. *D. visas* sp. n.

Introduction

Mitochondrial sequence data (COI) of 254 individuals of the montane caddisfly species *Drusus discolor*, covering its entire distributional range, have been analysed to reveal the population genetic structure (PAULS et al. 2006). High level of genetic differentiation was detected between mountain range populations with remarkable haplotype endemism. The uncorrected pairwise divergence reached the value of 6.3% between mountain ranges. However the differentiation within mountain ranges was low. Final conclusion was drawn that genetic distances between populations of different mountain ranges exceed those measured between other closely related species of *Drusus*. Diverged molecular endemic haplotypes were measured but their phenomics was not described taxonomically. Preliminary observations on male genital morphology revealed no morphological divergences among the haplotype groups. The few adults available for study showed higher morphological variability within than between populations. At the same time the significant genetic differentiation between and low genetic diversity within mountain range population was interpreted as speciation events. Further study was suggested using more molecular and morphological markers to elucidate whether they represent cryptic species or subspecies (PAULS, 2004).

In the present study we have applied our speciation trait approach (OLÁH et al. 2015) with fine structure analysis to find initial split criteria in *Drusus discolor* populations by empirical presentation of the diverged trait matrices. In the *Drusus bolivari* species complex we have found that the paraproct is the decisive speciation trait. Many species in the Chaetopterygini

and Stenophylacini tribes evolved incipient sibling species by divergences in the microstructure of the phallic organ. Sclerotized or soft microstructures on the aedeagus or on the paramere diverged as product of adaptive processes in sexual selection (OLÁH et al. 2015). In *Drusus* the paraprocts evolve in non-neutral, non-random adaptive genomic processes under sexual selection and the miniaturised parameres are more variable due to neutral, random non-adaptive mechanisms being more exposed to stochastic processes of gene flow, genetic drift and recombination.

With focus on paraproct and paramere fine structures we have examined 1119 specimens of *Drusus discolor* species complex collected almost from the entire distributional area (Map 1). We have detected, delimited and delineated four new incipient species. Those populations from the Pyrenees, Massif-Central, and Western Alps were distinguished as morphologically diverged incipient sibling species which were most differentiated and highly diverged also by neutral molecular marker (PAULS et al. 2006). Here we diagnose these new species and present the detailed diverged trait matrices both for paraproct and paramere just to demonstrate for visual experience how stable are the paraproct speciation trait on the huge distributional area of the ancestral species *Drusus discolor* and how it evolved stably and subtly delicate and consistent in the isolated new siblings.
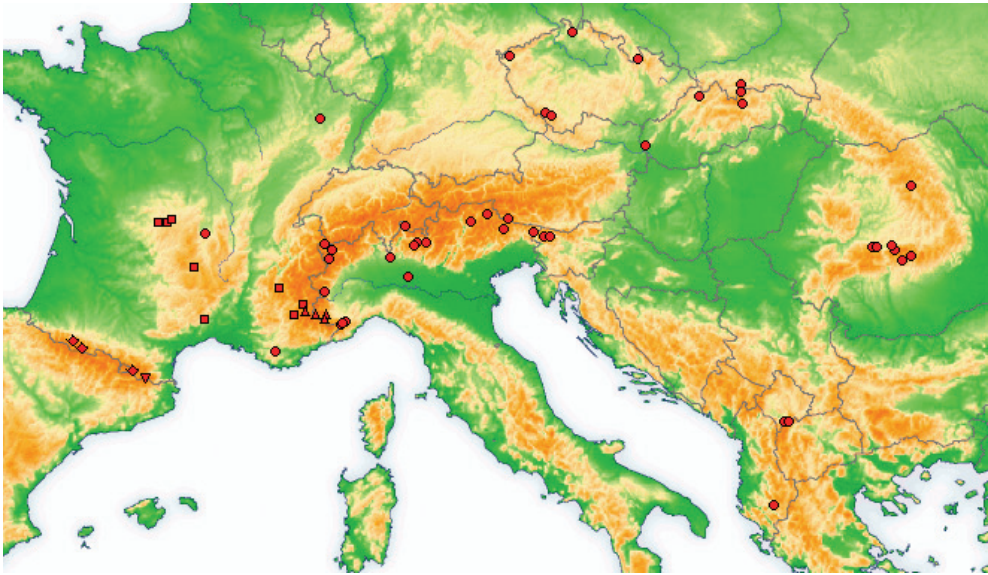
Map 1. Distribution of *Drusus discolor* species complex. Circle = *D. discolor,* square = *D. ferdes* sp. n., downward triangle = *D. kupos* sp. n., upward triangle = *D. leker* sp. n., twisted square = *D. visas* sp. n.

Theoretical part

Without target research we have found contact populations with hybrid effect under reinforcement processes among the examined samples of the new sibling species. First we present some theoretical details of this speciation process getting more and more importance in the

final stage of speciation. We survey some details on the fluctuating asymmetry, an important indicator of paramere variability and an effective procedure to quantify developmental instability of parameres by geometric morphometrics.

Reinforcement to complete speciation

Character displacement, a term coined by BROWN & WILSON (1956), is a speciation process by which traits evolve in respond to selection to lessen resource competition and reproductive interactions between species (PFENNIG & PFENNIG 2009). The idea originates from GAUSE (1934). It was demonstrated experimentally that two species cannot coexist if they overlap in resource requirements. Later the competitive exclusion principle was formulated (HARDIN 1960). Interacting taxa evolve differences in resource use and reproductive traits to prevent local extinction through exclusion. Individuals, populations or taxa most dissimilar are expected to be more successful. Character displacement in traits associated with resource use is the ecological character displacement and that in traits associated with reproduction is the reproductive character displacement. If reproductive character displacement evolves from selection to avoid hybridization, this process is referred to as reinforcement.

Reinforcement is a speciation mechanism that may contribute to reproductive isolation by strengthening barrier building between populations as a result from selection against unfit hybrid offspring (BUTLIN et al. 2011). If hybrids have reduced fitness, reinforcement may lead to divergence between two taxa in the region of overlap. As a result taxa more prone to experiencing character displacement will be more diverse, especially if interacting species are phenotypically variable and abundant in standing genetic variation. Divergence rate depends on gene flow into the sympatry of overlap from the allopatry.

Character displacement is vital in completing the process of speciation when selection lessens resource completion or reproductive interaction by causing sympatric species to diverge in traits associated with resource or mate acquisition. It can finalize speciation between already divergent taxa. However character displacement can initiate divergence and reproductive isolation between conspecific populations that differ in their interactions with heterospecifics. Conspecific population in sympatry and in allopatry are expected to diverge either in resource use or in reproductive traits and differentiation between them in sympatry *versus* allopatry occurs if character displacement generates fitness trade-offs in local adaptation (PFENNIG & PFENNIG 2009, RICE & PFENNIG 2010).

Strong reinforcement process of premating isolation (divergent sexual selection by plumage and vocalization) has been detected in *Ficedula* flycatchers with strong intrinsic postzygotic isolation by female hybrid sterility following Haldane's rule of more severe fitness reduction from genetic incompatibilities in hybrids of the heterogametic female sex (SÆTRE & SÆTHER 2010). Similar premating isolation occured among neighbouring conspecific populations of spadefoot toads by reproductive character displacement of the reinforcement (PFENNIG & RICE 2014). Trait evolved in response to selection to minimize deleterious reproductive interactions with heterospecifics.

Fluctuating asymmetry

Fluctuating asymmetry is measured by variation in the differences between right and left sides of bilateral traits, like parameres and used to assess developmental instability. There are two perspectives on symmetry and three types of asymmetry (KLINGENBERG 2015). (1) *Matching*

symmetry where the structure is present as two mirror imaged separate copies, like human hands, insect wings or the parameres on insect phallic organ. Symmetry axis runs between the two copies. Insect parameres have matching symmetry. (2) *Object symmetry* where the structure is symmetric in itself, like the human face. Symmetry axis runs through the structure.

There are three types of asymmetry detected mostly with scalar measurement (length, angles, and counts of structures like setae), but applicable also to multidimensional formation of shapes. (1) *Antisymmetry* has bimodal distribution of left-right differences with clear left- or right-biased asymmetry of "left-sided" and "right-sided" individuals or dextral and sinistral morphs. (2) *Directional asymmetry* has left-right differences distributed around a mean of different from zero, asymmetric in a consistent way like most internal organs. (3) *Fluctuating asymmetry* has left-right differences of bell-shaped distribution with a mean of zero. These usually small differences are random imprecisions in developmental processes deviating from the expectation of target phenotype and expressed under genomic and environmental control. However, normal distribution would require many small additive and independent random effects, but fluctuating asymmetries are rather non-linear, non-additive with mutual interdependences although random in their directions. Fluctuating asymmetry is effectively applicable to study the developmental origin of integration within and between morphological structures. Development is not completely deterministic.

Paramere asymmetry
Most paramere exhibit fluctuating asymmetry with imprecision measures of developmental instabilities correlated or caused either by adverse environmental conditions or by genetic challenges. Genomic molecular integration with chromosomal rearrangement rather than selection influences this fluctuating asymmetry in polygenic mechanisms comprised of pleiotropic, epistatic, dominance and various "selfish" genetic processes. Gene flow, genetic drift, mutations, recombination and standing genetic variation all may further influence the effective power of the integrative mechanisms in asymmetry. Developmental processes are inherently stable and could be remarkably precise depending on balance between genomic or environmental noise and buffering by developmental stability and canalization (KLINGENBERG 2015).

We have experienced very high stability in the complex structure of the parameres in all European populations of the spring-dwelling caddisfly species *Potamophylax nigricornis* (OLÁH et al. 2013) as well as in other limnephilid genera (OLÁH et al. 2014, 2015). At the same time we have recorded significant fluctuating asymmetry in the count of terminal setae on the parameres of the *Chaetopteryx rugulosa* species group (OLÁH et al. 2012). Similarly less stability and more fluctuating asymmetry was experienced during our studies on parameres in the *Drusus bolivari* species complex (OLÁH et al. 2015).

Paramere variability in *Drusus discolor* complex
In the present study on the *Drusus discolor* new species complex we have examined the parameres of over one thousand specimens (total number of examined specimens is 1119 in the 5 taxa). Drawings of relevant paramere structure are prepared with focus on the articulation region of the long filiform terminal modified seta. At lower magnification of the stereomicroscope it seems that this long and thin terminal filiform structure is the apical section of the paramere shaft and the variously developed spur-like structure is a modified seta. At higher magnification of compound microscope it is clearly visible that the spur formation

is the apical part of the shaft and terminal filiform long structure is a modified seta attaching with alveolus to the spur-shaped shaft head subapicad. Beside the terminal rigid filament and the apical spur of the shaft there are no more setae or spine formation developed on the paramere of the *Drusus discolor* species complex. The apical spur formation sometimes may disintegrate into various substructures.

In most of the examined limnephilid genera we have detected and documented that parameres are the target trait of the divergence due to their central function acting during the copulatory processes in various mechanisms of sexual selection. As a result the parameres are the most diverged traits and their divergence evolved most stable. The high comparative rate of diversity of parameres suggests some kind of runaway coevolution coupled and enforced by cryptic female choice operating on an unknown genomic segment in the sexual selection processes. High rate and stability of divergences confirm that parameres were generated selectively by non-neutral and non-random adaptive mechanisms. We have concluded that these delicate structures of limnephilid genera directly involved in copulatory processes are speciation traits evolved under sexual selection and confirmed or completed with various reinforcement mechanisms in local adaptation (OLÁH et al. 2012, 2013, 2014, 2015).

In subfamily Drusinae the parameres are miniaturised into a slim and slender rod-like structure with some modified setae present in various numbers in various shapes and on various regions of the paramere shaft. Paramere size-reduction induced probably some kind of function alteration manifested in reduced selective power and resulted in increased variability. Due to this reduction in size the parameres were almost neglected in the diagnosis of species descriptions. Usually paramere drawings are lacking or if present, prepared from images of low magnification. Very few paramere drawings have been prepared from proper preparations and with higher resolution (MURGOCI & BOTOSANEANU 1954).

Paraproct divergence

In *Drusus* genus the paraproct with increased direct function in copulatory processes during sexual selection has taken over most selectivity of the parameres. As a result the paraproct diverged under non-neutral, non-random, that is under directed selective and adaptive mechanisms. That is clearly demonstrated as self-evidence of the high stability that we have demonstrated earlier in the diverged trait matrices (OLÁH et al. 2015). The lateral profiles of both the ancestral paraproct in *Drusus discolor* as well as of the diverged paraprocts in the four new incipient sibling species are remarkable stable. The divergences in the paraproct shape of all the four new siblings are rather subtle, but consistent. However even subtle modifications in the head shape of the dorsal branch of the paraproct may result in significant alternative stimulatory effect during copulation in the cryptic female choice. The black dorsoapical head of the paraproct are strongly sclerotized and densely packed with peg-like pointed short and black cuticular outgrowths (spinules or tubercles of Schmid and Botosaneanu). The short pointed processes of micro-sculpture are non-cellular in origin and composed entirely of heavily sclerotized cuticula and are fixed to and confluent with the exoskeleton. The serrated pointed sculpture of the paraproctal head may have decisive stimulatory function. Any divergence in shape and direction of this enlarged heavily sclerotized structure with particularly patterned surface must have a dominant copulatory function of barriers in reproductive isolation.

Besides the speciation trait of the paraproct we have found morphological divergences among siblings also in the paramere microstructure and in the gonopod shape. However variability is high due to the stochastic processes dominating in their genetic or epigenetic formation.

There are several possible drawing planes to document the evolved shape divergences in the paraproct structure. We have selected the lateral view that is most simple to align and more reliable to reproduce. SCHMID (1956) already emphasized that lateral view of the paraproct is very characteristic in the genus *Drusus*. The paraproct was adjusted in lateral view with an arrangement having the apical margin exactly in vertical position. This has prime importance to view and draw the paraproctal head in proper direction to image the real lateral head profile and the serrated pattern of pointed peg-like micro-sculpture.

Material and methods

Practical problems for analysis of microstructures

Studies on microstructures of the speciation traits focus on divergences that are fairly subtle. When building and composing diverged trait matrices (structure matrix of diverged speciation traits; visualized structure matrix, as a graphical copy of population reality) we work nearby the structural diverging point of the initial split criteria (OLÁH et al. 1914). We have to pay particular attention to artefacts (KLINGENBERG 2015): (1) from museum specimen stored dry or in liquid; (2) from specimen preparations of clearing and cleaning procedures; (3) from removing setae (hairs); (4) from exposition of internally withdrawn phallic organ by pulling out with super fine forceps; (5) from distortions and injuries frequently occur in nature during mating or copulation processes; (6) from aligning or positioning to microscope when collecting 2D data of 3D structures and loss of information; (7) from lacking clear orientation of front and back, up and down, left and right; (8) from variation in the level of focus or inaccurately focused images; (9) from distortions by low-quality lenses; (10) from enlarged parallax of an oblique angle; (11) from low-quality light with unequal illumination. Moreover there are systematic errors affecting all measurements of any observer with given equipment, magnification especially at higher resolution power. Random errors affect each individual observation differently; produce deviations from the reality in all directions. However the main question remains how much greater divergence is than the artefact error and the range of individual trait variation together.

Depositories: Constantin Ciubuc Private Collection, Sinaia, Romania (CCPC), Coppa Private Collection, Villers-sur-Bar, France (CPC), Hungarian Natural History Museum, Budapest, Hungary (HNHM), Museo Civico di Scienze Naturali "E. Caffi", Bergamo, Italy (MCSNBG), National Museum, Prague, Czech Republic (NMPC), Oláh Private Collection under national protection by the Hungarian Natural History Museum (OPC).

Taxonomical part

Drusus discolor (Rambur, 1842) (Figs 1–190)

Rediagnosis – After a preliminary throughout examination of genital fine structure of all populations we have recorded cerci non-diverse and stable, gonopods diverse and variable, paramere non-diverse and variable. The paraproct exhibited the most stable and most diverse state of trait. These findings confirm the speciation function of the paraproct supported by the selective non-random, non-neutral *versus* random, neutral trait comparative principle, realized in adaptive process of sexual selection and resulted in reproductive barrier building and character displacement.

We have examined 1119 specimens and found 1030 specimens having the ancestral shape of the paraproct, 5 specimens diverged to *Drusus kupos* sp. n. in Pyrénées-Orientales, Err; 39 specimens diverged to *D. ferdes* sp. n. in Massif-Central and in Alpes-de-Haute-Provence; 26 specimens diverged to *D. leker* sp. n. in Piemonte, Italy and Alpes-de-Haute-

Fig. 1. *Drusus discolor* (Rambur, 1842) male genitalia without phallic organ
and with eight tergite profile in left lateral view

Provence, France; 19 specimens diverged to *D. visas* sp. n. in Pyrénées-Orientales, and Hautes-Pyrénées.

The ancestral shape of the paraproct in *Drusus discolor* (Rambur) is characterized by a lateral profile of flat convex serrated head, horizontal and rounded quadrangular relative to the vertically positioned apical margin. The serrated head is rounded truncated. This shape is very stable in all the populations inhabiting huge distributional area from France to Albania through Italy, Slovenia, Czech Republic, Slovakia, Poland, Romania, Bulgaria, and Kosovo. We have to emphasize however what we have experienced during the tedious cleaning procedures as well as during the detailed microscopic examination and drawings of so many specimens, that as it was detailed among the practical problems of microstructure analysis, the alignment of the cleared genitalia may have dramatic effect on the image what
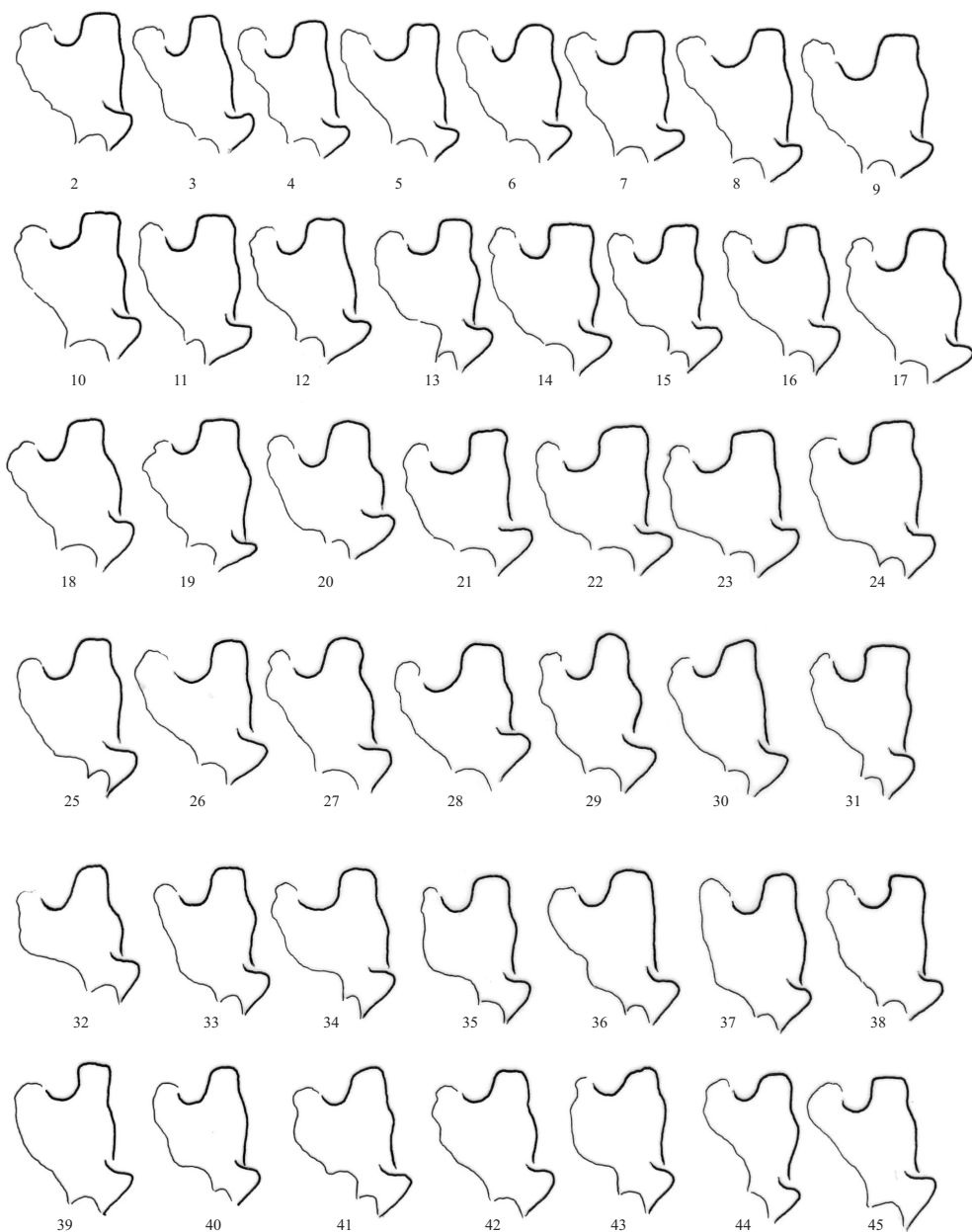
Figs 2–45. *Drusus discolor* (Rambur, 1842) paraproct (superanal complex without cercus) in lateral view.
France: 2–3 = Vosges, 4–7 = Alpes Maritimes (contact population with *D. leker* sp. n), 8 = Savoie.
Italy: 9–10 = Briga Alta, 11–16 = Piemonte, Crissolo, 17–19 = Piemonte, Ceresole Reale,
20–25 = Valle d'Aosta, 1700 m, 26–29 = Valle d'Aosta, 1650 m, 30 = Mendatica, 31 = Madisimo,
32 = Ronobello, 33 = Vigilio, 34 = Valbondione, 1940 m, 35 = Valgoglio, 36 = Valbondione, 1862 m,
37–38 = Fiume Po, 50 m, 39–41 = Telve, 42 = Claut, 43–45 = Sappado
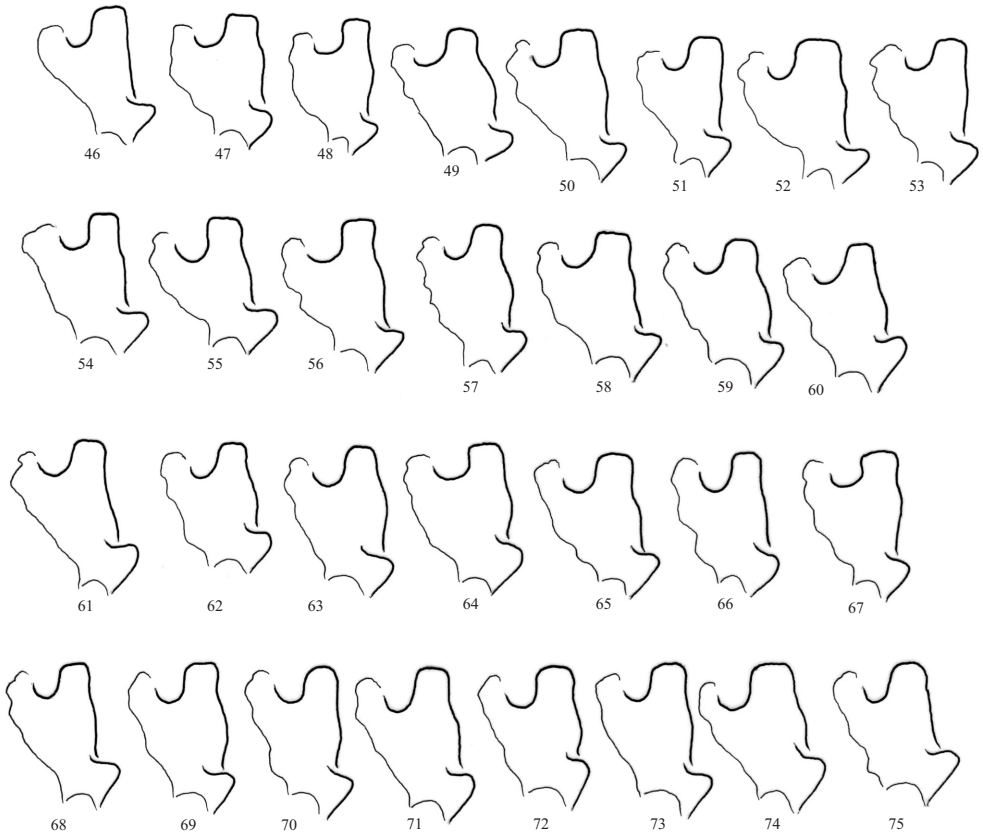
Figs 46–75. *Drusus discolor* (Rambur, 1842) paraproct (superanal complex without cercus) in lateral view.
Italy: 46–48 = Resia. **Slovenia**: 49–52 = Julian Alp.
Slovakia: 53 = Pleso nad Skokem, 54 = Biela Voda, 55 = Mlynica, 56–58 = West Tatra, Jamycky Stream.
**Poland**: 59–61 = Gorce Mts, **Romania**: 62–64 = Lacu Rosu, 65–66 = Bucegi Mts,
67–68 = Fagaras Mts, 69 = Cibin Mts, 70 = Tarcu Mts
Kosovo: 71 = Lepenc, 72 = Prevall. **Albania**: 73–75 = Ostrovice Mts

we see under the microscope and what we draw with the drawing tube. The serrated head of the paraproct is composed of the two free, not fused dorsal branches of the paraproct. The branches are flat in sagittal plane and positioned oblique from anterolaterad to posteromesad. As a result a very tiny alteration in observation view from exact lateral may change dramatically the length (not the height!) of the head on the images what we see under microscope and consequently the drawings what we prepare.

The spur formation, the actual terminal ending of the paramere is developed into a slightly upward curving and narrowing pointed structure with a variously produced dorsosubapical uprising or more frequently without any such outgrowth. The variation within or between populations is almost the same, due to dominating stochastic processes including fluctuating asymmetry, as it was discussed in the theoretical part.

Material examined – **Albania**, Skrapar district, Ostrovicë Mts, Backë, brook and spring NE of the village, N40°31.346', E20°25.096', 1650 m, 29.05.2013, P. Juhász, T. Kovács, G. Magos, G. Puskás (4♂, 11♀, OPC). **Bulgaria**, Blagoevgrad province, Belasica Mts, Petrič, sping of Lesniska Stream SW of the city, N41°21.021', E23°10.767', 1025 m, 27.10.2013, J. Kontschán, D. Murányi, T. Szederjesi (1♀, HNHM). **Czech Republic**, S Bohemia, Šumava Mts, Hamerský potok stream below Horská Kvilda, N49°03'25", E13°32'06", ca. 1000 m, 27.07.1991, P. Chvojka (1♂, NMPC). S Bohemia, Šumava Mts, Hamerský potok stream below Horská Kvilda, 49°03'25"N, 13°32'06"E, ca. 1000 m, 12.06.1992, P. Chvojka (1♂, NMPC). W Bohemia, Krušné hory Mts, Rájecký potok stream above Støíbrná, N50°22'38", E12°32'21", 640 m, 05.06.1993, P. Chvojka (3♂, 1♀, NMPC; 3♂, 1♀, OPC). N Bohemia, Jizerské hory Mts, Jedlová stream above Josefùv Dùl, N50°47'17", E15°14'32", 650 m, 05.06.2002, F. Krampl (6♂, 4♀, OPC). N Moravia, Králický Snì ník Mt., Morava River, N50°11'59", E16°50'36", 1100 m, 16.07.2001, P. Chvojka (2♀, OPC). N Moravia, Králický Snì ník Mt., Morava River, N50°11'59", E16°50'36", 1100 m, 09.07.2003, P. Chvojka (12♂, OPC). **France**, Department Vosges, Le Valtin, la Meurthe, 02.07.2008, G. Coppa (2♂, OPC). Department Alpes-Maritimes, Guila affluent Macruera ancien sentier, 08.07.2008, G. Coppa (4♂, 1♀, OPC). Department Savoie, Bramans, Granges de Savine, 16.08.2009, G. Coppa (1♂, 1♀, OPC). **Italy**, Lombardia, Valbondione (BG), Lago Barbellino, N46.0630°, E10.0482°, 1862 m, 16.07.2003, light trap, R. Calandrina (5♂, 2♀, MCSNBG). Madesimo (SO), Montespluga, torrente, N46.4852°, E9.3538°, 1917 m, 23.07.2004, light trap, M. Valle (8♂, 2♀, MCSNBG; 5♂, 1♀, OPC). Valgoglio (BG), Val Sanguigno, torrente vicino al lago Prespontino, N45.9789°, E9.8457°, 1910 m, 07.08.2009, light trap, S. Cerea (24♂, 10♀, MCSNBG; 10♂, 7♀, OPC). Roncobello (BG), Baite di Mezzeno, N45.9652°, E9.8084°, 1600 m, 08.07.1995, light trap, Bertuetti (22♂, 12♀, MCSNBG). Roncobello (BG), Baite di Mezzeno, N45.9652°, E9.8084°, 1600 m, 08.07.1995, light trap, Bertuetti (22♂, 12♀, MCSNBG). S. Rocco al Porto (LO), Fiume Po, N45.0611°, E9.7042°, 50 m, 29.07.1997, light trap, F. Carrara, O. Lodovici (1♂, MCSNBG). Valbondione (BG), Baite di Sasna, N46.0177°, E10.0446°, 1940 m, 24.07.1995, light trap, Albrici (16♂, 4♀, MCSNBG). Piemonte, Crissolo (CN), Pian della Regina fiume Po, N44.7000°, E7.1163°, 1700 m, 02.09.1997, light trap, M. Valle (74♂, 37♀, MCSNBG; 10♂, 8♀, OPC). Briga Alta (CN) affluente torrente Negrone, N44.1109°, E7.7252°, 1600 m, 18.07.2001, light trap (7♂, 19♀, MCSNBG). Ceresole (TO), Torrente Orco sopra Chiapili, N45.458°, E7.1630°, 1800 m, 21.08.1965, A. Viganò (3♂, 1♀, MCSNBG). Liguria, Mendatica (IM), affluente torrente Tanarello c/o Valcona Sottana, N44.0977°, E7.7509°, 1230 m, 18.05.2001, light trap (1♂, MCSNBG). Mendatica (IM), rio delle Salse, N44.1105°, E7.7367°, 1300 m, 18.07.2001, light trap (1♀, MCSNBG). Trentino Alto-Adige, Telve (TN), torrente Maso, N46.1497°, E11.4496°, 1370 m, 08.08.2001, light trap, L. Bianchi, O. Lodovici (16♂, 18♀, OPC). Marebbe (BZ), San Vigilio Marebbe Val de Rit, N46.6740°, E11.9444°, 1600 m, 27.07.1994, light trap, Becci, Pisoni (7♂, 4♀, MCSNBG). Valle d'Aosta, Courmayeur (AO), torr. Ferret – la Vachey, N45.850°, E7.024°, 1650 m, 11.07.1964, A. Viganò (4♂, MCSNBG). Cogne (AO), Pra Suppiaz – torr. Valnontey, N45.575°, E7.145°, 1700 m, 13-24.08.1970, A. Viganò (13♂, 1♀, CNSMB; 10♂, OPC). Veneto, Sappada, (BL), fiume Piave, N46.5847°, E12.7269°, 1400 m, 08.08.2002, (7♂, 1♀, MCSNBG). Friuli Venezia Giulia, Claut (PN), torrente Cellina M.ga Casavento, N46.2689°, E12.5991°, 950 m, 16.09.1996, light trap, P. Pantini, M. Valle (7♂, 5♀, MCSNBG). Resia (UD), Uccea Rio Uccea, N46.3056°, E13.4168°, 550 m, 20.09.1996, light trap, P. Pantini,
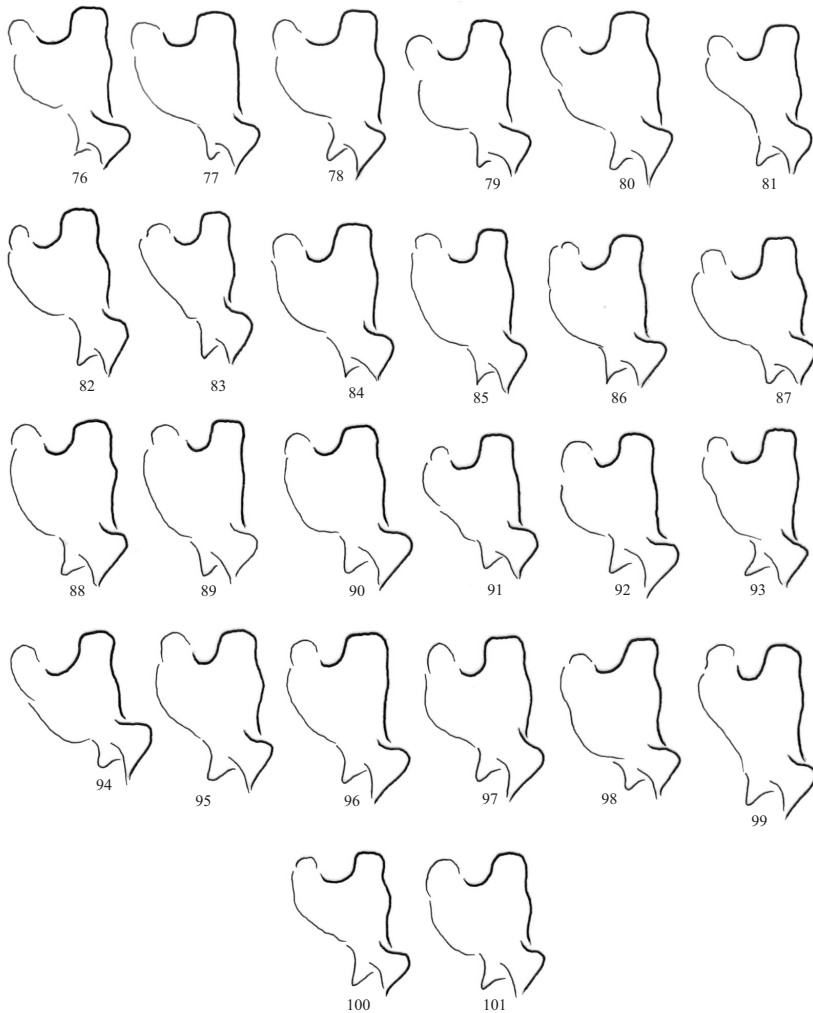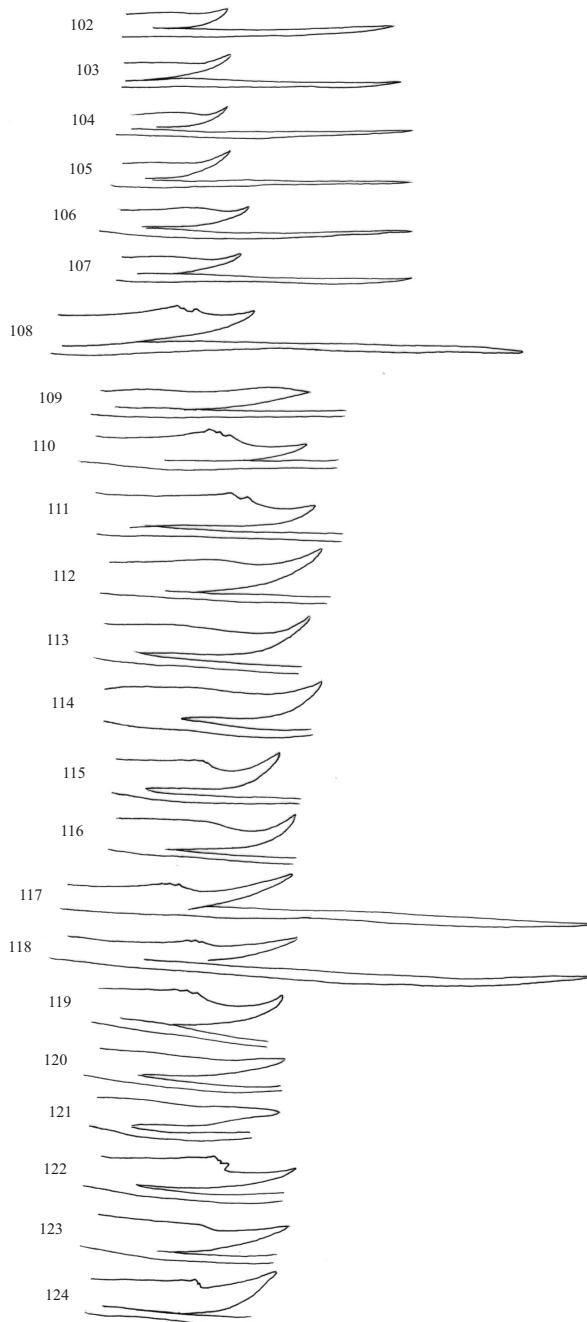
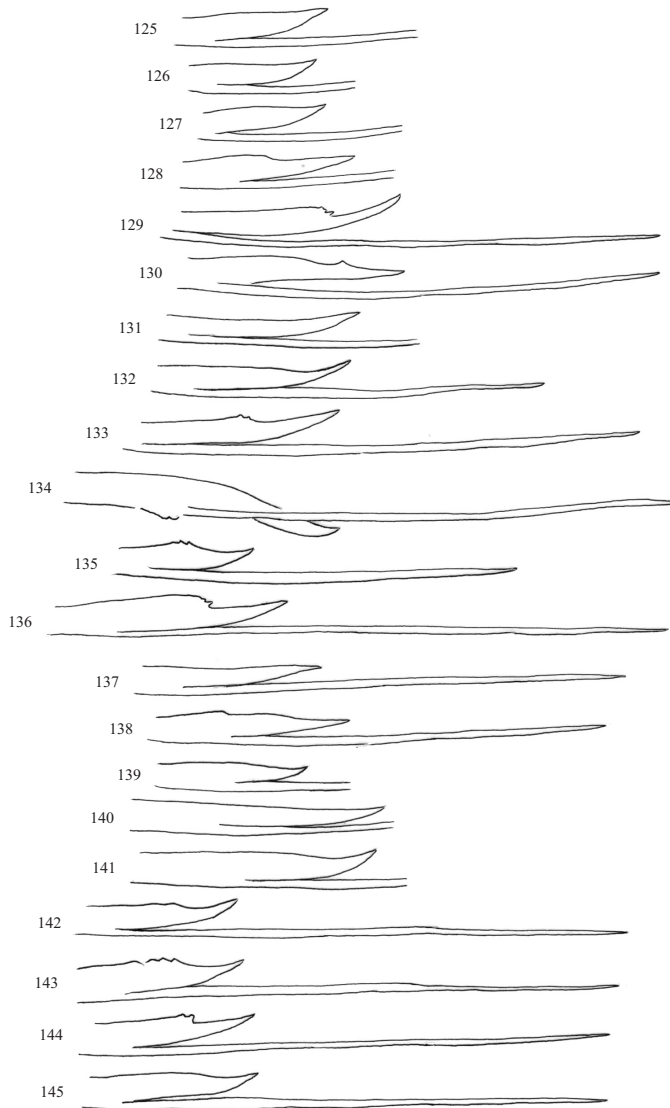**Figs 76–101.** *Drusus discolor* (Rambur, 1842) paraproct (superanal complex without cercus) in lateral view. **Czech Republic**: 76–87 = Kralicky Snéznik Mts, 88–93 = Krusné Hory Mts, 94–99 = Jizerské Hory Mts, 100–101 = Sumava Mts

M. Valle (5♂, 2♀; MCSNBG). **Kosovo**, Lepenc, 21.06.2012, H. Ibrahimi (1♂, 1♀, OPC). Prevall, 21.06.2012, H. Ibrahimi (1♂, OPC). **Poland**, Gorce Mts, Kamienice stream, 26.06.1985, light, J. Oláh (3♂, OPC). **Romania**, Lacul Roşu, Valea Cupas, 19.06.1981, light, L. Peregovits, L. Ronkay (1♂, OPC). Lacul Roşu, Valea Cupas, 09.07.1981, light, L. Peregovits, L. Ronkay (1♂, OPC). Lacul Roşu, Valea Cupas, 17.07.1981, light, L. Peregovits, L. Ronkay (1♂, OPC). Caraş-Severin county, Ţarcu Mts, Poina Mãrului, upper section of Sucu Stream, S of the village, N45°20.907', E22°31.073', 955 m, 08.06.2011, T. Kovács, D. Murányi, G. Puskás (2♂, HNHM). Bucegi Mts, Coteanu Padina, N45°22'35.33",
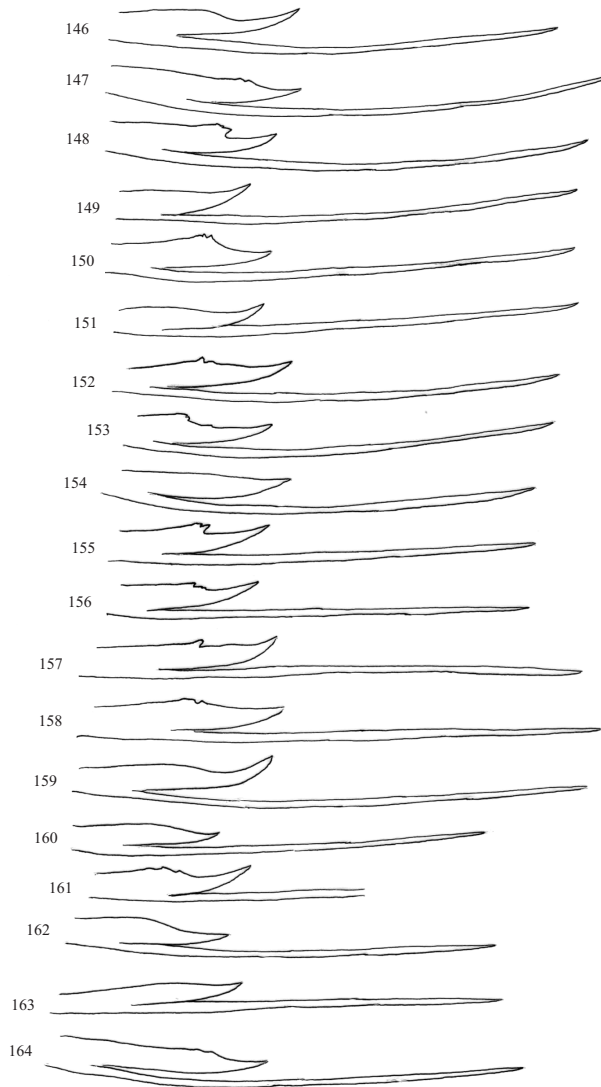
Figs 102–124. *Drusus discolor* (Rambur, 1842) left paramere sections in lateral view.
France: 102–103 = Vosges, 104–107 = Alpes Maritimes (contact population with *D. leker* sp. n), 108 = Savoie.
Italy: 109–110 = Briga Alta, 111–16 = Piemonte, Crissolo, 117–118 = Piemonte, Ceresole Reale,
119–124 = Valle d'Aosta, 1700 m

Figs 125–145. *Drusus discolor* (Rambur, 1842) left paramere sections in lateral view.
Italy: 125–28 = Valle d'Aosta, 1650 m, 129 = Mendatica, 130 = Madisimo, 131 = Ronobello, 132 = Vigilio, 133 = Valbondione, 1940 m, 134 = Valbondione, 1862 m, 135–136 = Fiume Po, 50 m, 137 = Telve, 138 = Claut, 139 = Sappado, 140–141 = Resia. **Slovenia**: 142–145 = Julian Alp.

E25°26'07.96", 1485 m, 29.06.2007, C. Ciubuc (2♂, CCPC). Bucegi Mts, Coteanu Padina, N45°22'35.33", E25°26'07.96", 1485 m, 06.07.2007, C. Ciubuc (1♂, CCPC). Bucegi Mts, Coteanu Padina, N45°22'35.33", E25°26'07.96", 1485 m, 13.07.2007, C. Ciubuc (1♂, CCPC). Bucegi Mts, Coteanu Padina, N45°22'35.33", E25°26'07.96", 1485 m, 03.08.2007,
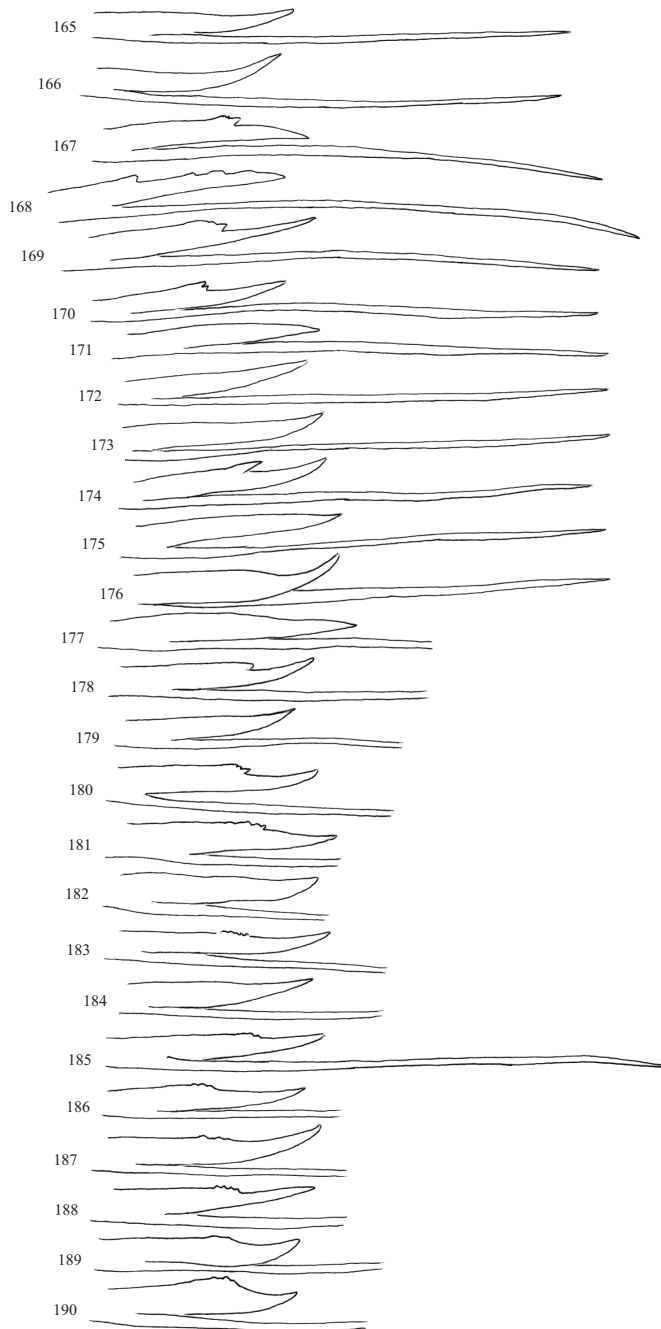
Figs 146–164. *Drusus discolor* (Rambur, 1842) left paramere sections in lateral view.
Slovakia: 146 = Pleso nad Skokem, 147 = Biela Voda, 148 = Mlynica, 149–151 = West Tatra, Jamycky Stream.
Poland: 152–153 = Gorce Mts, **Romania**: 154 = Lacu Rosu, 155–156 = Bucegi Mts, 157–158 = Fagaras Mts,
159 = Cibin Mts, 160 = Tarcu Mts, **Kosovo**: 161 = Lepenc, 162 = Prevall. **Albania**: 163–164 = Ostrovice Mts

C. Ciubuc (1♂, CCPC). Cindrel Mts, Râul Mic afl. stg. am Cabana Forestrierã, N45°40'26",
E23°49'15", 22-23.06.2009, C. Ciubuc (4♂,1♀, CCPC). Cindrel Mts, Râul Mic afl. stg. am
Cabana Forestrierã, N45°40'26", 23°49'15", 24-25.06.2009, C. Ciubuc (6♂, CCPC). Cindrel
Mts, Crãciuneasa, Râul Mare, N45°40'22", E23°51'53", 28-29.07.2009, C. Ciubuc (1♂,
CCPC). Cibin Mts, Crãciuneasa, Râul Mare, N45°40'22", E23°51'53", 29-30.07.2009,

Figs 165–190. *Drusus discolor* (Rambur, 1842) left paramere sections in lateral view.
Czech Republic: 165–176 = Kralicky Snéznik Mts,
177–182 = Krusné Hory Mts, 183–188 = Jizerské Hory Mts, 189–190 = Sumava Mts

C. Ciubuc (2♂, CCPC). Cindrel Mts, Crãciuneasa, Râul Mare, N45°40'22", E23°51'53", 07-08.06.2010, C. Ciubuc (4♂, CCPC). Cibin Mts, Crãciuneasa, Râul Mare, N45°40'22", E23°51'53", 08-09.06.2010, C. Ciubuc (107♂, CCPC). Cibin Mts, Crãciuneasa, Râul Mare, N45°40'22", E23°51'53", 09-10.06.2010, C. Ciubuc (226♂, 21♀, CCPC; 3♂, 2♀, OPC). Fãgãraş Mts, Sâmbãta de Sus, N45°40'06.5", E24°47'32.5", 12-13.07.2011, C. Ciubuc (10♂, CCPC; 2♂, OPC). Fãgãraş Mts, Sâmbãta de Sus, N45°40'06.5", E24°47'32.5", 13-14.07.2011, C. Ciubuc (2♂, CCPC). Fãgãraş Mts, Sâmbãta de Sus, N45°40'06.5", E24°47'32.5", 14-15.07.2011, C. Ciubuc (2♂, CCPC). Bucegi Mts, Lalomiţa stream, N45.416957°, E25.416958°, 1837 m, 15.07.2015, Z. Baczó, J. Kecskés (4♂, 7♀, OPC). Bucegi Mts, Lalomiţa stream, N45.402125°, E25.443147°, 1680 m, 16.07.2015, Z. Baczó, J. Kecskés (3♂, 12♀, OPC). **Slovakia**, Mlynica stream, 21.07.1966, J. Oláh (1♂, OPC). Pleso nad Skokom, 21.07.1966, J. Oláh (1♂, OPC). Biela voda, 22.07.1966, J. Oláh (1♂, OPC). West Tatra, Jamnicky stream, 12.07.1978, light trap, Nagy (4♂, OPC). **Slovenia**, Julian Alp, Vrsic Pass, Soca spring, 22.06.1988 (4♀, OPC). Julian Alp, Radovna stream, 23.06.1988 (15♂, 2♀, OPC).

Drusus ferdes sp. n. Oláh et Coppa (Figs 191–237)

Diagnosis – The divergence of this new incipient sibling species is realized in the speciation trait of the modified paraproct. The lateral profile of the paraproctal head is characterized by the slant serrated dorsoapical margin that is sloping downward obliquely from posterad to
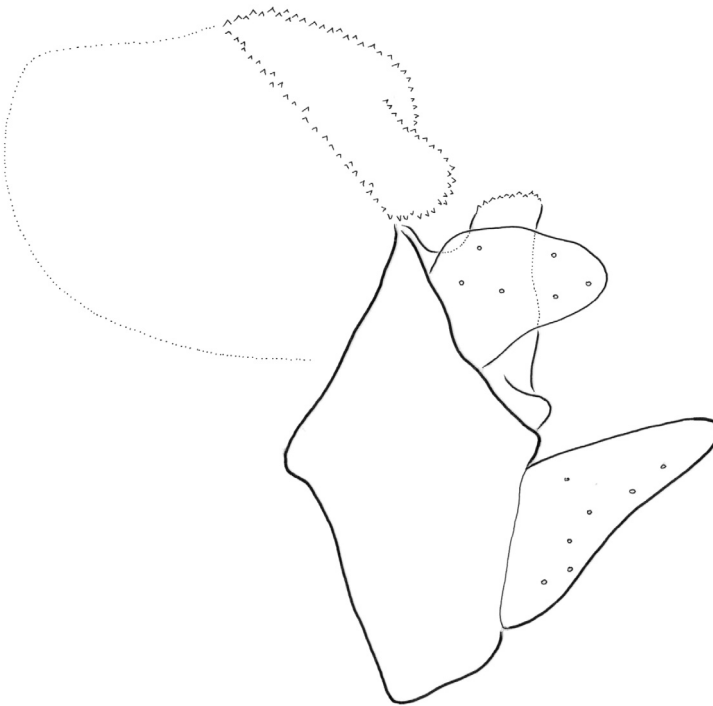

Fig. 191. *Drusus ferdes* sp. n. male holotype genitalia without phallic organ and with eight tergite profile in left lateral view

anterad. This divergence is stable in all of the examined population from Massif Central and from Alpes-de-Haute-Provence. It seems that the function of this modification on the serrated head of the paraproct works effectively alone or in combination with other premating barriers in mate recognition or in postmating prezygotic barriers of cryptic female choice or others, like gametic isolation. We have found no contact zone populations with hybrid effect.
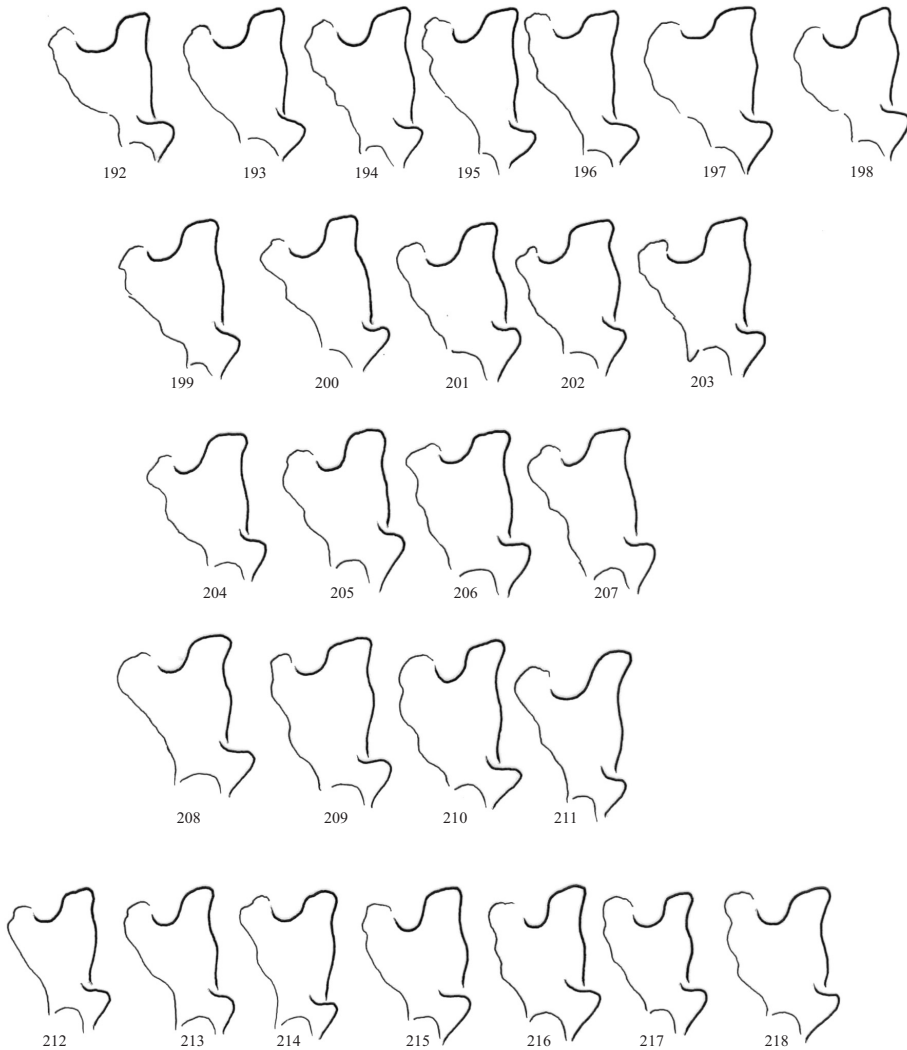
Figs 192–218. *Drusus ferdes* sp. n. paraproct (superanal complex without cercus) in lateral view.
France: 192–198 = Chambon sur Lac, Ru Derriere la Dent de la Rancune Ru Granit,
199–207 = Chambon sur Lac, Couze de Chaudefour Cascade Aval Reserve, 208–209 = Cantal, Brezons,
210–211 = Lozere, Meyrueis, 212–214 = Chastreix, 215–216 = Alpes de Haute Provence, Uvernet Fours,
217–218 = Alpes de Haute Provence, Larche
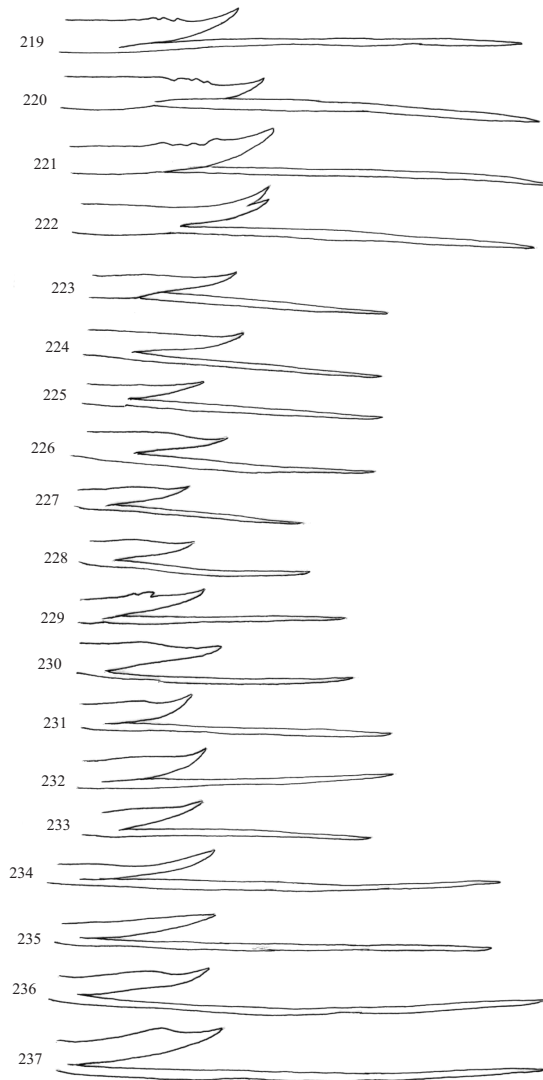
Figs 219–237. *Drusus ferdes* sp. n. left paramere sections in lateral view.
France: 119–222 = Chambon sur Lac, Ru Derriere la Dent de la Rancune Ru Granit,
223–226 = Chambon sur Lac, Couze de Chaudefour Cascade Aval Reserve, 227–228 = Cantal, Brezons,
229–230 = Lozere, Meyrueis, 231–233 = Chastreix, 234–235 = Alpes de Haute Provence, Uvernet Fours,
236–237 = Alpes de Haute Provence, Larche

The gonopod is rather slender relative to the ancestral species, *Drusus discolor*, but the range of variability is rather high compared to the selective speciation trait and very plane sensitive in drawing process.

The spur formation, the actual terminal ending of the paramere is developed into a slightly upward curving and narrowing pointed structure with few dorso-subapically produced uprising.

The variation within or between populations is almost the same, due to dominating stochastic processes including fluctuating asymmetry, as it was discussed in the theoretical part.

Material examined – Holotype. **France**, Department Puy-de-Dôme, Chambon-sur-Lac, near la Dent-de-la-Rancune, 23.06.2012, G. Coppa (1♂, CPC). Paratypes. Same as holotype (3♂, CPC; 3♂, OPC). Department Puy-de-Dôme, Chambon-sur-Lac, pont de Sainte-Anne, 21.07.2012, G. Coppa (1♀, OPC). Department Puy-de-Dôme, Chambon-sur-Lac, Couze de Chaudefour, waterfall Nature Reserve, 25.05.2009, G. Coppa (8♂, OPC). Department Puy-de-Dôme, Chastreix, Ru du Névé, 22.08.2015, G. Coppa (4♀, OPC). Department Puy-de-Dôme, Chastreix, Ru du Névé, 24.09.2015, G. Coppa (3♂, 1♀; OPC). Department Cantal, Brézons, sur le Brézons, Saut de la Truite, 27.06.2010, G. Coppa (2♂, OPC). Department Lozère, Meyrueis, Combe de Else, 22.06.2014, G. Coppa (2♂, 1♀, OPC). Department Alpes-de-Haute-Provence, Uvernet-Fours, Torrent Braissette, 25.08.2009, G. Coppa (2♂, 5♀, OPC). Department Alpes-de-Haute-Provence, Larche, Torrent Ornaye, 2380 m, 27.07.2008, G. Coppa (2♂, 1♀, OPC).

Etymology – *ferdes*, from "*ferde*" oblique or slant in Hungarian, refers to the direction, to the deviation from horizontal of the serrated dorsal margin of the paraproctal head.

Drusus kupos sp. n. Oláh et Coppa (Figs 238–248)

Diagnosis – The divergence of this new incipient sibling species is realized in the speciation trait of the modified paraproct. The lateral profile of the paraproctal head is characterized by narrowing conical dorsoapical margin. This divergence is stable in the single population


Fig. 238. *Drusus kupos* sp. n. male holotype genitalia without phallic organ and with eight tergite profile in left lateral view

examined. The spur formation, the actual terminal ending of the paramere is developed into a slightly upward curving and narrowing pointed structure. Some sign of dorso-subapically produced uprising present in a single paratype.

Material examined – Holotype. **France**, Department Pyrénées-Orientales, Err, Ru d'Err Aiguanein, 24.08.2011, G. Coppa (1♂, CPC). Paratypes. Same as holotype (2♂, CPC; 2♂, OPC).

Etymology – *kupos*, from "*kúpos*" conical in Hungarian, refers to the narrowing conical shape of the serrated dorsal margin of the paraproctal head.
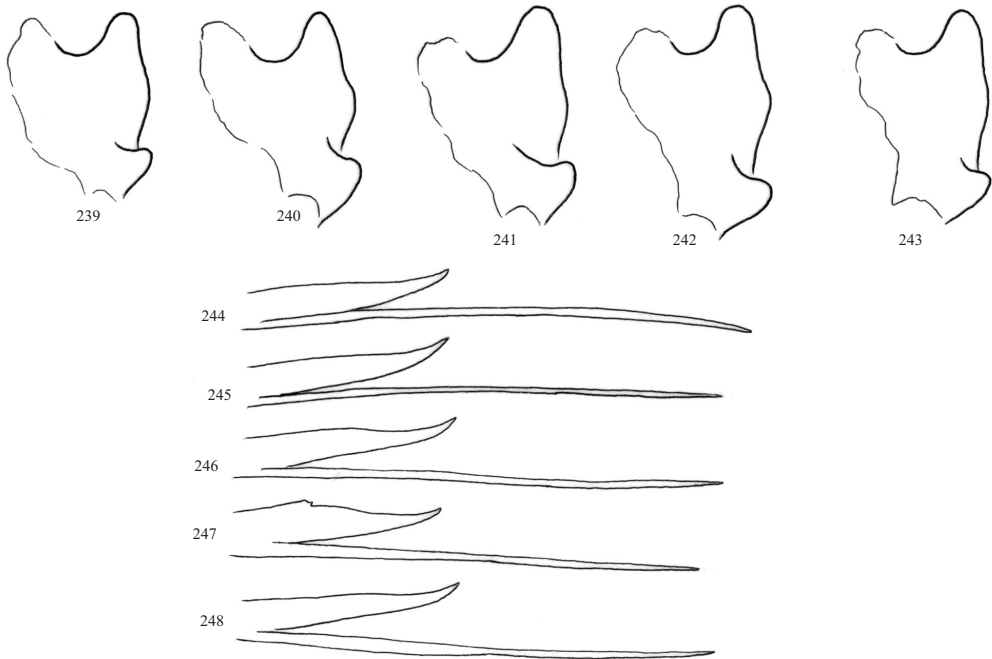
Figs 239–248. *Drusus kupos* sp. n. 239–243 = Paraproct (superanal complex without cercus) in lateral view. **France:** Department Pyrénées Orientales, Err. 244-248 = Left paramere sections in lateral view. **France:** Department Pyrénées Orientales, Err.

Drusus leker sp. n. Oláh (Figs 249–281)

Diagnosis – The divergence of this new incipient sibling species is realized in the speciation trait of the modified paraproct. The lateral profile of the paraproctal head is characterized by the rounded serrated dorsoapical margin. This divergence is stable. It seems that the function of this modification on the serrated head of the paraproct works effectively alone or in combination with other premating barriers in mate recognition or in postmating prezygotic barriers of cryptic female choice or in others, like in gametic isolation. We have found sign of contact zone populations with possible hybrid effect.

The spur formation, the actual terminal ending of the paramere is developed into a slightly upward curving and narrowing pointed structure with or without few dorso-subapically produced uprising.

Fig. 249. *Drusus leker* sp. n. male holotype genitalia without phallic organ and with eight tergite profile in left lateral view

Material examined – Holotype. **Italy**, Piemonte, Vinadio (CN), 2250 m, Col de la Lombarda lago, N44.20°, E7.14°, 18.08.1964, A. Viganò (1♂, MCSNBG). Paratypes. Same as holotype (2♂, 2♀, MCSNBG; 3♂, 2♀, OPC). Piemonte, Vinadio (CN), Santuario di S. Anna, 1880 m, N44.238°, E7.106°, 19.07.2012, G. Pezzi, I. Bendazzi (3♂, 1♀, MCSNBG; 2♂, OPC). **France**, Department Alpes-de-Haute-Provence, Saint-Paul-sur-Ubaye, Affluent Ubaye, 2000 m, 27.06.2008, G. Coppa (1♂, 3♀, OPC). Department Alpes-de-Haute-Provence, Uvernet-Fours, Sanguinerette Amont, 25.08.2009, G. Coppa (4♂, 2♀, OPC; contact population with *D. discolor*).

Etymology – *leker*, from "*lekerekitett*" rounded in Hungarian, refers to the rounded shape of the serrated dorsal margin of the paraproctal head.
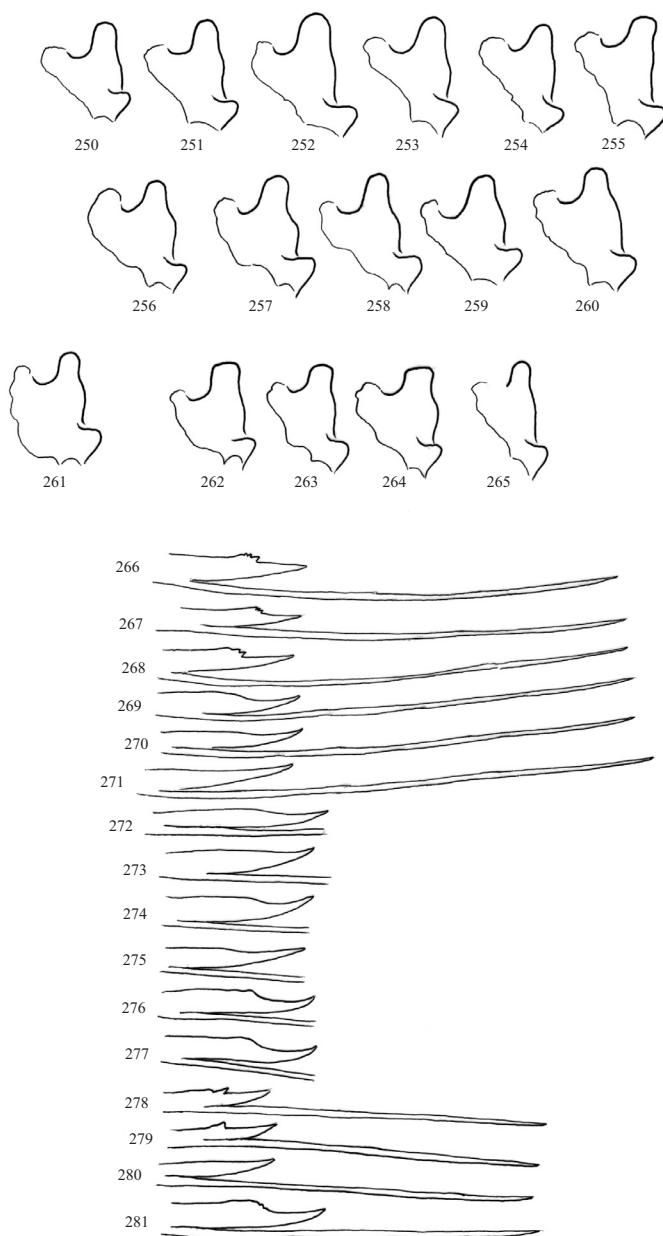
Figs 250–281. *Drusus leker* sp. n. 250-265 = Paraproct (superanal complex without cercus) in lateral view. **Italy:** 250–255 = Piemonte, Vinadio, 2250 m, 256-260 = Piemonte, Vinadio, 1880 m, **France:** 261 = Alpes de Haut Provence, Saint Paul sur Ubaye, 262–265 = Alpes de Haut Provence, Uvernet Fours (contact population with *D. discolor*). 266–281 = Left paramere sections in lateral view. **Italy:** 266–271 = Piemonte, Vinadio, 2250 m, 272–276 = Piemonte, Vinadio, 1880 m, **France:** 277 = Alpes de Haut Provence, Saint Paul sur Ubaye, 278–281 = Alpes de Haut Provence, Uvernet Fours (contact population with *D. discolor*)

***Drusus visas* sp. n. Oláh et Coppa (Figs 282–310)**

Diagnosis – The divergence of this new incipient sibling species is realized in the speciation trait of the modified paraproct. The lateral profile of the paraproctal head is characterized by backward, anterad directed anterior corner of the serrated head of the paraproct. This divergence is stable in the examined populations. It seems that the function of this modification on the serrated head of the paraproct works effectively alone or in combination with other premating barriers in mate recognition or in postmating prezygotic barriers of cryptic female choice or others, like gametic isolation. We have found no contact zone populations with hybrid effect.

The gonopod is rather slender relative to the ancestral species, *Drusus discolor*, but the range of variability is high compared to the selective speciation trait and very plane sensitive in drawing process.

The spur formation, the actual terminal ending of the paramere is developed into a slightly upward curving and narrowing pointed structure with or without few dorso-subapically produced uprising. The variation within or between populations is almost the same, due to dominating stochastic processes including fluctuating asymmetry, as it was discussed in the theoretical part.
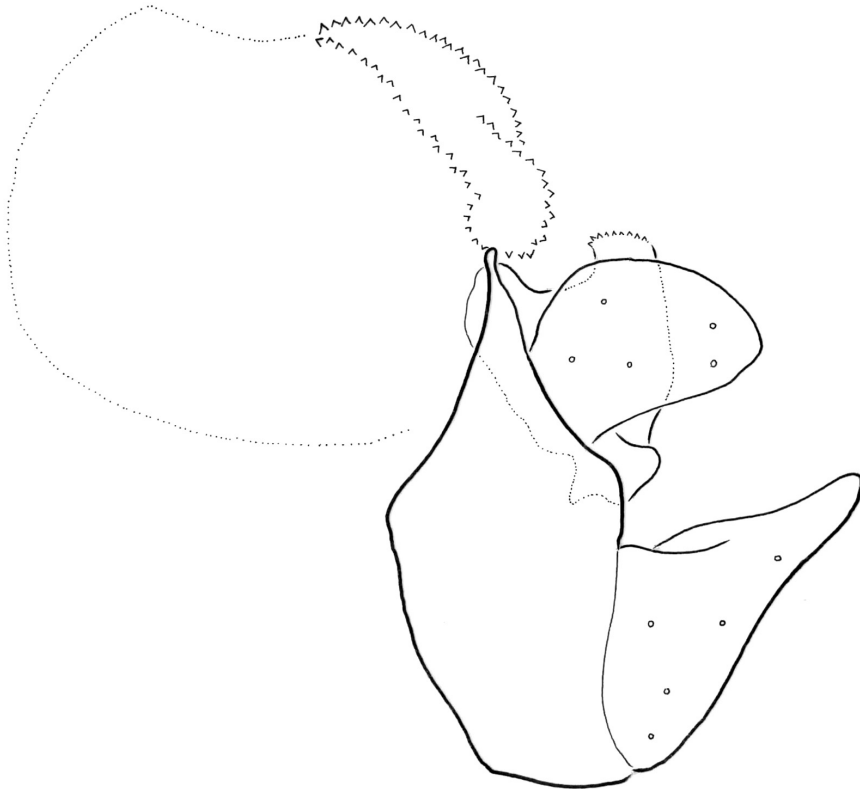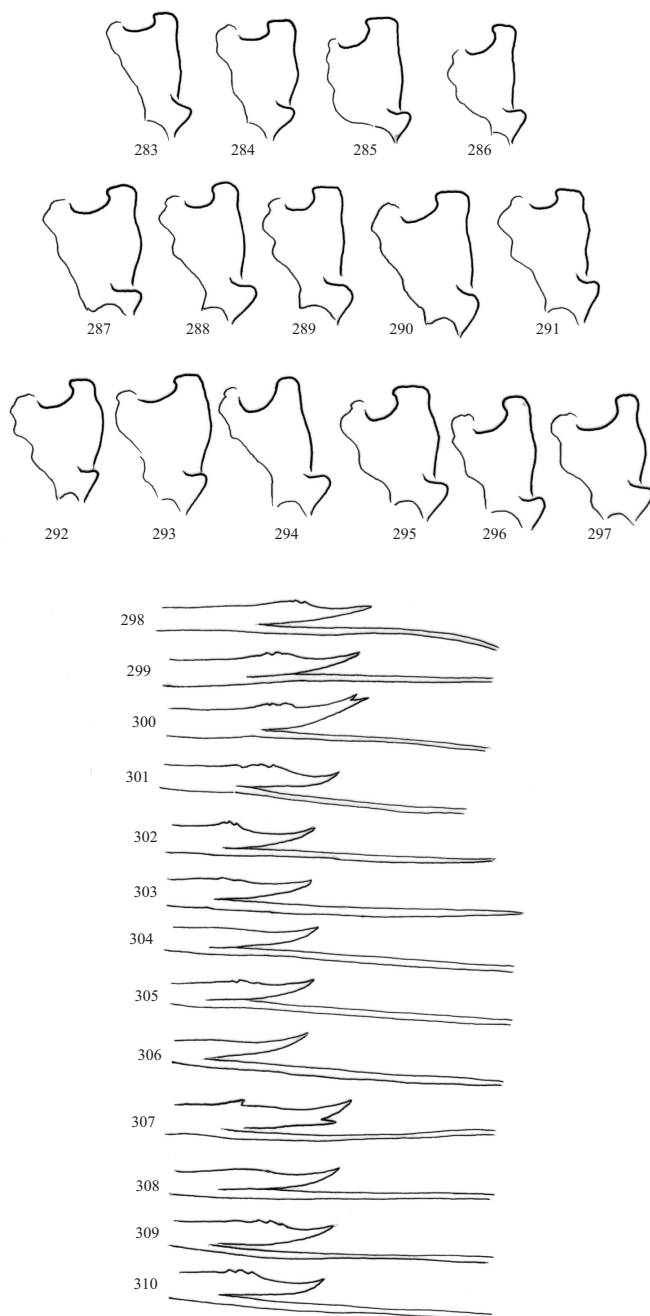

Fig. 282. *Drusus visas* sp. n. male holotype genitalia without phallic organ and with eight tergite profile in left lateral view

Figs 283–310. *Drusus visas* sp. n. 283–297 = Paraproct (superanal complex without cercus) in lateral view.
France: 283–286 = Department Pyrénées Orientales, Port Puymorens, 287–291 = Department Pyrénées Orientales, Cauterets, 292–297 = Haut Pyrenées, Gedre. 298–310 = Left paramere sections in lateral view.
298–301 = **France:** Department Pyrénées Orientales, Port Puymorens, 302-306 = Department Pyrénées Orientales, Cauterets, 307–310 = Haut Pyrenées, Gedre

Material examined – Holotype. **France**, Department Pyrénées-Orientales, Porte Puymorens, Ru de l'Orris, 21.08.2011, G. Coppa (1♂, OPC). Paratypes. Same as holotype (3♂, 4♀, OPC). Department Hautes-Pyrénées, Cauterets, Gave Cambasque, 16.07.2010, G. Coppa (5♂, OPC). Department Hautes-Pyrénées, Gèdre, Pont Sauge, Gave, 27.07.2010, G. Coppa (6♂, OPC).

Etymology – *visas*, from "*vissza*" back in Hungarian, refers to the backward, anterad directed anterior corner of the serrated dorsal margin of the paraproctal head.

References

BROWN, W. L. & WILSON, E. O. (1956): Character displacement. – Systematic Zoology, 5: 49–64.

BUTLIN, R., DEBELLE, A., KERTH, C., SNOOK R. R., BEUKEBOOM, L. W., CASTILLO CAJAS, R. F., DIAO, W., MAAN, M. E., PAOLUCCI, S., WEISSING, F. J., van de ZANDE, L., HOIKKALA, A., GEUVERINK, E., JENNINGS, J., KANKARE, M., KNOTT, K. E., TYUKMAEVA, V. I., ZOUMADAKIS, C., RITCHIE, M. G., BARKER, D., IMMONEN, E., KIRKPATRICK, M., NOOR, M., MACIAS GARCIA, C., SCHMITT, T. & SCHILTHUIZEN, M. (2012): What do we need to know about speciation? – Trends in Ecology and Evolution, 27(1): 27–39.

GAUSE, G. F. (1934): The struggle for Existence. – The Williams and Wilkins Company, Baltimore, 192 pp.

HARDIN, G. (1960): The competitive exclusion principle. – Science, 131(3409): 1292–1297.

KLINGENBER, C. P. (2015): Analysing fluctuating asymmetry with geometric morphometrics: concepts, methods, and applications. – Symmetry, 7: 843–934.

MURGOCI, A. & BOTOSANEANU, L. (1954): Contributii la studiul genului Drusus Steph. (Trichoptera) in R.P.R. – Buletin Stiintific Sectiunea de Stiinte Biologice Agronomice, Geologice si Geografice, 6(3): 967–979.

OLÁH, J., KOVÁCS, T., SIVEC, I., SZIVÁK, I. & URBANIČ, G. (2012): Seven new species in the *Chaetopteryx rugulosa* species group: applying the phylogenetic species concept and the sexual selection theory (Trichoptera: Limnephilidae) – Folia historico-naturalia Musei Matraensis, 36: 51–79.

OLÁH, J., ANDERSEN, T., CHVOJKA, P., COPPA, G., GRAF, W., IBRAHIMI, H., LODOVICI, O., PREVIŠIĆ, A. & VALLE, M. (2013): The Potamophylax nigricornis group (Trichoptera, Limnephilidae): resolution of phylogenetic species by fine structure analysis. – Opuscula zoologica, Budapest, 44(2): 167–200.

OLÁH, J., CHVOJKA, P., COPPA, G., GRAF, W., IBRAHIMI, H., LODOVICI, O., RUIZ GARCIA, A., SÁINZ-BARIÁIN, M., VALLE, M. & ZAMORA-MUÑOZ, C. (2014): The genus Allogamus Schmid, 1955 (Trichoptera, Limnephilidae): revised by sexual selection-driven adaptive, non-neutral traits of the phallic organ. – Opuscula zoologica, Budapest, 45(1): 33–82.

OLÁH, J., CHVOJKA, P., COPPA, G., GODUNKO, R. J., LODOVICI, O., MAJECKA, K., MAJECKI, J., SZCZĘSNY, B., URBANIČ, G. & VALLE, M. (2015): Limnephilid taxa revised by speciation traits: Rhadicoleptus, Isogamus, Melampophylax genera, Chaetopteryx rugulosa, Psilopteryx psorosa species groups, Drusus bolivari, Annitella kosciuszkii species complexes (Trichoptera: Limnephilidae). – Opuscula zoologica, Budapest, 46(1): 3–117.

PAULS, S. (2004): Phylogeny and phylogeography of the montane caddisfly Drusus discolor (Rambur, 1842) (Trichoptera: Limnephilidae, Drusinae). – PhD-Thesis, University of Duisburg-Essen, 164 pp.

PAULS, S. U., LUMBSCH, H. T. & HAASE, P. (2006): Phylogeography of the montane caddisfly Drusus discolor: evidence for multiple refugia and preglacial survival. – Molecular Ecology, 15: 2153–2169.

PAULS, S. THEISSINGER, U., K., UJVAROSI, L., BALINT, M. & HAASE, P. (2009): Patterns of population structure in two closely related, partially sympatric caddisflies in Eastern Europe: historic introgression, limited dispersal, and cryptic diversity. – Journal of the North American Benthological Society, 28(3): 517–536.

PFENNIG, K. S. & PFENNIG, D. W. (2009): Character displacement: ecological and reproductive responses to a common evolutionary problem. – The Quarterly Review of Biology, 84(3): 253–276.

PFENNIG, A. S. & RICE, A. M. (2014): Reinforcement generates reproductive isolation between neighbouring conspecific populations of spadefoot toads. – Proceedings of the Royal Society B, 281: 20140949.

Rice, A. M. & Pfennig, D. W. (2010): Does character displacement initiate speciation? Evidence of reduced gene flow between populations experiencing divergent selection. – Journal of Evolutionary Biology, 23: 854–865.

Sætre, G.-P. & Sæther, S. A. (2010): Ecology and genetics of speciation in Ficedula flycatchers. – Molecular Ecology, 19: 1091–1106.

Schmid, F. (1956): La Sous-Famille des Drusinae (Trichoptera, Limnephilidae). – Mémoires Institut royal des Sciences naturelles de Belgique, deuxieme serie, 55: 1–92+18 plates.

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