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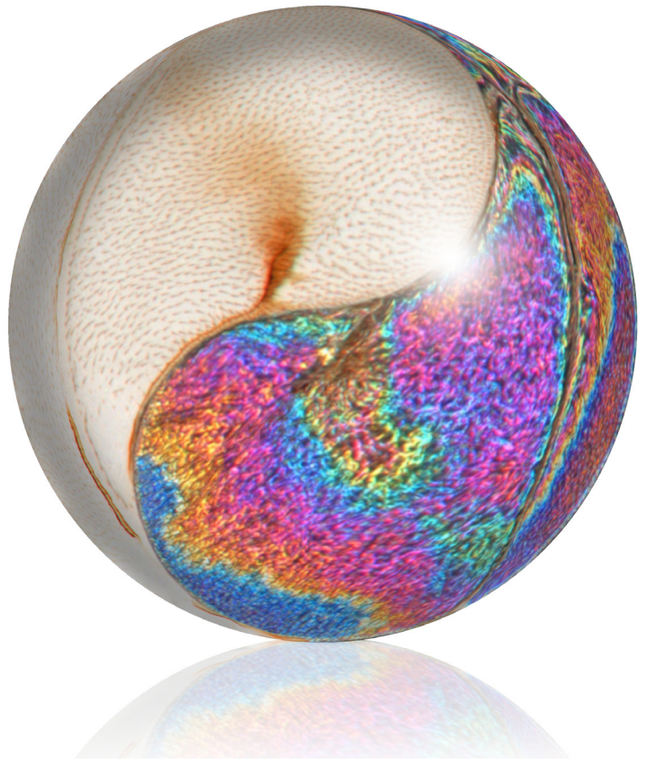

Ekaterina Shevtsova

Department of Biology
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Ekaterina Shevtsova

Doctoral Thesis

LUND UNIVERSITY
Faculty of Science

Department of Biology
Lund, February 2012

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Abstract

The remarkably thin transparent wing membranes in tiny wasps may appear to have a simple structural design, but hide a largely unexplored complex of micro-morphological features that serve aerodynamics and may also function in visual signaling. I found that when such small transparent wings are viewed against a dark background they display vivid structural color patterns due to thin film interference, and named them Wing Interference Patterns (WIPs). Areas of different thickness across the wing membrane reflect specific interference colors and all together produce a specific color pattern, offering a new way to map the wing micro-morphology through direct observations. The color sequence is very characteristic and lacks pure red but may contain UV light. Hence, it fits the UV-blue-green trichromatic color vision of most small insects, strongly suggesting that the biological significance of WIPs lies in visual signaling. WIPs are optically stabilized by corrugations in the wing membrane and are essentially noniridescent over a large range of light incidences. These patterns show a high diversity in small Hymenoptera and are often species-specific, which makes this new morphological character useful in taxonomy. Several sympatric species of parasitic wasps were found to display sexually dimorphic WIPs, suggesting sexual selection as one of the driving forces for their evolution. The significance of wing membrane micro-morphology and the origin of the specific color sequence observed in WIPs are discussed, using *Achrysocharoides* and *Omphale* as model taxa. Several new findings are reported in addition to those in my five publications. A comprehensive study of the wing cuticle ultra-structure, based on analyses of wing membrane cross-sections by transmission electron microscopy, revealed asymmetrical organization of the dorsal and ventral cuticles. Presence of ultraviolet light reflections in WIPs is indirectly demonstrated through fluorescence microscopy, further strengthening the signaling function of WIPs.

Key words

Chalcidoidea, parasitic wasps, structural colors, visual signalling, cryptic species, sexual dimorphism, wing membrane thickness, wing micro morphology, wing cuticle ultrastructure

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Cover image: Forewing of *Neochrysocharis formosa* (Hymenoptera: Eulophidae) is seen as transparent against a white, and as colorful against a black background. *Seemingly contrary forces are interconnected and interdependent in the natural world, and they give rise to each other in turn. Opposites thus only exist in relation to each other and interact within a greater whole, as part of a dynamic system.* This description of the Yin & Yang concept taken from Wikipedia can also raise a philosophical view on the evolutionary interplay between the transparent and colorful dualities of the wings of small insect. Their wing membranes are as thin as a soap bubble and yet strong and flexible to provide the insect with efficient flight. In this duality between fragile daintiness and functional strength enfolds a sphere that embraces the invisibility and display in a new evolutionary perspective.

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"*You have formed a theory, then*?"

"*At least I have got a grip of the essential facts of the case. I shall enumerate them to you, for nothing clears up a case so much as stating it to another person, and I can hardly expect your co-operation if I do not show you the position from which we start.*"

Watson and Holmes, in "Silver Blaze"

The Memoirs of Sherlock Holmes (1894)

Contents

List of publications

Paper I Shevtsova, E., Hansson, C., Janzen, D.H. and Kjaerandsen, J. (2011) Stable structural color patterns displayed on transparent insect wings. – *Proceedings of the National Academy of Sciences of the United States of America*, 108 (2): 668-673

Paper II Hansson, C. and Shevtsova, E. (2010) Three new species of *Achrysocharoides* Girault (Hymenoptera: Eulophidae) parasitoids of *Phyllonorycter* spp. (Lepidoptera: Gracillariidae) on *Acer platanoides* and *Robinia pseudoacacia*. – *ZooTaxa*, 2388: 23-43

Paper III Shevtsova, E. and Hansson, C. (2011) Species recognition through wing interference patterns (WIPs) in *Achrysocharoides* Girault (Hymenoptera: Eulophidae) including two new species. – *ZooKeys*, 154: 9–30

Paper IV Hansson, C., Shevtsova, E. and Godfray, H.C.J. Do Wing Interference Patterns (WIPs) in Minute Parasitoid Wasps Show Reproductive Character Displacement? *Manuscript submitted to the Journal of Evolutionary Biology*

Paper V Hansson, C. and Shevtsova, E. Evolution of features in two newly introduced character sets for European *Omphale* Haliday (Hymenoptera: Chalcidoidea: Eulophidae: Entedoninae). *Manuscript*

Contributions

Paper I E.S. discovered WIPs and conceived the idea. E.S., C.H. and J.K. designed research and investigated WIP diversity across Hymenoptera (E.S. and C.H.) and Diptera (J.K.). E.S. performed the micromorphological study of the wing membranes in parasitic wasps. J.K. contributed with the Newton series scale metering as a new tool to measure the wing thickness. E.S., C.H., D.H.J. and J.K. performed research and analyzed data. E.S., C.H., D.H.J. and J.K. wrote the paper.

Paper II C.H. conceived the idea and acquired the material. E.S. found WIPs with distinct blue spot in male specimens which were described as a new species *A. platanoidae*. C.H. and E.S. analyzed and illustrated external morphology of all species. E.S. documented and analyzed WIPs of four cryptic species discussed. C.H. wrote the paper.

Paper III E.S. came up with the idea and discovered two new species from North America. E.S. and C.H. designed research. E.S. documented and analyzed intraspecific variation in WIPs of all species discussed. E.S. contributed with new method for visualization of uneven thickness of the wing membrane in SEM. C.H. wrote the species descriptions and identification keys. E.S. and C.H. wrote the paper.

Paper IV C.H. formulated the hypothesis and wrote the initial manuscript. H.C.J.G. designed and performed the statistical analysis. The manuscript was then adapted to include the findings of the analysis, and all authors contributed. E.S. and C.H. both furnished the illustrations.

Paper V E.S. documented and analyzed WIPs, made slide preparations and photos of male genitalia, and discovered the merged apices of the aedeagal apodemes in *versicolor* species group. C.H. came up with the ideas, designed and performed the phylogenetic analysis and wrote the manuscript. E.S. gave valuable input to the manuscript. E.S. and C.H. both collected and processed the large material of Omphale, and both also contributed with illustrations.

1. Introduction to Wing Interference Patterns (WIPs)

1.1. Transparent wings and their secret identity in Hymenoptera

We used to look, but now we can see.

The knowledge about insect biology, morphology and structural organization accumulated for the last two centuries is immense. Indeed, it might seem that there is nothing left to pioneer in this field ... unless of course it has been very well hidden. The majority of species in Hymenoptera and Diptera are small to minute with much simpler organized wings compare to those of Lepidoptera which are covered with thousands of scales to produce stunning color patterns. The wings of a wasp or a fly are transparent airfoils with venation patterns of various designs and are often covered with tiny hairs. Some wings display characteristic pigment patterns that can vary from modest to elaborate. This generalized picture of a small insect wing has been changed only recently. The transparent wings of small wasps and flies were discovered to display a variety of stable and taxon specific structural color patterns, which are now no longer a privilege of moth and butterfly wings (Paper I). The addition of a new colorful morphological dimension also revealed unexplored signaling channels in insect wings.

The seemingly transparent and remarkably thin wings of small insects display vivid structural color patterns due to Thin Film Interference (TFI) when viewed against dark backgrounds. We named them WIPs and some of us also like to call the type of wings that display WIPs – iWing, where "i" reflects Intelligent Insect Interference. WIPs are stable from various angles of view and show striking diversity across smaller Hymenoptera (Fig.1) and Diptera.

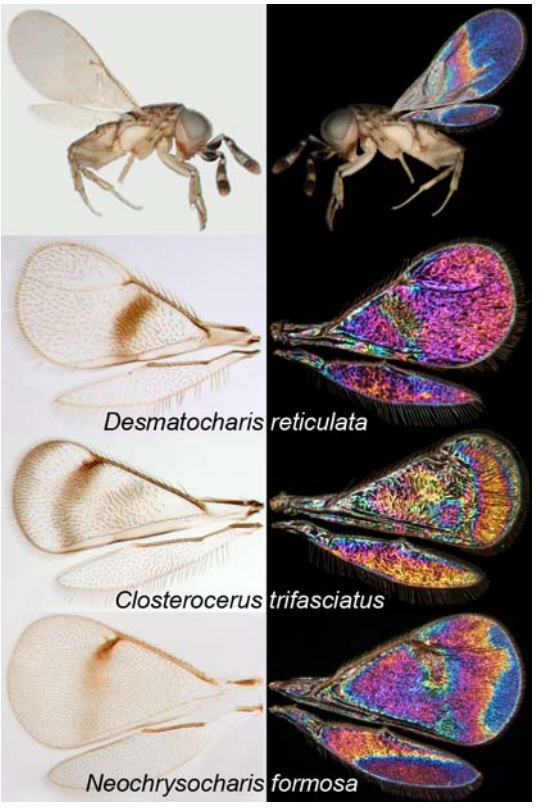

Fig.1 Composite images of tiny parasitic wasp *Metaphycus sp.* (Encyrtidae) and wings of three Eulophidae species (wings removed from the specimens) illustrate the dramatic changes in the visual appearance of the wings as an effect of switching the background from white to black. The left side wings display patterns of pigmentation and may appear to have a simple structural design due to their transparency. The right side wings display elaborate structural color patterns, WIPs, which emphasize a largely unexplored complex of micro-morphological features of the wing membrane. The photos of each species are of the same wings viewed against white and black backgrounds.

WIPs display a very characteristic color palette due to TFI, which reminds of the oil slicks on water or soap bubble iridescence coloration. The unstable thin film of soapy water constantly changes its thickness while the thickness in the membranous wings is consistent. In addition they possess microstructures that provide optical stabilization to WIPs and eliminate the iridescence effect over a large range of light incidences (Paper I). The stability of WIPs indicates their possible role in visual signaling and also makes them a reliable morphological character in taxonomy. Elaborate wing pigment patterns are common in Diptera where they contribute as frames to admirable WIPs (Paper I).

Not all transparent wings display WIPs. Wings of some large insects are too thick to produce bright TFI colors, e.g. 1-6 µm thick wings in locusts and dragonflies (Combes, 2010; Hooper et al., 2006). For instance, transparent cicada wings have special antireflective structures on the surface to eliminate color reflections (Stoddart, 2006). The iWings, on the other hand, are extremely thin and their thickness usually range between 100 and 600nm (Paper I). Thus they meet a thin film thickness comparable to the wavelengths of light required for TFI (Chapman, 1998). In Chalcidoidea (Hymenoptera) the wing venation is greatly reduced and veins are confined to the anterior wing margin, leaving the wing membrane as a seemingly large empty space. Coupled with lack of pigment patterns in most species such wings have been regarded as non-informative neutral entities (Paper III). And yet they are capable of transformation into rich colorful posters when viewed against light absorbing backgrounds. Due to the structural origin of colors in WIPs they do not fade with time as pigmentation but keep the same brightness and color distribution as we have observed in hundred years old museum specimens (Paper I). To obtain data about WIP distribution, diversity and stability a wide survey of WIPs was carried across major chalcid families (Agaonidae, Aphelinidae, Encyrtidae, Mymaridae, Pteromalidae, Torymidae, Trichogrammatidae) with main focus on the Eulophidae, and with a glance into WIPs of Braconidae and Ichneumonidae (Ichneumonoidea). A fascinating world of widespread colorful WIPs with high interspecific and low intraspecific variation was revealed (Paper I). WIPs are often taxon specific and have already proved useful in recognition of cryptic species (Papers I, II, and III) and on genus level classification (Hansson, 2011).

1.2. To see or not to see: how to make WIPs appear

How it comes that WIPs are not illustrated in entomological textbooks? Why are they not used as a routine character in taxonomy? The structural duality of iWings might explain how they managed to keep this colorful secret from us for so long (Fig. 2). The iWings change appearance reversibly from colorless to colorful depending on the optical properties of the background or illumination conditions. The texture of wings is so fine that reflections from below, e.g. from a white background that largely reflects the incident light, illuminate the wing and overwhelm the wings own reflectance. Similar to the bright field microscopy in this condition the wing

appears transparent and shows the venation and pigmentation patterns, and arrangement of setae. Since these morphological features have been the desired subjects of scientific interest tiny insects with transparent wings used to be observed on a white paper card or in a white dish filled with alcohol.

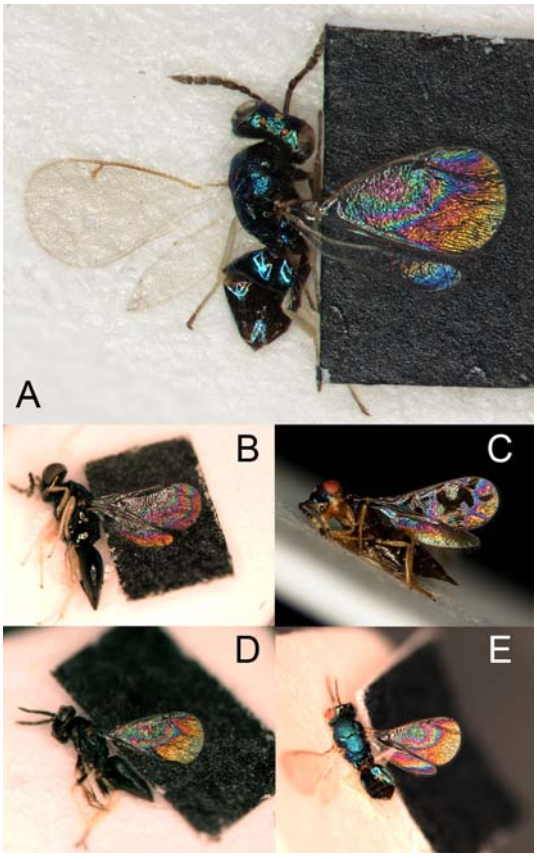

Fig.2 Rough "working" images of WIPs observed on intact specimens glued on white paper cards. The specimens in entomological collections are mounted like this, or pinned through the thorax. In the latter case the wings are left freely arranged on both sides (Paper III). Here I demonstrate that observation of WIPs does not require the removal of wings and it is very easily done. Valuable specimens, e.g. type material, can be examined and WIPs documented. With a small piece of black paper placed under the wing (A, B, D, E), or with specimen arranged against a remote dark background (C) wings appear structurally colored and reveal hidden morphological information. The contrast between transparent wings and WIPs is remarkable. Indeed, nothing is dull in the insect world. (A, D) *Chrysocharis pubicornis* (B) *Horismenus opsiphanis (*C) *Astichus arithmeticus,* (E) *Chrysocharis pentheus.* (Hymenoptera: Eulophidae)

Optically absorbing substrates enhance the saturation of structurally colored systems (Vukusic et al., 2004). A black paper absorbs unscattered light transmitted through the wing (80%) and eliminates reflections from below and the wavelengths reflected by the wing (20%) become visible (Paper I). Similar to dark field microscopy in this condition the wing appears colorful and shows its bright WIP surrounded by the black field. Dry wings can switch from transparent to structural mode and back, but when the wing is embedded in an oil-based medium in e.g. slide preparations – all structural colors disappear (Paper I). In a sense it is unfortunate as museum collections of small Hymenoptera, e.g. Mymaridae or Trichogrammatidae, largely consist of slide mounted specimens including their wings. The minute species often express a deficit of characters in the external morphology that can be used in taxonomy. WIPs as a new character system is an invaluable source of additional information for such species and the practice of mounting the entire insect on slide, at least type specimens, should be argued against. Dry specimens are often pinned or glued on one side with freely arranged wings. Thus such type material has potential for reexamination and WIPs can be added as a reference to aid in species identification.

2. Are there preconditions to WIPs?

2.1. Wing micromorphology and optical stabilization of WIPs

To demonstrate the diversity of insect wing morphology, i.e. venation and pigment patterns, two-dimensional photos of flattened wings against a white background are used. Structural colors and spatial dimensions of wings become invisible. Such photos can be misleading, as they suggest that insect wings are flat and rigid; but in reality, wings are three-dimensional structures that change shape dynamically during flight (Combes, 2010). The shape changes of the wing are largely passive, determined by the aerodynamic and inertial forces associated with flapping flight. Flexibility and controlled deformations are both beneficial and necessary for many aspects of wing functioning (Combes, 2010). SEM micrographs may give some ideas about dimensions of wings in tiny parasitic wasps (Fig. 3). The wings are not planar, as one could imagine, but their membrane is wrinkled or corrugated with crests and troughs that serve aerodynamics and perhaps also play a role in signaling (Paper I). Even in very small insects the forewings has an arched profile which increases its rigidity to bending forces from below (Wootton, 1992). The wings in larger insects also possess corrugations but mainly on a larger scale in association with veins forming the ridges. Such a corrugated wing profile has all the advantages of low mass, high stiffness and low membrane stresses in bending (Rees, 1975). The micro corrugations in small wings lacking venation likely provide similar benefits, increasing membrane stiffness in particular.

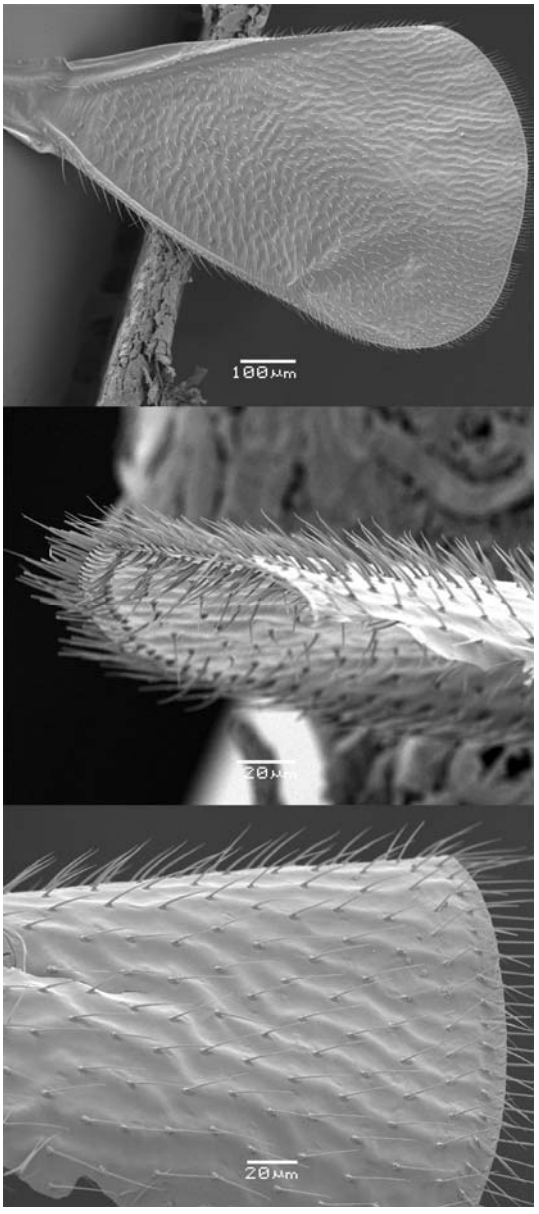

Fig.3 SEM micrographs of the wing surface of metal coated forewings of different eulophids. Despite the size of the whole wing, compatible to a single wing cell in e.g. a damselfly, the wings of parasitic wasps show a complex micro

morphological organization. The wing membranes are highly corrugated or wrinkled with the series of convex ridges and concave valleys that are reversed when seen on the opposite side of the wing. The wing membranes are also covered with rows of setae confined to the top of the ridges on both sides of the wing. The venation in chalcids is greatly reduced, leaving a thin wing membrane without a framework of supporting veins. Instead the stiffness of the wing membrane is due to dense micro corrugations.

WIPs can be used to visualize the complex micro-morphology of wings. The color reflections are confined to the convex ridges of corrugations which form series of color stripes emphasizing the topography of the wing (Fig. 4). The arrangement of corrugations plays a structural role. Akin to a thin paper with series of longitudinal folds that remains flexible longitudinally, but becomes resistant to transverse bending. In chalcidoid wasps the wing is corrugated in two nearly perpendicular directions: anterior-posterior in the basal part and proximal-distal in the apical part. In this way it becomes rigid and resistant to forces from opposite directions. The thick vein along the fore margin in the basal part of the wing prevents transverse bending of the wing while corrugations prevent longitudinal bending along the wing axis. This makes the basal part of the wing stable to withstand the inertial forces in flight. The apical part of the wing lacks veins and therefore the corrugations are arranged to prevent transverse bending, thus leaving the membrane flexible to longitudinal bending which occurs reversibly during flapping flight. In most chalcid wings the entire wing surface is corrugated (Figs. 3, 4 A-C) and shows a wing topography akin to desert dune fields. In some chalcid wings the basal part of the membrane is thick and lacks corrugations (Paper I). It also displays a camber with its upper surface convex (Fig. 4, D). Such wings are corrugated only in the thin apical part.

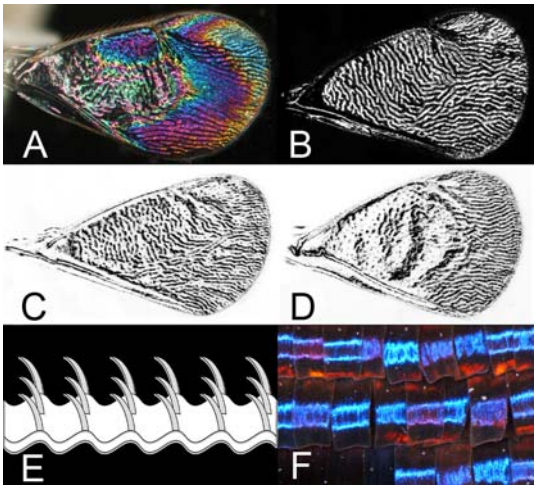

Fig.4 (A) Achrysocharoides sp. forewing. WIPs emphasize the wing membrane micro-morphology through the color reflections that are confined to the top of the corrugation ridges and thus produce alternation of thin colored and black stripes. (B-D) Duotone composite images of metal-coated wings prepared for SEM. The complex topography of wings is clearly visible and these images represent a “wing print” in analogy with a fingerprint. (B, C) Forewing of Chrysocharis spp. The arrangement of corrugations changes gradually from anterior-posterior in the basal part of the wing to radial in the apical part providing resistance to longitudinal and transverse bending of the wing respectively. (D) Forewing of Achrysocharoides latreilleii, female shows another topographic plan. The wing membrane is smooth and thick in the basal part of the wing and also slightly cambered. The apical part of the wing membrane possesses extensive radial corrugations as in B-C. (E, F) Courtesy of Jostein Kjaerandsen (E) Due to corrugations the wing profile in a cross-section is not a straight but a wavy line with alternation of crests and troughs. (F) Wing scales of day active moth Chrysiridia croesus, Uraniidae. These scales are bent unlike the flat iridescence scales of butterflies like Morpho. The urania wing scales arranged in rows form convex ridges with structural color reflections on top. Underneath the structural scales there is a layer of flattened pigmented scales that form the dark background. Thus the overall wing pattern consists of color stripes with dark areas in between, similar to WIPs.

The colorful pattern in the wings of a diurnal moth (Fig. 4, F) may seem homogeneous from a distance but a closer look reveals that they are also composed of series of color stripes. Similar to the corrugated iWing, the color reflections in moth wings are confined to the top of curved wing scales. These structural scales form an upper layer on the wing surface covered below with additional layer of dark and flattened pigmented scales. The TFI color reflections from the upper scales are thus reinforced and their spectral purity is enhanced by the background that absorbs transmitted light (Vukusic et al., 2004; Chapman, 1998). A principally similar optical system that incorporate a dark background, but implemented differently, is present in the iridescence hind wings of the damselfly *Neurobasis chinensis chinensis* (Vukusic et al., 2004). The dorsal wing surface reflects bright green color at normal incidence, while the ventral surface is dark brown due to presence of pigments, assumed to be melanin. The cuticle of the wing shows distinct laminations. The layers on the ventral side form a highly absorbing background which is the reason for the spectrally pure color of a high contrast reflected from the dorsal layers as a result of optical interference. The damselfly and moth wings represent more complex optical effects compared with iWings. However WIPs are displayed only when the same optical requirements are met. The difference is that iWings lack a permanent dark background produced by their own membrane. Thus WIPs are not an obligate, but optional property of the wings and can be switched on/off depending on the current surroundings chosen by their bearers.

Structures that reflect light of a certain wavelength due to TFI are thin and transparent. In wasp and moth wings they also express curvature. In case of planar thin films the viewing from a more oblique angle is equivalent to increasing the distance between upper and lower surfaces. Hence the light beams travel longer path inside the film that affects the wavelength of reflected light (Chapman, 1998). Thus the color changes together with the angle of view which appears as a play of colors on the surface, i.e. an iridescence effect. Contrary to this, in WIPs the convex ridges of a corrugated wing membrane reflect TFI colors from the top, similar to curved butterfly scales. When such a wing is tilted these “tops” move along the ridge and show the same interference color. That is because they keep nearly horizontal at each point and maintain the same distance for the light beams that travel inside the wing. Thus the corrugations of the wing membrane eliminate the iridescence effect and provide optical stabilization to WIPs (Paper I). Therefore when the iWings are tilted they show the same colors as in horizontally arranged wings.

The wing structure is partly optimized as a compromise between two requirements, maximum stiffness in flight and minimum weight to optimize energy costs. The thinner the wing membrane the better, as the wing becomes lightweight, but how thin can it get? Simultaneously the wing has to be stiff enough to be aerodynamically efficient when it is under inertial or aerodynamic load (Rees, 1975). The mechanical strength of a wing stands in direct relation to its thickness, the thicker the wing the stiffer it gets, but the wing mass cannot exceed the optimum for the operating muscles. The solution is brilliant - a thinning of the wing is compensated by the membrane corrugations that indirectly increase its stiffness. Thus the wing membrane may become significantly thinner when it is corrugated, compared to a flat airfoil, and

more aerodynamically efficient when in possession of cambers. Such a construction of the wing provides small insects with very light and very thin but yet strong airfoils optimized for flight. Additionally, wings acquire sparkling TFI colors when they become thinner than 1 µm.

Does it mean that stable colorful WIPs are an optical side effect of very thin and corrugated wing membranes? To avoid the interference colors specific antireflective structures or wax coatings on the wing surface could have evolved, but no such are present in the iWings. So if you got it and don't mind it, why not using it? For instance lepidopterans evolved highly organized micro scales covering the wings and providing color patterns used in mating and territorial signaling (Meyer-Rochow, 1991; Allen et al., 2011) and predator avoidance (Oliver et al., 2009). The visual signaling enables conspecific and interspecific communication and is an integral part of insect evolution. In small wasps the same abilities are potentially given at low cost, no extra structures are produced, basically as an advantage of the small size and optimized wing thickness. Therefore the iWings may be one possible explanation for the evolutionary success of small winged insects. There are more indications for the biological significance of WIPs in visual signaling. So far four species with sexually dimorphic WIPs have been found as well as distinct and species specific WIPs in sympatric species in the genus *Achrysocharoides* (Paper III) and also displays of transparent wing have been encountered in fig wasps and microgastrine braconid wasps (Paper I) and *Pteromalus cassotis* (Chalcidoidea: Pteromalidae) (Part 4.1).

2.2. Two-beam Thin Film Interference hypothesis

The surfaces in transparent materials which lack pigments may appear colored due to their structural organization. When the material is thin enough most of the incoming light is transmitted, but certain wavelengths are refracted, reflected and superposed by the upper and lower surfaces of the thin film. The reflected waves that have traveled different paths interfere and may reinforce each other so that a stronger reflection of this particular wavelength occurs. Thus the optical properties of the surface depend on its physical structure. This consistency enables modeling and reciprocal calculations of the structures and corresponding colors (Fig. 5). The light beams reflected from a single or a series of superimposed thin transparent layers produce different interference colors (Zawischa, web). TFI occurs when the distances between layers and also their thickness are comparable with the wavelengths of light (Chapman, 1998). The TFI colors are displayed in a definite sequence (known as Newton's series) in correlation with the number of layers and also depend on the refractive index of the material forming the layers. In a single thin film the interference occurs between two beams reflecting from the upper and the lower surfaces and is therefore called two-beam TFI (Fig. 5, A). Structurally colored lepidopteran scales comprise layers of cuticle and air (Vukusic et al., 2004), thus interference occurs between more than two light waves reflected from the subsequent layers and is called multiple-beam TFI (Fig.

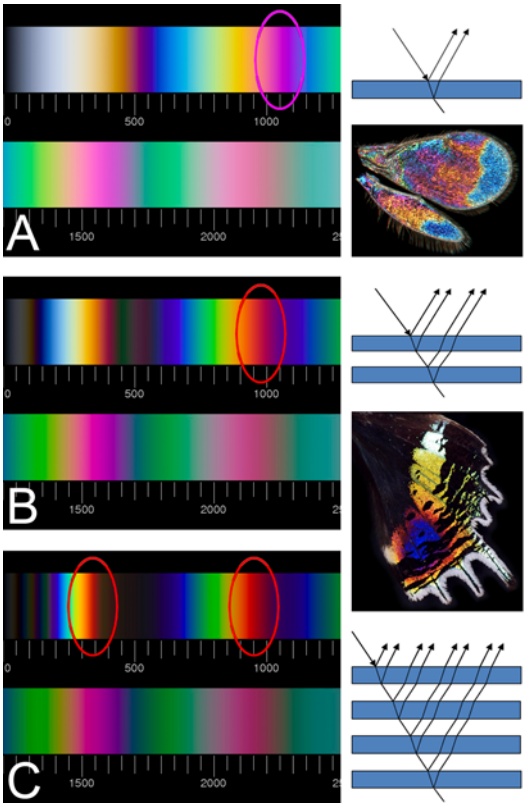

Fig.5 Three computed sequences of specific TFI colors (courtesy of Dietrich Zawischa) illustrate the differences between two-beam and multiple-beam TFI. The sequences of colors are very characteristic and follow the increasing thickness of the film. Most of the incoming light is transmitted through the thin film and only two small fractions are refracted, reflected and superposed by the upper and lower surfaces. The resulting color wavelength depends on interference of beams that have traveled different paths. (A) Two-beam TFI color sequence from a single transparent layer as seen in soap bubbles and WIPs (wings of an aphelinid wasp). This sequence lacks pure red but displays fringes of magenta, an extra-spectral color which is a mixture of red and blue ends of the light spectrum (Chapman, 1998). (B, C) Color sequences that includes red, produced by several transparent layers. In highly organized lepidopteran scales with a tree-like shape in cross section, cuticle layers are separated by air gaps. Each layer produces two reflected beams as illustrated in (A), but here they undergo additional refraction when traveling through extra layers. The number of layers in the stack and the distance between them affect the color fringes. The pure colors of wing patterns in some species of *Urania* (Uraniidae) (courtesy of Jostein Kjaerandsen) are due to multiple reflecting surfaces in the scales with a constant spacing between them. (B) Four-beam TFI color sequence produced by two layers of thin film. Note the presence of one red fringe. (C) Multiple-beam TFI color sequence that includes two fringes of vivid red color. It is produced by four layers separated by air (the more layers in the stack, the more saturated the colours become).

5, B-C). The colors in WIPs are similar to the colors on the surface of a soap bubble where the wall of soapy water is a single thin and transparent film. So the origin of the structural colors in WIPs is readily explained by the two-beam TFI. However, this hypothesis might seem controversial because the insect wing is a more complex structure formed by two membranes, dorsal and ventral. Thus the wing theoretically might have a sandwich-like construction with a gap between the two wing walls. If this was the case, a four-beam TFI sequence with more saturated colors, including a red fringe, would be produced, but this is not observed in WIPs. To resolve this question a TEM study of the wing ultrastructure of tiny wasp *Asecodes congruens* (Eulophidae) was performed. The wing cross section revealed that the two membranes are fused solidly without a gap between them and thus form a single thin film around 300 nm thick (Paper I). The structural organization of the iWing together with an exact match of colors to the original color sequence for two-beam TFI (Fig. 5A) confirms the two-beam hypothesis as the origin of colors in WIPs.

The wings of the giant (about 5 cm in body length) wasp *Megascolia procer javanensis* (Scoliidae) are opaque and iridescent (Sarrazin et al., 2008). They are not strictly speaking iWings, but utilize the same principles as in WIPs. The wings are few micrometers thick with dorsal and ventral surfaces covered by a single homogeneous

transparent chitin layer (about 300 nm thick) with refractive index 1.76. The thickness of this layer is constant therefore the two-beam TFI result in a uniform blue-green hue over the entire wing surface. This thin cuticle layer covering all four wings functions as an optical filter. The bulk of the wing in the middle between two thin (upper and lower) cover layers is black and very absorbant due to high melanin content. The thickness of this thick layer also varies and constitutes the mechanical core of the wing. The presence of a black background allows for the highly visible structural blue-green coloration on both wing surfaces. Sarrazin et al. suggest that this simple and very effective system is among the most elementary interference filters. This is true, but the production of structural coloration in wings using a minimal number of interference waves, i.e. two-beam TFI, occurs in WIPs as well. The black background integrated into the wing membrane in *Megascolia* makes such a wing more sophisticated compared to the iWing; hence the iWing is the most elementary interference filter in insect wings described so far. The chitin/melanin layer in *Megascolia* wings assures the constant in time color reflections. Since the wings are opaque the dorsal and ventral surfaces are also divided in space. These are possibly preconditions in insect wing evolution to develop color patterns on both wing surfaces independently, by simply producing uneven thickness of the structural cover layer. This might have raised the opportunity for divergence in patterns on dorsal and ventral surfaces under natural and sexual selection as in e.g. butterfly wings.

The multiple-beam TFI system is more complicated and involves many variable parameters besides the film thickness, e.g. the number of layers and the distance between them which also depends on their regular or irregular spacing. In the case of WIPs the one layer organization of the wing provides a major advantage for those studying them. The optical system for two-beam TFI is very simple and includes only few parameters: the membrane thickness, refractive index, and reflected colors that depend on the other two. The refractive index of chitin (1.57) and the readily observed colors in WIPs enable an estimation of the wing thickness (Paper I). The Newton series scale metering is based on the scale that was calibrated for the refractive index of chitin; this scale gives an approximate thickness of the wing membrane in nanometers (Fig. 6).

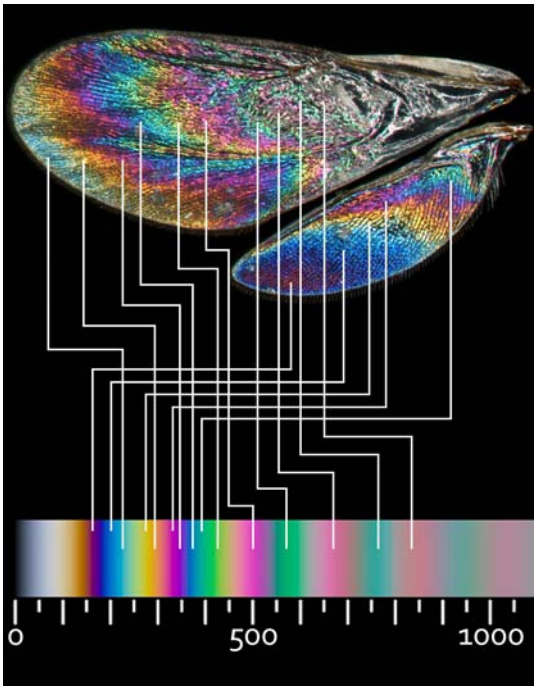

Fig.6 Mapping of wing thickness in *Microterys sp.* (Encyrtidae) through WIP. The Newton series scale for two-beam TFI colors (courtesy of Dietrich Zawischa) calibrated for the refractive index of chitin (1.57) gives approximate thickness of the wing membrane in nanometers. The thickness of the wing membrane gradually increases from the thin apical margin to the thick basal part. The forewing is approximately 225-

840 nm thick, and the thickness of the hind wing is around 160-390 nm.

The thickness metering is basically the matching of WIP colors with the color fringes in the scale where each of them corresponds to a certain membrane thickness. Thus WIPs not only visualize the uneven wing thickness in general but also provide values for the WIP based mapping of wing thickness. The precision of the method was tested on the forewing of *Asecodes congruens* (Paper I). The membrane thickness in the apical part of the wing, which was magenta in WIP, was estimated to 300nm according to the Newton series scale. The cross section of the wing was documented in TEM and the calculated thickness of the wing membrane was, in fact, 300nm. Thus the estimated wing profile based on the Newton series scale metering accurately matches the actual thickness of wings. The wing thickness distribution pattern invisible in transparent wings is an important but overlooked part of insect morphology. The wing thickness profile can now be estimated through direct observations of WIPs. This new tool will aid in the studies of the wing membrane mechanical properties and its adaptations to the overall forces in flight. The mapping of the thickness in transparent wings may also be useful in the fabrication and design of artificial biomimetic wings for micro air vehicles. Usually a planar polymer film of uniform thickness is used as an artificial wing membrane (Shang et al., 2009) despite the thickness distribution pattern that affects the wing performance in flight.

3. What is inside the wing?

3.1. Wing membrane ultrastructure. Two in one thin film

The insect cuticle organization in wings has been mainly studied in larger species of Orthoptera (Banerjee, 1988) and Odonata (Hooper et al., 2006), and focused on elytra in Coleoptera (Jewell et al., 2007) and wing scales in Lepidoptera (Vukusic & Sambles, 2003). The complex venation architecture and flight adaptations of dragonfly and locust wings as well as photonic structures that produce structural coloration in butterflies, moths and beetles have long attracted scientific attention. The transparent wings of tiny parasitic wasps, on the other hand, have greatly reduced venation and lack pigment patterns, so they may seem quite boring. The situation has changed and iWings raise many fascinating and new questions. The study of the wing ultrastructure in parasitic wasps is challenging because the wings can be smaller than 1mm and only a few hundred nanometers thick (Paper I), compared with few micrometers in larger insect wings (Combes, 2010). To get a better comprehension of how thin the iWings really are, let's compare them with the transparent cicada wings (Stoddart et al., 2006). Cicada wings are about 8, 4 µm thick with a highly modified superficial cuticle layer that forms papillae and provides antireflection properties to the surface. This is the thinnest layer of the wing cuticle and is approximately 100-340 nm thick. The hind wing composed of two whole membranes in *Microterys sp.* (Fig. 6) is 160-390 nm thick, i.e. on the same range as a single cuticle layer of cicada wing alone! The insect cuticle is a complex composite material with highly organized structure. It is produced as a layered, extracellular secretion by the epidermis (Andersen et al., 1995). The cuticle consists of an outer thin *epicuticle*, containing lipids and proteins and lacking chitin, and a thicker *procuticle*, consisting mainly of chitin (a polysaccharide similar to cellulose) and proteins. In the procuticle the chitin microfibrils are embedded in a matrix of cuticular proteins and water molecules (Klowden, 2007). The mechanical properties of the cuticle largely depend on the protein compound and are also influenced by the chitin architecture and the degree of hydration. The sclerotized cuticles that contain heavily cross-linked proteins are more rigid while the cuticles with reduced cross-linking of the proteins are softer (Chapman, 1998; Andersen et al., 1995).

The ultrastructure of the wing cuticle in small insects was only hypothesized by Wootton (1992, p. 124): "*Because this measurement includes the apposed cuticles of the dorsal and ventral sides, each layer must be remarkably thin and may perhaps in some cases consist of epicuticle alone*". Through cross section studies I have found that this is not the case and despite extreme finesse of iWings the procuticle is present in both the dorsal and ventral membranes.

The iWing cross section studies were first aimed at finding the number of optical layers that produce TFI colors in WIPs. Next, a calibration test was performed in order to compare the actual wing thickness with the estimated thickness based on a Newton series scale metering. The results confirmed the two-beam TFI hypothesis and the

significance of the new method for measurements of the wing thickness (Paper I). TEM images of the wing cross section (Fig. 7) also revealed features of the wing ultrastructure that are worth a more thorough analysis. The wing is formed by two united cuticles of the dorsal and ventral wing membranes. The wing construction is sandwich-like and the upper and lower cuticles are secreted outwards from the two inner epidermal cell layers before their death/retraction after eclosion. For each cuticle the inner most part is close to the membrane junction and the outer most part is close to the wing surface. The dorsal cuticle consists of three main regions (after Klowden, 2007): (1) **procuticle**, the thick inner part which can be divided further for two layers: the inner ***endocuticle*** and the outer ***exocuticle***; (2) **epicuticle**, the thin layer covering the procuticle; (3) **envelope**, the outermost layer of the cuticle. The differentiation of the procuticle is due to degree of sclerotization, where the exocuticle is hard and rigid, while the endocuticle is undifferentiated and soft (Chapman, 1998). The thin ventral cuticle lacks the endocuticle and consists of only one procuticle layer, the exocuticle, in addition to the epicuticle and the envelope. This corresponds to the known ultrastructure of thicker wings, e.g. the forewings of Orthoptera, where the wing membrane always contains exocuticle, but endocuticle is sometimes absent from large areas (Wootton, 1992).

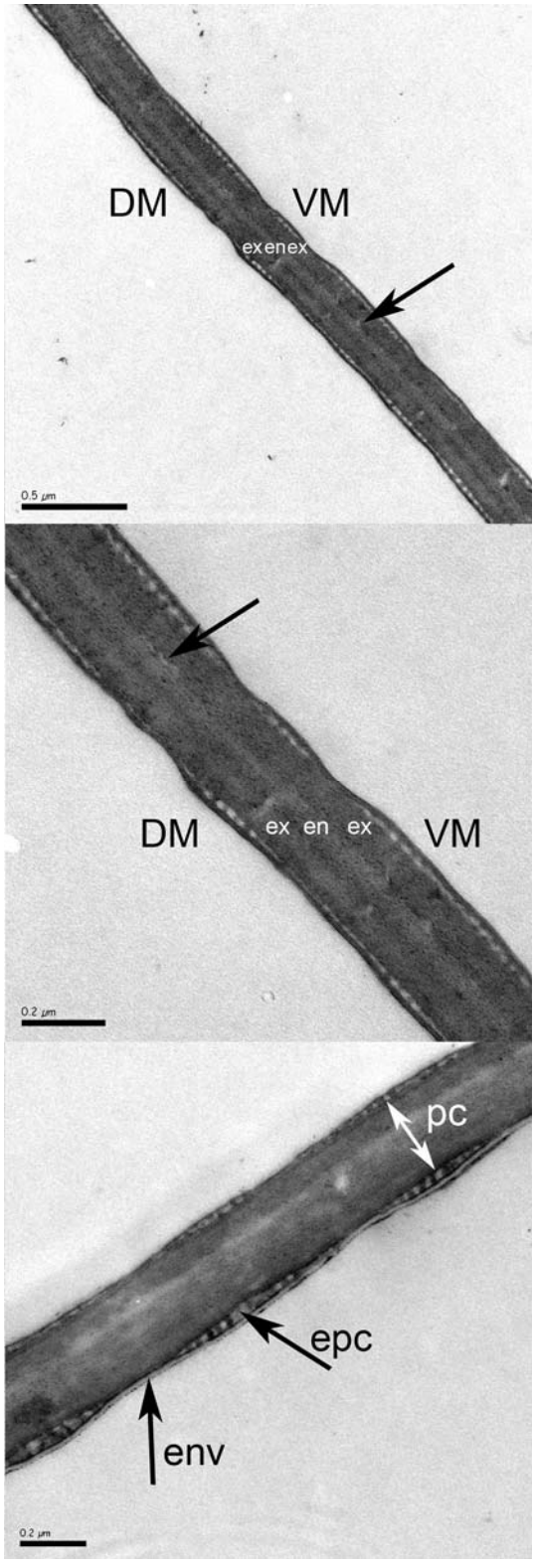

Fig.7 Forewing cross-section of female *Asecodes congruens* (Chalcidoidea: Eulophidae) by TEM. The upper and lower wing walls are fused together tightly and form a solid wing membrane with a barely discernible border-line in between (black arrow). The micrographs reveal unequal ultrastructural organization of the dorsal (DM) and the ventral (VM) wing membranes that consist of four and three cuticular layers, respectively. The envelope (env) is the finest and outermost

cuticular layer that covers both sides of the wing. Below this the epicuticle (epc) reveals a heterogeneous ultrastructure with dark and light stripes that probably represent struts with spaces between them. The next layer is the procuticle, which is the one that differs between the dorsal and ventral membranes. The dorsal procuticle contains two layers, the outer exocuticle (ex) and inner endocuticle (en). The ventral procuticle consists of the exocuticle alone. The procuticles of both wing walls constitute the wing procuticle (pc) in the middle which forms the bulk of the wing and is the thickest layer of the wing membrane. The thickness of the whole wing is in the range 250-350nm where the dorsal membrane makes up approximately 55-65%. The living specimen was fixed directly in the primary fixation solution for TEM to prevent degradation of proteins which may cause artificial structural changes in the wing membrane. Thus these micrographs can be used as a prime reference for TEM of the cross section of wings that were kept in alcohol or dried prior to fixation.

The observations of the iWing membrane ultrastructure by TEM were confirmed by cross section studies of a balloon wing (Fig. 8). This is a rare artificial condition of wings that may occur when a newly emerged imago young specimen is preserved in alcohol before eclosion is completed. In the studied specimen the left pair of wings retained a natural shape while the right forewing became a wing-sack inflated by alcohol. This unique specimen enabled me to carry out TEM studies of the two discrete cuticles separately, and then compare them with a whole wing membrane of the same specimen (Fig. 9). These micrographs show unequal organization of the dorsal and ventral wing membranes, similar to Fig.7. Two procuticle layers, the endocuticle and exocuticle, and two outer layers, the epicuticle and envelope make up the dorsal membrane. The ventral membrane is thinner and consists of a single procuticle layer, the exocuticle, and also outer layers, the epicuticle and envelope. My concern was about possible negative influence of alcohol and long preservation time on the ultrastructure of the wing cuticle. However, in the second study it remained the same compared to the first preparation where the living insect was fixated instantly. The only difference found is in the left wing where the border line between cuticles is discernible. A gap between the upper and lower cuticles occurs because the membranes are not entirely fused and alcohol could penetrate in-between even though the wing has retained its shape.

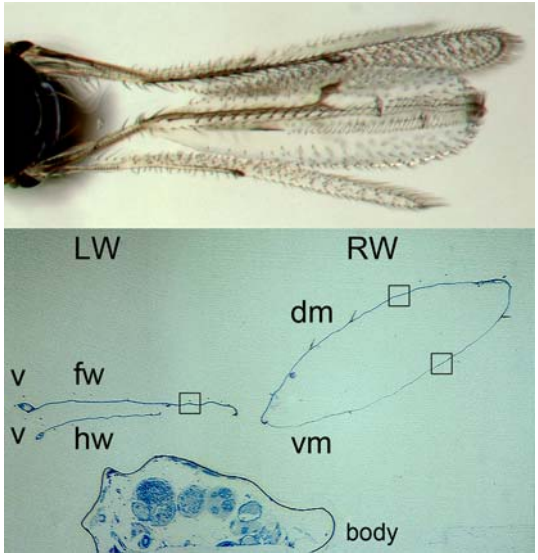

Fig.8 Cross section of the wings of a female *Omphale chryseis* (Chalcidoidea: Eulophidae), prepared for TEM (Fig. 9). In a young insect alcohol can penetrate between the wing walls that are not yet fused properly, thereby inflating the wing like a balloon. This rare specimen had a condition with one balloon and one normal wing, and was considered to be an optimal model for the comparative analysis of the wing ultrastructure by TEM. The dorsal and ventral membranes can be observed individually and compared with the whole wing membrane of the same specimen. Here the left wings (LW) are normal while the right forewing (RW) is inflated. The left forewing (fw) and hind wing (hw) appear as two single lines with rings of hollow veins (v) on the edge. The right forewing appears as an oval due to separated dorsal (dm) and ventral (vm) wing walls. The locations for TEM micrographs are marked with squares. Note the body cross section below the wings.

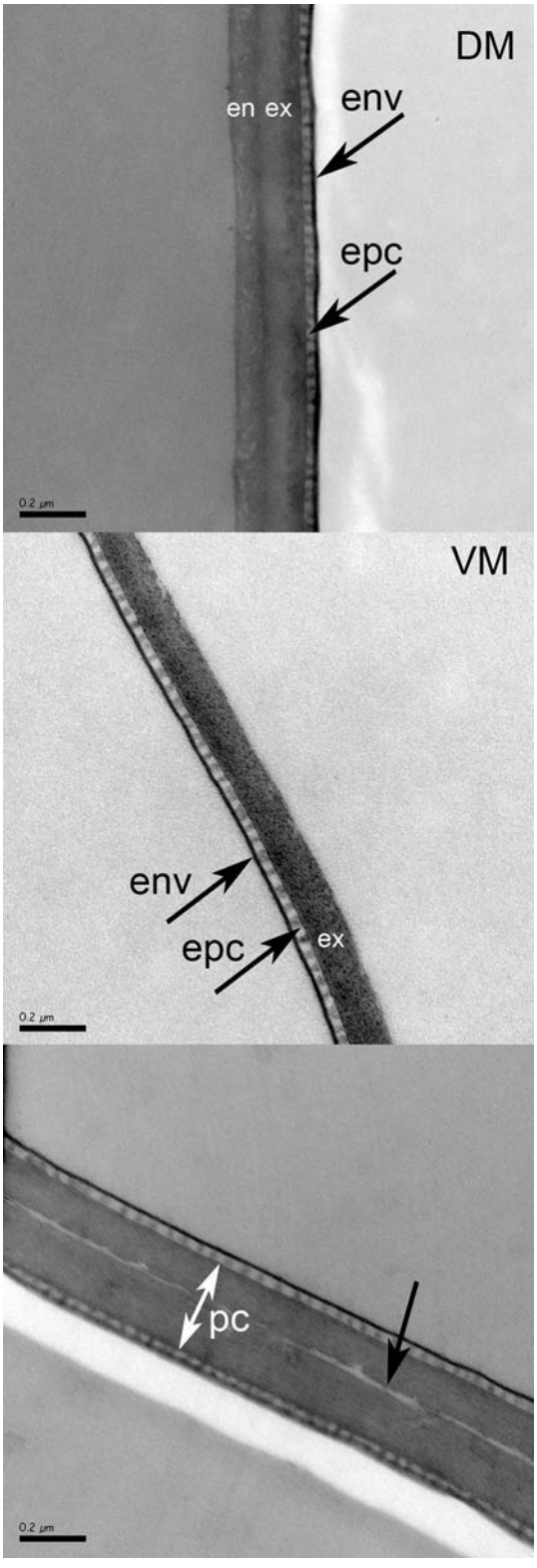

Fig.9 TEM cross sections micrographs of the wing from Fig.8 (squares). The dorsal (DM) and ventral (VM) membranes of the right forewing differ in the ultrastructure i.e. in the number of cuticle layers and thickness. The superficial cuticle layer is the envelope (env) that covers both surfaces of the wing. The layer below is the epicuticle (epc) with alternation of the dark and light stripes that look like struts. The procuticle in the dorsal membrane consists of two layers, the outer exocuticle which is more resistant to tearing i.e. rigid, and the inner and soft endocuticle (en) which is easily damaged. The ventral procuticle is thin and consists of exocuticle alone. The procuticle (pc) of the entire left forewing is the thickest part of the wing formed by two fused procuticles of the dorsal and ventral wing membranes. The border line between two wing walls is still clearly visible due to the short time passed since eclosion. The approximate wing thickness is 350 nm of which the dorsal and ventral membranes makes up 200 nm and 150 nm, respectively. This specimen was kept in alcohol prior the preparation for TEM. The ultrastructure of the wing cuticle is the same as in Fig.7, indicating that fixation in alcohol does not influence the ultrastructures and layering.

The sample preparation procedure for TEM wing cross sections included: primary fixation in 3% glutaraldehyde in a 0,1 M Na-cacodylic buffer; secondary fixation in 1% osmium tetroxide in buffer; dehydration through acetone series and 50% to absolute ethanol; embedding in EPON Resin and polymerization at 60° C. Sectioning was 50nm thick and grids staining with 2% uranyl acetate with post-staining with lead (Pb) citrate.

3.2. Open wing WIPs on dorsal and ventral membranes

With the discovery of WIPs we can see transparent wings of tiny insects from a new functional perspective. For instance the structural colors in lepidopteran wings produced by external structures, the wing scales, cannot be directly correlated to the ultrastructure of the wing membranes. But WIPs, besides being a colorful wing pattern, also visualize the uneven thickness of a wing membrane. Thus the synthesis of visual signaling, adaptations for flight and wing development, apparent through WIPs, uncovers different aspects of wing evolution. Morphological studies of wings always concern “normal” wings where the upper and lower wing walls are fused to form a single transparent membrane. This might be a reason why the individual morphological organization of the dorsal and ventral wing membranes has long been overlooked. In open balloon wings their “transparent” morphology as well as their WIPs, and hence the thickness of the cuticles, can be analyzed (Fig. 10). An interesting question is how each membrane contributes to the WIP in the whole wing? My initial hypothesis was that the dorsal and ventral patterns were equal, but that they differed in their hues due to thicker dorsal and thinner ventral cuticles. In this case when membranes become fused their patterns would overlap and reinforce each other. The colors would change also due to almost doubled thickness.

However, open wings revealed that the dorsal and ventral WIPs are asymmetric (Fig.10). The thick dorsal membrane has a leading role in the pattern formation while the thin ventral membrane appears as a supporting membrane in a final WIP (Paper I). So, the initial hypothesis did not work, but why not? The lepidopterans display different patterns on the dorsal and ventral surfaces of the wings, which can be used alternatively for mate signalling or predator avoidance (Allen et al., 2011). But in parasitic wasps the dorsal and ventral WIPs are not spatially separated thus cannot function as separate units in visual signalling. The same (united) WIP is reflected on both surfaces of the iWing and therefore the finding that WIPs are different between the upper and lower membranes is rather peculiar.

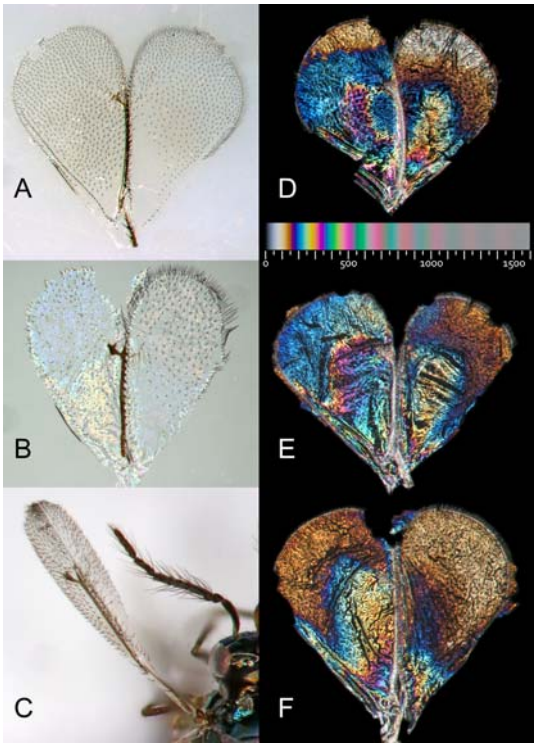

Fig.10 Morphology of the dorsal (left) and ventral (right) wing membranes in parasitic wasps. All photos are of the left wings in the original layout. The wings, inflated in alcohol, were opened and flattened. The resulting heart-shaped open wings allow observations of the morphology and WIPs of each membrane individually. (A-B) Open forewings, transparent mode. The veins are confined to the dorsal membrane which is largely covered with short setae. The marginal fringe of

longer setae is attached on the ventral membrane which has a bare basal part. Thus the upper and lower wing walls differ in morphological organization and are not symmetrical. (A) *Chrysocharis sp*. (B) *Omphale chryseis*. (C) Balloon wing of *Omphale sp.* in alcohol, in frontal view, showing that veins are confined to the dorsal membrane. (D-F) WIPs of open forewings. The wing walls display asymmetrical WIPs. The dorsal WIPs are distinct and match the WIPs as seen when wings have the dorsal and ventral membranes fused. The ventral WIPs are more similar between species with general thickening of the basal part of the membrane. The Newton series scale can be used to see the relative thickness of the membranes. (D) *Achrysocharoides platanoidae*, male. The thickness of the dorsal membrane is 140-490nm while ventral membrane is 100-400nm thick. See the original *A. platanoidae* WIP in Figs. 13 & 15 and note the shift of colors that occurs in the dorsal WIP due to thickness reduction. The blue apical spot (210-250nm thick) outlined by the yellow border in the normal WIP becomes the yellow spot (140-160nm) with the magenta border in the dorsal WIP. (E) *Achrysocharoides sp.* The dorsal membrane is 200-440nm thick and ventral membrane is 150-310nm. (F) *Chrysocharis sp.* The dorsal membrane is 150-350nm and ventral membrane is 120-300nm.

Nevertheless, the different WIPs of dorsal and ventral wing walls might suggest a trend in common with butterfly wing pattern evolution. In butterflies the dorsal surface characters evolve with higher rates than ventral surface characters and also display sex-based differences more often (Oliver et al., 2009). This matches the situation in WIPs where elaborate dorsal patterns also display sex-based details in some species, e.g. the apical blue spot in male WIPs of *A. platanoidae* while the ventral patterns are not specific (Paper I). One possible explanation for the difference in evolutionary rates of the dorsal and ventral wing surfaces is developmental constraints that prevent changes on some wing surfaces, but not others (Oliver *et al.* 2009). However Oliver et al. (2009) have shown that the main drivers in *Bicyclus* butterflies are not the developmental constraints, but different selective forces. In case of small insects with transparent wings a visual selection that would affect the dorsal and ventral membranes differently seems unlikely, as they cannot be seen individually. Instead, the different number of cuticle layers in the dorsal and ventral wing membranes suggests the differences in development and possible constraints that affect the ventral membrane and limit its pattern formation.

In wings of butterflies the signals between dorsal (mate signaling) and ventral (predator avoidance) surfaces are separated in space. The differences in the dorsal patterns in sister species are especially evident in sympatric species pairs where they function as isolating mechanism (Meyer-Rochow, 1991). Tiny parasitic wasps in the genus *Achrysocharoides* species that co-occur on the same host also display specific and very distinct WIPs (Papers III and IV). Since the pattern formation in WIPs is mainly due to the dorsal membrane, it means that differences in sympatric species WIPs, in fact, reflect the structural differences in their dorsal membranes in parallel with dorsal surfaces in butterfly wings. In butterflies the characters in forewings, involved in mate signaling, display higher rates of evolution than the characters in hind wing (Oliver et al. 2009). Again, in *Achrysocharoides* there are four species with sexually dimorphic WIPs *A. platanoidae*, *A. butus*, *A. robiniae*, and *A. latreillei* (Paper III). The distinct and species specific male WIPs are displayed by forewings, while WIPs in hind wings are similar in both sexes and also between species. It is very interesting to see that the model integrating natural and sexual selection in butterfly wing pattern evolution fits evolution in WIPs.

4. Visual signaling and species recognition.

4.1 Sexually dimorphic WIPs. What is the story behind a blue spot?

Most insects have three visual pigments with maximum sensitivity to wavelengths in the green, the ultraviolet (UV), and the blue region (Chapman, 1998). The color sequence in WIPs lacks pure red (Fig. 5, A) and thus fits the trichromatic color vision in small insects. Correlation between color vision and the colors in WIPs suggests a biological significance of WIPs in visual signaling (Paper I, and IV). Some Lepidoptera possess additional red-sensitive photoreceptors (Chapman, 1998) and notably red color can be found in butterfly wing patterns e.g. *Heliconius* and *Papilio* spp. In addition to the color spectrum visible to a human eye, UV reflection is also produced as an interference color (Chapman, 1998, Ghiradella et al., 1973). Insects exploit UV sensitive photoreceptors and UV for them is as "normal" as red color is for us, hence the UV reflections in WIPs (invisible to us but visible to insects) is not a qualitatively different signaling channel. Does this mean that insects see WIPs differently than we do? For sure! To better understand how insects might perceive WIPs it is interesting to get some ideas about the UV pattern and how it is integrated in WIPs (Fig. 11). Illumination of wings with shortwave and UV radiation causes fluorescence i.e. emission of longer wavelengths than those of the incident light. Fluorescence in a visible spectrum overwhelms the actual UV reflectance that can only be seen with use of a UV-pass, and visible-cut filter on the camera (Savazzi, 2011). Without such images that contain pure UV information we can use the UV-induced fluorescence pattern (Fig. 11, B) to

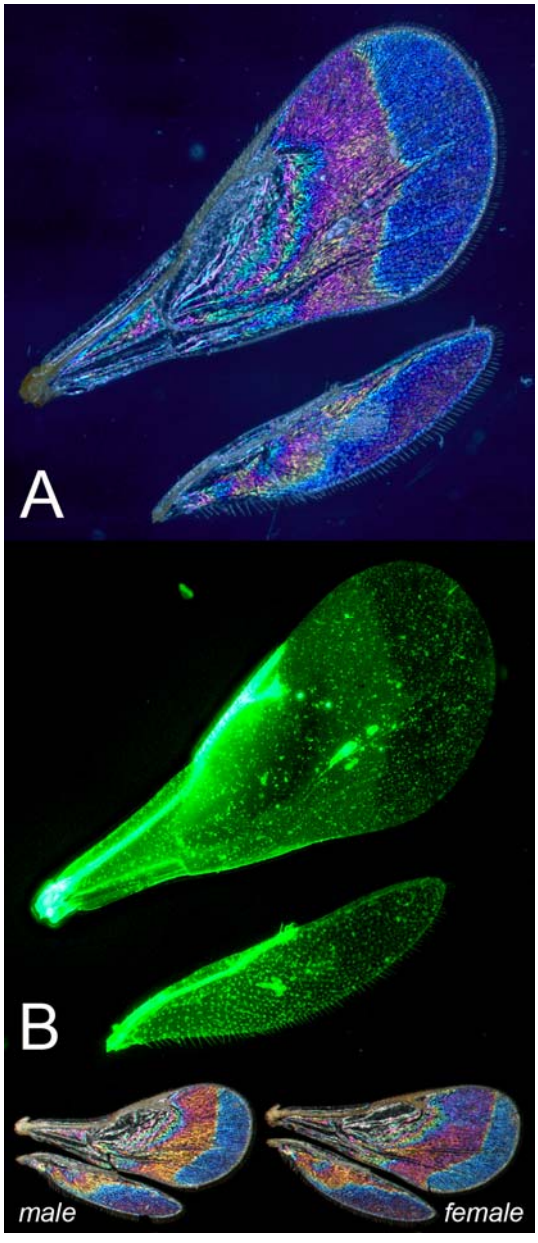

Fig.11 Transparent wings of female of *Idiomacromerus sp*. photographed in light microscope with two different illumination techniques (courtesy of Peter Lindelöf, LRI

Instrument AB). The images provide complementary data. (A) Dark field microscopy, WIP. The WIP in the forewing can be divided into three transverse bands: the basal area, middle band (magenta) and apical spot (blue). The hind wing WIP also displays the basal area and a blue apical spot. (B) Fluorescence microscopy (UV-excited fluorescence, FITC). The wings were illuminated with blue light of shorter wavelength near-UV which caused fluorescence and emission of bright green light of longer wavelength. The strongest signal is from the veins and the adjacent area. In relation to WIP only the middle band in forewing and the basal area in hindwing have strong fluorescence (green). The apical spots of both wings lack fluorescence (black) and also the basal area in the forewing. These images suggest the presence of reflections in the UV complementary to and superimposing the WIPs. Thereby the signalling WIPs fit even better to the blue-green-UV trichromatic color vision in small insects.

speculate about the actual UV patterns in iWings. The energy emitted through fluorescence corresponds to the energy that was absorbed in the UV, so the fluorescence pattern can be regarded as a negative image of the reflective-transmissive pattern in the UV. Hence, the UV pattern likely to be seen by insects is the inverse of the fluorescence pattern (Savazzi, 2011; E. Savazzi, pers. comm.). This suggests the UV reflectance in the apical spots of the wings, those that are blue in WIPs, and the UV absorption in the rest of the wing. Such evidences strengthen further the signaling function hypothesis of the blue spots in WIPs (Paper I). In species of *Idiomacromerus* used for UV study (Fig. 11) both sexes display large and distinct blue apical spots in their WIPs. Such WIPs are not sexually dimorphic and were found in several species. The presence of a blue spot in males and females indicates a possible mutual signalling e.g. during courtship. The width of the blue spot differs between hypothesized species (in unidentified material) and is also similar in males and females that are potentially conspecific (unpublished data). If these species will be confirmed then it suggests that the blue spots in forewing WIPs are under selection pressure for a specific shape and size. The origin of a blue TFI color in WIPs is the same in all iWings and caused by a corresponding membrane thickness. Therefore the UV reflection indicated in *Idiomacromerus* spp. is very probably a phenomenon that occurs generally in WIPs as the UV is one of the TFI colors. Consequently *Achrysocharoides* species with a blue spot only in male WIPs (*A. platanoidae* and *A. butus*) also display the UV dimorphism which provides the basis for sexual recognition in courtship (Ghiradella et al., 1973).

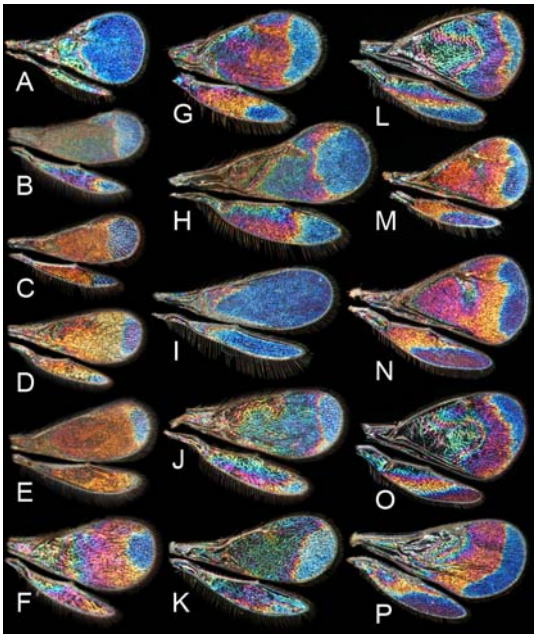

Fig.12 Blue spots in WIPs of various Chalcidoidea (Hymenoptera). (A) Trichogrammatidae. (B-K) Aphelinidae. (L-O) Eulophidae. (P) Torymidae. (A-K) unident. spp. (L) *Achrysocharoides butus*, male (M-N) male and female of *Neochrysocharis formosa*, (O) *A. platanoidae*, male (P) *Idiomacromerus* sp., female.

The WIP diversity in Chalcidoidea I have seen so far reveals that distinct blue spots of different shapes and sizes can be found in

several families and are especially common in small aphelinids (Fig. 12). Likely these spots also reflect UV indicating a behavior that involves visual signals in wings. In many parasitic Hymenoptera copulation is preceded by elaborate courtship displays which include species-specific characteristics (Van den Assem, 1975). In Chalcidoidea conspecific display of males to females prior to mating is well known (Van den Assem and Jachmann, 1982), but the research is focused on other behavioral aspects than visual wing display, such as courtship cycles (Van den Assem, 1975), courtship songs by wing vibrations (Villagra et al, 2011), or movements of the antennae (Romani et al, 2008). The main reason for it, I think, is that before the concept of WIPs was introduced transparent wings in minute wasps were generally regarded as colorless and uninformative entities and their use in visual signalling seemed unlikely. With WIPs this view is about to change and many species with blue spots in WIPs will raise new behavioral studies aimed to explore their potential role in visual signaling.

Two cases of tiny parasitic wasps that display their seemingly pattern-free transparent wings in different manners were reported in Paper I and another case is reported here. The evolution of such peculiar behavior is difficult to explain without also taking their WIPs into account. Newly eclosed 2-3 mm long microgastrine braconid wasps raise their wings and wave them when encountering a sibling in the rearing container. The WIP may be part of the signal (Paper I). Females of pollinating fig wasps hold their wings in a very unusual position – straight up in the air, like sails or billboards – when walking on the fig (Michaloud & Devez, 1982). When entering the fig's flower structure (syconium) through a very tight opening (ostiole) to lay eggs, females shed their wings which become fixed in the ostiole and are visible outside of the syconium. These wings might function as a species-specific signal for other females advertising that the flower is now occupied (Paper I). In a video recorded by Edith Smith at the Shady Oak Butterfly Farm in Florida (Smith, web.) the males of the parasitic wasp *Pteromalus cassotis* (Chalcidoidea: Pteromalidae) are seemingly engaged in a vigorous male-to-male wing display. Wasps are leaving the chrysalis of a Monarch butterfly (*Danaus plexippus*) through a single hole. The males emerge before the females and wait outside of the chrysalis for females to emerge. Meanwhile the males actively open their wings and hold them horizontally or wave, seemingly to display their wings to each other while females are still inside and cannot see them. When the females emerge, one by one, from the same hole they are instantly grabbed by males for copulation. So, in this species the females do not choose the males. It rather seems that males compete for the nearest place to the exit hole by displaying their wings. The ones that get close enough have increased chances to grasp an emerging female. Although difficult to explain in terms of selection, the wing displays among males in *P. cassotis* suggest, in my opinion, that visual signals include WIPs, which are noticeably flashing on transparent wings against a dark green chrysalis in a video record. Wing movements also produce vibrations which can be perceived as sounds (Van den Assem, 1975). The fact that males prefer to hold their horizontally spread wings motionless while walking is in favor of the visual signaling hypothesis in case of *P. cassotis*.

It is not uncommon that males and females in insects display different pigment or structural color patterns on their wings,

including differences in the UV reflections, e.g. in some Lepidoptera species (Ghiradella et al., 1973; Meyer-Rochow, 1991; and Allen et al, 2011). Sexually dimorphic and species specific wing patterns in general, and WIPs in particular, suggest that their evolution is at least partly driven by sexual selection that involves visual signaling, e.g. wing display in courtship. We hypothesized a male-to-female wing display in species where WIPs are sexually dimorphic (Papers I, IV). To date four species in *Achrysocharoides* (*A. platanoidae*, *A. butus*, *A. latreilleii*, and *A. robiniae*) are found to have sexually dimorphic WIPs. In all four cases distinct and species specific patterns are seen only in the males while females have WIPs that are similar between species (Paper III). The genus *Achrysocharoides* is an interesting case for evolutionary research in WIPs since the species are unusually host specific, not only to their primary host, species of leaf mining moths, but to the host plant species as well. The comprehensive knowledge about their hosts and ecology gave us an opportunity to broaden the study of WIPs in an attempt to answer the question why species specific and sexually dimorphic WIPs have evolved in this genus (Paper IV). The comparative analysis of WIPs and host records in respective species revealed that sympatric species (Fig. 13) are more likely to display divergent and sexually dimorphic WIPs compared with allopatric species. These results indicate that visual signaling by use of WIPs evolved as an isolating mechanism only when two or more species co-occur. In such cases WIPs are probably used for species recognition and the sexually dimorphic WIPs indicate that WIPs in the males are under selective pressure through female choice.

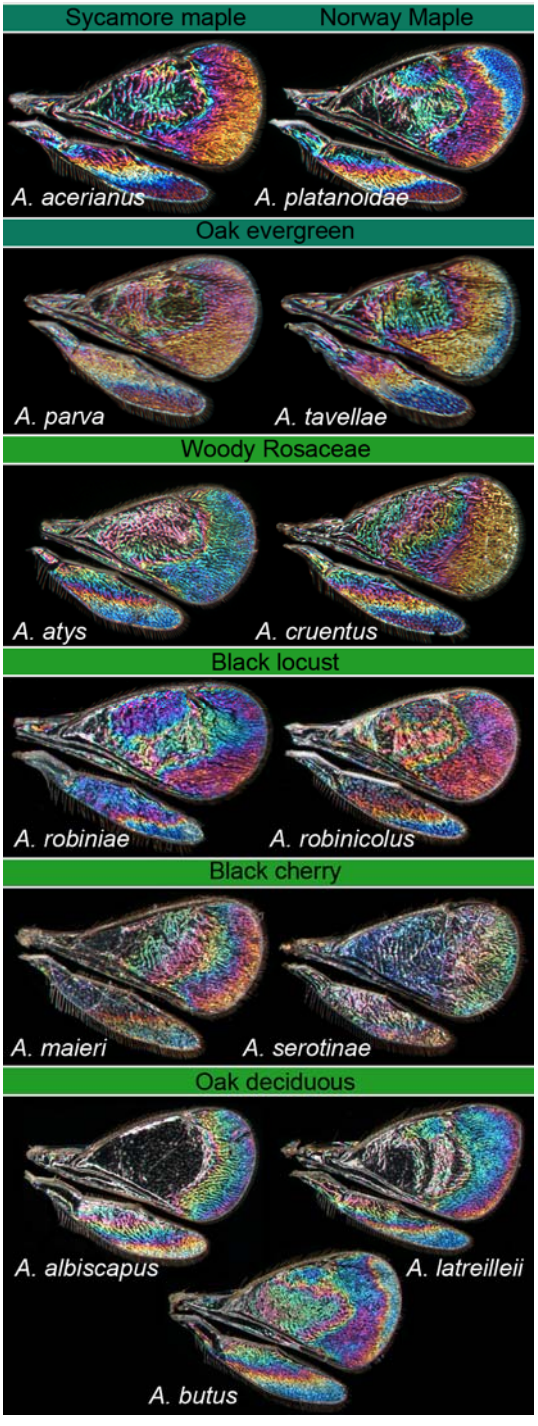

Fig.13 Divergent male WIPs in sympatric species of *Achrysocharoides* and the host plant with which they are associated. Photos of *A. parva*, *A. tavellae*, and *A. cruentus* are courtesy of Christer

Hansson. There are two cases of species that at the present time are not sympatric but nevertheless show character displacement in WIPs. They were hypothesized in Paper IV to having co-occured during their history, but then became isolated in space (*A. acerianus* and *A. platanoidae*) or in time (*A. parva* and *A. tavellae*). *A. acerianus* and *A. platanoidae* are host-specific on two different species of the same plant genus: *Acer*. *Achrysocharoides parva* and *A. tavellae* occur on the same plant, but parasitize different stages of the same host, therefore do not occur simultaneously as adults. Nevertheless, *A. platanoidae* and *A. tavellae* retain a divergent wing pattern to different degree. The blue apical spot on the fore wing margin in *A. platanoidae* is very distinct. The spot in *A. tavellae* is smaller, less pronounced and without a border line. After *A. tavellae* became allopatric the blue spot was no longer selected for and hence may be in the process of gradually fade out and go back to the ancestral pattern. Possibly the allopatric separation in *A. acerianus* and *A. platanoidae* is more recent and the blue spot in *A. platanoidae* have not had time to drift away as much as seen in *A. tavellae*.

4.2 WIPs in closely related and cryptic species. New character system.

To "read" the WIP one may wonder whenever the wing thickness was optimized for flight or for visual signaling. In some species WIPs are elaborate with curved lines and spots while in others they are quite featureless and reflect one color, indicating the same thickness throughout the entire wing. The wings with unicolored WIPs, e.g. in some *Omphale* (Paper V), are perhaps optimized for visual signaling since the uniform thickness may reduce the wing stability in flight. In taxonomy, however, all WIPs provide additional morphological information regardless their biological significance and are useful in many ways. WIPs visualize the thickness distribution patterns in transparent wings and these patterns often vary between species, but show little variation within species (Paper III). As a new morphological character WIPs are useful for species discrimination (Fig. 14) and in some cases species can be identified by their distinct WIPs alone (Fig. 15). The WIP data may confirm a current species hypothesis, thus strengthen the case, or contradict with it, suggesting that case should be reconsidered. Conclusions about usefulness of WIPs in taxonomy are mainly based on the WIP investigation in the two eulophid genera, *Achrysocharoides* and *Omphale*. WIPs greatly aided the species identification and were particularly useful for the discrimination of cryptic species (Papers I, II) and morphologically similar species within the same species-group (Paper V). In *Achrysocharoides* species specific WIPs can be used to differentiate sympatric species (Fig. 16), while species with an allopatric distribution all share the same, ancestral, pattern (Paper IV). WIPs in *Omphale* are diverse and often species specific within respective species-group (Paper V). Another and very important application of WIPs in this field is linking conspecific males and females that are difficult to associate when species show a pronounced sexual dimorphism in other morphological characters (Papers III, V). In *Omphale* WIPs are the same in both sexes thus basically can be used to link males and females in all species with distinct and specific WIPs. So far in *Achrysocharoides* four sympatric species were found to have sexually dimorphic WIPs (Papers I, III) therefore in this genus not in all species but in majority of species linking conspecific males and females is possible through WIPs (Fig. 16). Furthermore, in a newly described

eulophid genus *Cornugon* (Hansson, 2011) a derived WIP, with a blue apical spot on the hind wing, is shared by all species, but such WIP is not found outside the group, thus can be used as an autapomorphy indicating the monophyly of this genus. The taxonomic analysis aimed for species identification, description of new species or classification will benefit from the addition of WIPs into the system of morphological characters.

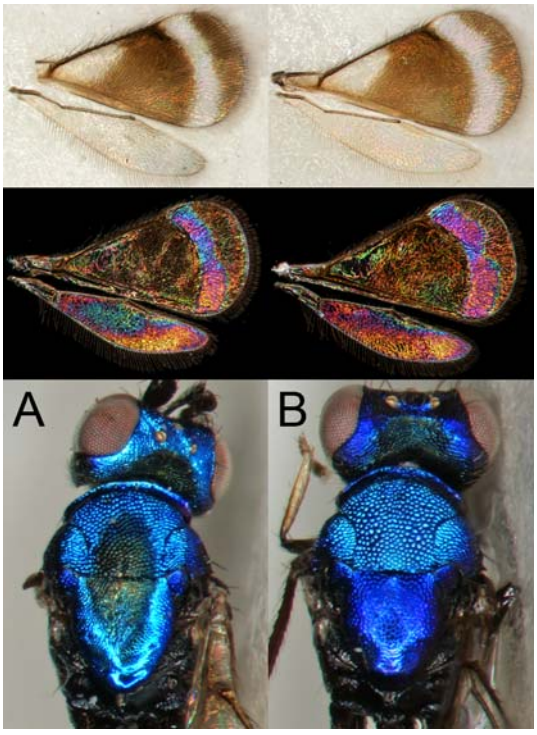

Fig.14 *Closterocerus* spp. Species A and B: the wings, WIPs and the mesosoma, dorsal view (note the differences in coloration and reticulation). The same wings are viewed in transparent and structural mode. The forewings show elaborate pigment patterns while the hind wings are transparent and featureless.Forewing WIPs correspond to the pigment pattern and strongly emphasize the different shape of the transverse transparent band. It can be overlooked if the wings are viewed on a white background or considered as intraspecific variation. Hind wing WIPs show a transition of different thickness in species A, compared to species B, which is invisible against a white background. These specimens can be recognized as two different species through other external morphological characters, but very similar pigment patterns in the forewings can mislead to opposite conclusion. However easily observed WIPs display additional morphological features that strengthen the differences between these species.

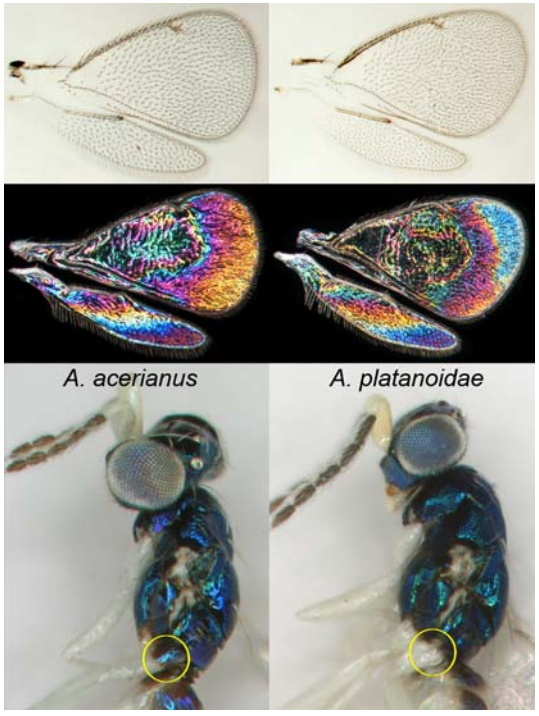

Fig.15 Male wings and their WIPs in two cryptic *Achrysocharoides* species. Morphologically these two species appeared at first very similar and difficult to separate. Their transparent wings appeared featureless and were practically identical between the species. However, when the same wings were viewed in structural mode a distinct WIP with a blue apical spot was detected in *A. platanoidae*, and this spot was absent in *A. acerianus* (Papers I and III). The males showed small differences in other external morphological characters, but eventually two morphological differences were found: hind coxae (marked with yellow circles) had different coloration, and the antennae showed some minor differences (Paper II). The females were very similar to one another; the only difference between the species was the color of hind coxae, which was the same as in the male. These species are hard to distinguish and prior to the discovery of different WIPs the presence of a cryptic species complex within *A. acerianus* was overlooked.

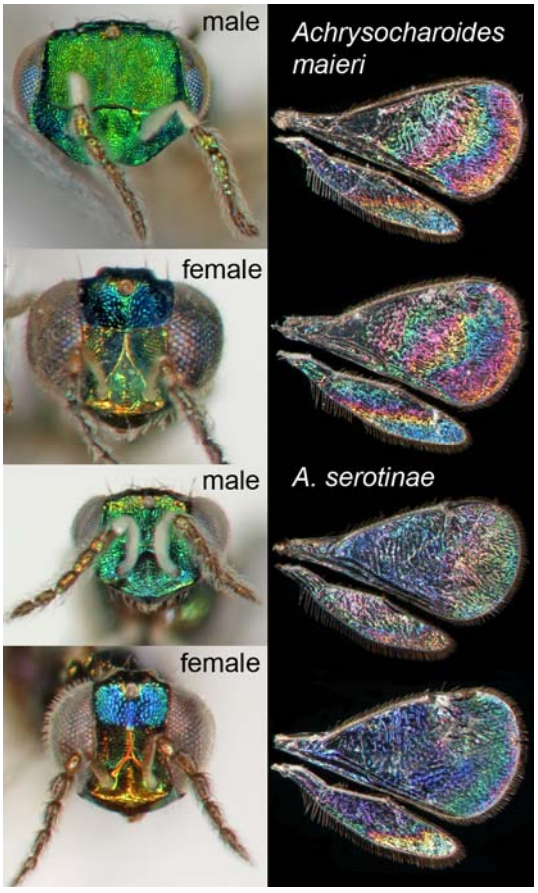

Fig.16 These *Achrysocharoides* species are similar but males can be distinguished by differently shaped heads: *A. maieri* with a peculiar flattened head and *A. serotinae* with a normal head (Paper III*)*. But females of the two species do not look like their conspecific male, and they are also similar to each other. Both sexes of the two species were reared together as they co-occur on the same host. So the problem was how to separate the females and also find out which male and female were conspecific. The transparent wings are featureless and similar between species and sexes. However, WIPs are distinct, species specific and the same in both sexes. *Achrysocharoides maieri* has several wide colorful bands diagonally across the wing, and *A. serotinae* has an almost unicolored WIP without any distinct lines or spots. Thus in this case WIPs provide very strong evidence to link respective males and females, and also proved useful in the identification of the otherwise hard-to-separate females.

Perspectives

A door to the world of colorful WIPs that used to be invisible for us is now open, and the key is accessible to everyone. My hope is that the discoveries presented in this thesis will encourage many of you to enter and see what you find. It is really cool stuff, I think, and if one keeps an open mind there is much more to discover ahead. Colorful WIPs are a striking part of the morphology in small insects and having seen the beauty of it once I was incapable of ignoring their existence.

WIPs form new and potentially very useful characters for species recognition, discrimination of cryptic species, and linking of conspecific males and females. While initially founded manly on taxonomic considerations my studies of WIPs gradually aimed more at building up a foundation for a new and wider interdisciplinary research program. I think that several other disciplines may benefit from recognizing WIPs as an integral part of insect morphology, ecology and evolution. The most obvious fields are: (1) **sensory biology** (visual signaling and wing displays involved in courtship, and possibly also antipredation behavior by the use of WIPs); (2) **Evo-devo**, evolutionary developmental biology (unequal development of the dorsal and ventral wing membranes, thickness profile formation to shape a specific WIP, and the genes and regulatory mechanisms involved); and (3) research on the **flapping flight** of insects (functional micro-morphology of wings visualized through WIPs and its relation to and effects upon flight performance).

More comprehensive data on WIP diversity and specificity provided by taxonomists will direct such new behavioral observations and experiments to new taxa and model groups. Millions of museum specimens still hide their colorful secrets in the darkness of the drawers and cabinets of entomological collections throughout the world. I see now a golden opportunity to join forces between taxonomists and ecologists, and together step forward towards a better understanding of evolution and adaptations in tiny insects with transparent wings.

Summary of papers (in chronological order)

Paper II

Two cryptic species complexes in the genus *Achrysocharoides* Girault (Chalcidoidea: Eulophidae: Entedoninae) are analyzed: *A. acerianus* (Askew) and *A. platanoidae* which both have an obligate association with the plant genus *Acer,* and the sympatric species *A. robiniae* and *A. robinicolus*, both associated with Black locust (*Robinia pseudoacacia*). Three of the species are newly described. These tiny parasitic wasps are larval endoparasitoids of leaf mining moths and also show unusual plant host specificity: *A. platanoidae* occurs on Norway maple (*Acer platanoides*) in Northern Europe, and *A. robiniae* (Central Europe and the U.S.A.) and *A. robinicolus* (the U.S.A.) occur on Black locust. The new species are very similar to some previously described species, but are shown to differ from their sibling species both in biology and in external morphology.

Paper I

The paper reports striking and stable structural color patterns — Wing Interference Patterns (WIPs) — in the transparent wings of small Hymenoptera and Diptera. Their wings are extremely thin and against a dark background they reflect vivid color patterns caused by thin film interference. Microstructures of the wing membrane provide an optical stabilization to the WIPs that practically eliminates the iridescence effect. WIPs often appear to be taxon-specific and the novel hypothesis that they may be used by the insects in visual signalling is put forward. The signalling hypothesis is further strengthened by the specific color sequence displayed by WIPs that lacks pure red and thus matches the color vision of most small insects. Species specific, and in some cases sexually dimorphic WIPs, are demonstrated to be useful characters for identification and to solve cases of cryptic species complexes. WIPs can also be applied to map the micromorphology of wing membranes through direct observations of the reflected colors and may prove useful in several fields of functional and evolutionary biology dealing with insect wings.

Paper III

The WIPs the genus *Achrysocharoides* Girault (Hymenoptera: Eulophidae) are analyzed and shown to be an important tool for species recognition. Two new species from North America, *A. maieri* and *A. serotinae* are described. The new species are sympatric and have distinct and specific WIPs that are similar between the sexes, which provide a strong link between conspecific males and females as they are otherwise difficult to associate. The WIPs of nine species, including the cryptic species discussed in Paper II, are illustrated and their intra- and interspecific variation is analyzed. Four species also display distinct sexually dimorphic WIPs. It is further found that the grey scale images of uncoated wings by scanning electron microscopy visualize the thickness distribution pattern in wing membranes, supplementing the colorful WIPs.

Paper IV

Intraspecific recognition involving WIPs and reproductive character displacement in WIPs are hypothesized for the genus *Achrysocharoides* Girault (Hymenoptera: Eulophidae). WIPs of all the 21 valid European species are surveyed and illustrated. The biology of this genus is better known in Europe compared with North America enabling the species to be classified into 16 functional groups according to biological host records. An ancestral WIP of outgroup was hypothesized. Derived, species specific WIPs are found in a minority (6/21) of the studied species and often occur only in the wings of males. A statistical analysis (Fisher's Exact Test) show significant support for the prediction that the sympatric species of this genus are more likely to show divergent WIPs than are the allopatric species.

Paper V

WIPs and male genitalia of 23 European species of the genus *Omphale* Haliday (Hymenoptera: Eulophidae) are illustrated and included in a phylogenetic analysis based on morphological characters. WIPs in *Omphale* are classified into four categories, and the derived states are shown to have evolved just in singular species or independently several times. Within species-groups WIPs are useful for species identification and also important in linking conspecific males and females. The male genitalia, on the other hand, exhibit an unusual extensive morphological diversity in *Omphale*, including both species-specific and group-specific characters. The analysis of male genitalia reports new apomorphies of importance for the classification of European *Omphale*.

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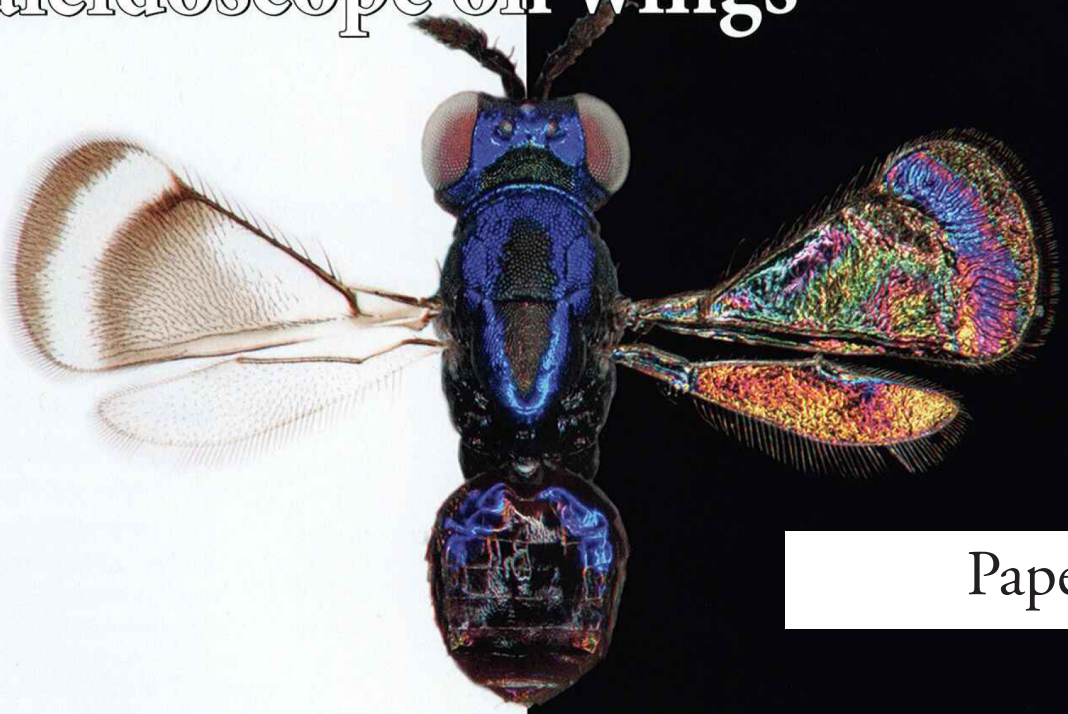
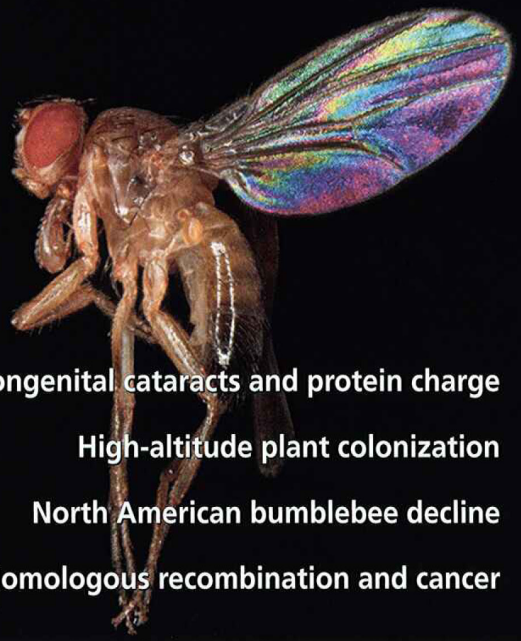
Kaleidoscope on wings



Paper I



Congenital cataracts and protein charge

High-altitude plant colonization

North American bumblebee decline

Homologous recombination and cancer

Stable structural color patterns displayed on transparent insect wings

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Contributed by Daniel H. Janzen, November 24, 2010 (sent for review October 5, 2010)

Color patterns play central roles in the behavior of insects, and are important traits for taxonomic studies. Here we report striking and stable structural color patterns—wing interference patterns (WIPs)—in the transparent wings of small Hymenoptera and Diptera, patterns that have been largely overlooked by biologists. These extremely thin wings reflect vivid color patterns caused by thin film interference. The visibility of these patterns is affected by the way the insects display their wings against various backgrounds with different light properties. The specific color sequence displayed lacks pure red and matches the color vision of most insects, strongly suggesting that the biological significance of WIPs lies in visual signaling. Taxon-specific color patterns are formed by uneven membrane thickness, pigmentation, venation, and hair placement. The optically refracted pattern is also stabilized by microstructures of the wing such as membrane corrugations and spherical cell structures that reinforce the pattern and make it essentially noniridescent over a large range of light incidences. WIPs can be applied to map the micromorphology of wings through direct observation and are useful in several fields of biology. We demonstrate their usefulness as identification patterns to solve cases of cryptic species complexes in tiny parasitic wasps, and indicate their potentials for research on the genetic control of wing development through direct links between the transregulatory wing landscape and interference patterns we observe in *Drosophila* model species. Some species display sexually dimorphic WIPs, suggesting sexual selection as one of the driving forces for their evolution.

Generation of complex pigmentation patterns by insects is currently an active research front (1–3), with insights into the morphogenetic control of pigment spots in wings of a *Drosophila* model species (4) (Fig. 1 *J* and *K*) underpinning principles for coloration and repeated regulatory evolution that are potentially broadly applicable beyond insects (5–7). Parallel studies of structural insect colors with repeated functional morphology and multiple functions of simple structures (8–10) have recently expanded into a major research area (11–14) that is predominantly focused on larger organisms such as butterflies (14–16), beetles (17), and damselflies (18). Here we merge these two fields by showing structural wing color patterns in the transparent wings of small wasps (Hymenoptera) and flies (Diptera). Given favorable light conditions, they display a world of brightly patterned wings (Fig. 1) that are apparently unnoticed by contemporary biologists. The color patterns are the effect of thin film interference; about 20% of incoming light beams are reflected from a single extremely thin and transparent layer with a refractive index of chitin (13). The remaining 80% of the light goes through the wing. Any animal with color vision can see these color patterns when the wing reflections are not overpowered by strong background reflections. The strength of their appearance in natural conditions depends on the balance between light reflections from the wing and from the background. The intensity of the background reflections in nature varies from 0% (pitch black background, Fig. 1 *A*, *D*, and *E*) to 100% (pure white background or toward a light source), but will normally be similar to a green leaf, where the wing reflections are readily observed (Fig. 1 *B*, *C*, and *F*). In laboratory conditions most wings are studied against a white background (Fig. 1 *G*, *H*, and *J*), or the wings are embedded in a medium with a refractive index close to that of chitin (e.g., ref. 19). In both cases the color reflections will be faint or invisible.

Insects are an exceedingly diverse and ancient group and their signal-receiver architecture of thin membranous wings and color vision was apparently in place before their huge radiation (20–22). The evolution of functional wings (Pterygota) that can be freely operated in multidirections (Neoptera), coupled with small body size, has long been viewed as associated with their extreme diversity (20). With selection acting to decrease the size of wing membranes that are reinforced for aerodynamic function by membrane corrugations, hair placement, and venation, there has been simultaneous reinforcement of an optically refracted and stable color reflection. This reflection, coupled with the early evolution of trichromatic UV-blue-green perception by the insect compound eye (22), has along with pigmentation (2, 4, 23), transformed wings into visual communication posters for those who can see their colors.

The color sequence reflecting from transparent insect wings was discovered and published before Darwin's theory of evolution (24), but it has later been disregarded as a soap bubble iridescence effect, with randomly changing colors flashing over the wing surface (25). Taxonomic monographs for Hymenoptera and Diptera typically describe wings as transparent, with or without pigmented areas, but with no mention of structural color patterns (e.g., refs. 26, 27). However, we have found that these small transparent wings almost universally display stable and essentially noniridescent structural color patterns that are often taxon-specific. The patterns are visible and stable at various angles of view in live insects in nature (Fig. 1 *A*–*F*) as well as on 100-year-old dry museum specimens (Fig. 1*K*).

Discussion

Two-Beam Wing Interference Patterns (WIPs). The wings of most insects are mainly composed of two layers of transparent chitin compressed to a single membrane (Fig. 2 *I*–*K*) with a refractive index of approximately 1.57 (14). In air, these dimensions are ideal for two-beam thin film interference, whereby light beams reflect from the upper and lower surfaces of the membrane (13). The thickness of the composite chitinous membrane varies with the topography of the wing, and the areas of different thickness reflect different interference colors that together produce a specific color pattern, the WIP. The sequence of colors in WIPs of Hymenoptera (Fig. 2 *F*, *G*, and *M*) and Diptera (Fig. 2*A*) is regular and identical to the Newton series reflected from a thin film

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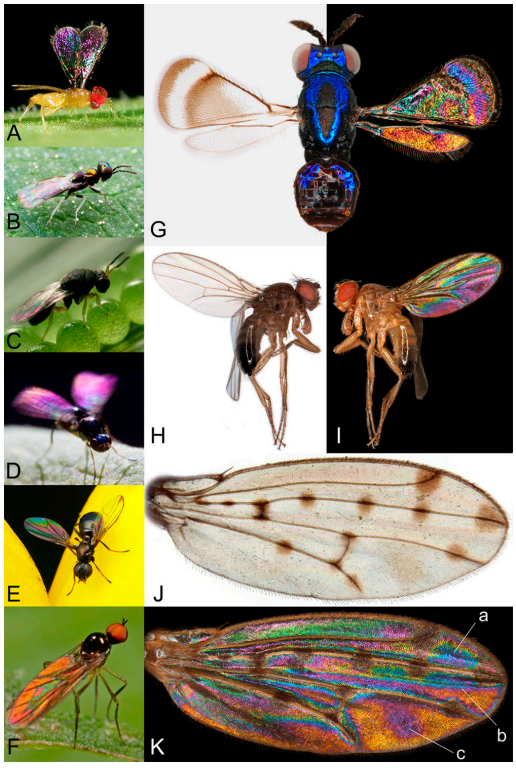

Fig. 1. WIPs in Hymenoptera and Diptera. (*A–F*) Examples of WIP displays under natural conditions. Note that the habit of the majority of small wasps and flies to fold their wings over each other and over the dark-colored abdomen at rest will aid to create a darker background for the wing on top. (*A*) This *Chrysonotomyia* sp. (Eulophidae) from Costa Rica exposes its forewings and displays the WIP against a black background. (*B*) A resting *Chrysocharis* sp. (Eulophidae), USA. (*C*) A resting *Neorileya* sp. (Eurytomidae), Costa Rica. (*D*) This *Archisepsis diversiformis* (Sepsidae) from Costa Rica creates a strong visual communicative signal in colors by active and specific wing movements, a typical behavior for members of the family. (*E*) Another unidentified Sepsidae from USA displaying a very different WIP. (*F*) A male *Ocydromia glabricula* (Hybotidae) from the Netherlands displaying its WIP in a green environment. (*G*) This greatly enlarged composite image of *Closterocerus coffeellae* (Eulophidae, female collected in Colombia) illustrates the dramatic effect of changing background reflections on WIP visibility. The left side wing displays its pigmentation pattern against a light reflecting white background whereas the right side wing displays its WIP reflection against a light absorbing black background. (*H–I*) A freshly killed wild male *Drosophila melanogaster* from Sweden shows the same effect. Only the background is changed between photos in (*H*) and (*I*) (reflected). (*J–K*) Right wing of the model taxon *Drosophila guttifera* (Drosophilidae). This wing is of the male holotype, collected in Florida and described by F. Walker in 1849 (Courtesy of NHM London). (*J*) The distinct spots along the veins and weak intervein color shades are currently being subjected to intensive morphogenetic research (4). (*K*) WIP image of the same wing as it appears simply by viewing it against a black background. A relevant question is whether the pigmentation is formed partly or mainly to control the WIP, such as the blue preapical spot (a) that is framed and demarcated by three pigment spots. The longitudinal division of the wing disc into anterior and posterior compartments associated with the regulators *engrailed* and *hedgehog* is visible as a distinct color transition (b). The *intervein shade cis*-regulatory element (see ref. 4) can be directly associated with the distinct magenta spot (c). (Photo *A*, *C*, and *D* courtesy of Kenji Nishida; *B* and *E* courtesy of Alex Wild; and *F* courtesy of Klaas van der Veen).

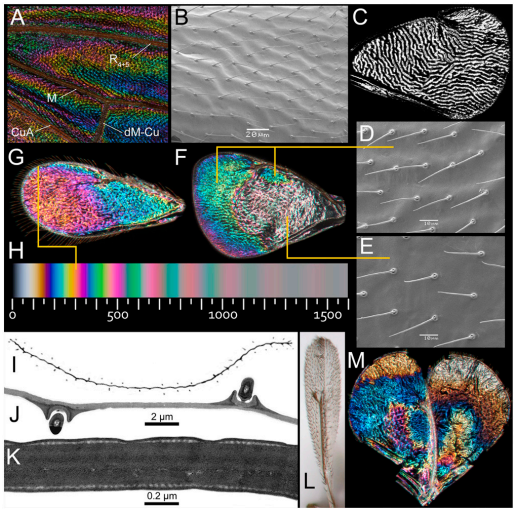

Fig. 2. Structural features of the wings in small chalcidoid wasps and *Drosophila* that produce strong noniridescent WIPs. (*A*) Midsection of wing membrane of *D. melanogaster* showing reflecting ridges along rows of microtrichia with nonreflecting troughs between them. Dense microtrichia produce a pebbled membrane surface. Vein abbreviations are taken from ref. 27. (*B–C*) Forewing of *Chrysocharis* sp. *B*. SEM image displaying its corrugation ridges with rows of setae. (*C*) Duotone image enforcing the topography of the membrane corrugations (as in a fingerprint). Note how the arrangement of corrugations gradually changes from anterior-posterior in the basal part to radial in the apical part—most likely for aerodynamic purposes and to give functional strength to the different wing parts. The corrugations simultaneously serve to reinforce the reflected WIP and eliminate the iridescence due to a dioptric stabilization of the convex ridges. (*D–F*) Forewing of a male *Achrysocharoides latreillei* demonstrating structural differentiation and the resulting WIP. (*D*) SEM image of corrugated parts. (*E*) SEM image of smooth central part. (*F*) Resulting WIP with colorful parts where the wing membrane is thin and corrugated, and the weakly reflecting central part where the wing membrane is thick and smooth. (*G*) WIP of female *Asecodes congruens*. The yellow line shows where the cross section was made for TEM images (*J–K*) and the corresponding match on the Newton scale (*H*). (*H*) Computer generated (Adobe® 1998 RGB rendered) Newton series scale of two-beam interference colors (28) calibrated for the refractive index of chitin (1.57) viewed in air at perpendicular angle of light incidence. The scale gives approximate thickness of a wing membrane in nanometres. (*I*) Composite duotone image of a whole apical cross section of the forewing of *Achrysocharoides atys*, showing the waves of sinuous corrugations with dots of crossed hairs on both sides. (*J–K*) TEM images of cross sections of the apical part of a forewing from the same species as in G, a freshly killed specimen was used for the TEM images to avoid possible artificial changes in the wing structure caused by drying or alcohol treatment. (*J*) Wing section showing how the wing membrane is uniform and extremely thin compared with two hair sockets on upper and lower membranes. (*K*) Enlarged section showing how the dorsal and ventral membranes are fused together to form a single thin film. The membrane thickness is between 308–317 nm, which is a perfect match with the color transition yellow-magenta as observed and in the Newton scale (compare to panels *G* and *H*). (*L*) Left "balloon" forewing of male *Omphale* sp. in frontal view. This condition sometimes occurs when recently emerged insects are killed in alcohol before the membranes are properly fused together and alcohol penetrates between them; this makes it possible to "open" the wing and observe the dorsal and ventral membranes separately. (*M*) Unequal organisation of dorsal (*Left*) and ventral (*Right*) membranes and resulting WIPs in an opened left forewing of a male *Achrysocharoides platanoidae*. The dorsal membrane produces the main WIP (as normally observed) whereas the ventral membrane has an unclear pattern that reinforces the main pattern when merged with the dorsal membrane. See Fig. 3*B* for a forewing of *A. platanoidae* in its natural condition where a distinct blue spot with yellow border is found along the apical margin. In the opened wing the color of the spot switches to yellow due to approximately half thickness of the dorsal membrane. A switch of colors occurs throughout the wing due to reduced thickness (compare to scale in panel *H*).

of oil on water (25, 28). The Newton series is a very characteristic sequence of repeated color bands grouped into orders. The first three Newton orders (up to 550 nm wing membrane thickness, see Fig. 2*H*) display a near complete scale of spectral colors, except for pure red, whereas the next higher orders (with increasing wing thickness) reflect a repeated sequence of nonspectral (to the human eye) magentas and greens that gradually fade into uniform pale gray. Those of the second and third order are the brightest in the scale. This ordered color sequence makes it possible to reciprocally calculate and map membrane thickness in the range between *ca.* 50 and 1500 nanometres when compared with a Newton series scale (Fig. 2 *A*, *F–H*, and *M*).

The fore- and hindwings of Hymenoptera are coupled together into one functional unit during flight and during what we suspect are WIP displays. The hindwing pattern usually forms an extension of the WIP from the forewing or sometimes displays its own characteristic details (Fig. 3), just as is the case with the pigment-based and scale-based patterns on Lepidoptera wings. In wings of small chalcidoid wasps (body length less than 3 mm) there are membrane corrugations that form regularly spaced parallel ridges about 20 μm apart (Fig. 2 *B* and *D*), with rows of setae along the tops of ridges. Diptera have only one pair of functional wings and the corrugation ridges usually occur in association with regular rows of microtrichia. These are typically spaced about 10–15 μm apart in the middle of spherical cell structures, as in *Drosophila* (29). The more pebbled interference patterns suggest spherical reflection around each microtrichium (Fig. 2*A*).

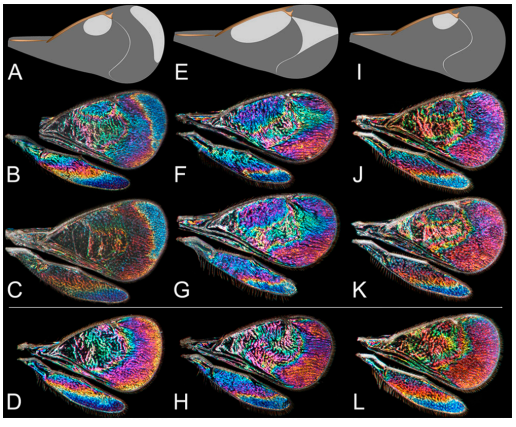

Fig. 3. WIPs of three species of genus *Achrysocharoides* (Hymenoptera: Eulophidae). Male wings (above the line) and female wings (below the line). While sorting a collection of *Achrysocharoides*, several males with a distinct blue spot in the WIP were discovered. Further investigations revealed a case of two cryptic species (*A. acerianus* and *A. platanoidae*) and extending the investigation resolved another case of three cryptic species (*A. gahani*, *A. robiniae*, *A. robinicolus*) (38). All species were initially separated using male WIPs, but other morphological differences in combination with acquired new biological data confirmed the hypothesis of species delimitation. *A. platanoidae* and *A. robiniae* have sexually dimorphic WIPs despite having transparent wings without any pigmentation and from the classical point of view males and females have identical wings. (*A–D*) *A. platanoidae*. (*A*) Schematic illustration of the distinctive small spot in the corner between the marginal vein and the stigmal vein and the larger marginal spot along the apical edge, which is blue in the WIP. (*B*) WIP of male (UK, 1999). (*C*) WIP of male (Sweden, 2007). (*D*) WIP of female (Sweden, 1981). (*E–H*) *A. robiniae*; all collected in Hungary, 2002. (*E*) Schematic illustration of the distinctive large spot along marginal vein and the extended spot in the apical part, which is green in the WIP. (*F–G*) WIP of males. (*H*) WIP of female. (*I–L*) *A. robinicolus*; all collected in USA, 2002. (*I*) Schematic illustration of the distinctive small spot in the corner between the marginal and the stigmal veins and lack of pattern in the apical part. (*J–K*) WIP of males. (*L*) WIP of female.

Whereas the microstructures of the wing membrane are somewhat different in Hymenoptera and Diptera, the resulting effect is the same: essentially noniridescent coherent scattering (cf. 8). The old report (25) of highly variegated colorings randomly mingled, with housefly wing changing color as the angle of vision changes, is wrong. We find almost no iridescence unless the light is narrowly concentrated in one direction at a slight angle to the surface. The stable noniridescent patterns that we see can be explained by the convex ridges of a corrugated (Fig. 2 *C* and *I*) or pebbled (Fig. 2*A*) wing membrane that act as diopters to stabilize the interference reflection and eliminate the iridescence effect over a large range of light incidences (8, 9). Contrary to the iridescence of a flat thin film, the strongly microstructured wing membrane appears noniridescent, both under different ring light illuminations and in natural outdoor light.

Pigmented areas and the rigidity of wing veins contribute to stabilize the wing color pattern, contributing frames for the WIPs of different wing segments and the wing overall. The WIPs may reciprocally display the vein system and emphasize the pigment patterns (Figs. 1, 4, 5, and 6). In species with smoky or semitransparent pigmented wings, the WIP loses its characteristic metallic shine (e.g., Fig. 6*G*), and it may not appear if pigments are capturing the light (e.g., Fig. 5 *P–R*). In species with large individuals, the reticulate system of veins compartmentalizes and supports the wing such that it remains strong while simultaneously being thin enough to produce WIPs in the areas framed by the veins. For example, wings of some Braconidae and Ichneumonidae wasps display cell-specific WIPs that are different from those of other adjacent compartments (Fig. 6 *N–P*). As a species evolves smaller individuals, the wing vein system is commonly reduced. In the smallest wasps, but those having a wing large enough to display a WIP, the veins are confined to the anterior wing margin, leaving the wing membrane as a seemingly large empty space (about the size of a wing cell on a larger wasp or fly). To stabilize such a vein-free wing there are extensive supporting corrugations and thickenings of the membrane (Fig. 2 *B–E*). These features form structural patterns that display WIPs based on the three first Newton orders, which are created by membrane thickness from 100 to about 600 nm (Fig. 2*H*). In sum a taxon-specific WIP reflects a complex of micromorphological features of the wing (uneven membrane thickness, corrugations, setae arrangement, pigmentation, venation) framed by a specific wing shape (Fig. 5, 6).

Genetic Control of the WIP. The complex black pigment patterns that are repeatedly evolved in many groups of Diptera are formed and controlled by a set of spatiotemporal on/off switches for the single gene *yellow* (6, 7) and sometimes also involve other genes and physical wing traits (2, 4). An increasing body of evidence demonstrates direct parallels between development and regulation of wing patterns in distantly related groups such as *Drosophila* and butterflies (2, 7, 23, 30).

WIPs add an additional dimension and morphological diversity palette to the now emerging "repeated regulating evolution" model (5). WIPs mean that wing pigmentation (4) is only a part of the story. Other morphogenetic elements are responsible for the regulation of membrane thickness, formation of membrane corrugations, hair placement (29), venation pattern (31), and other traits. The transregulatory wing landscape (32, 33) illustrates how different genes, *cis*-regulatory elements (33), and wing landmarks (4) (e.g., veins, bumps, troughs, slopes, hairs) may work together to form the wing and create/stabilize the size, location and nature of a specific WIP. A specific WIP may be the analogue to a pigment field or complex that performs a specific function, such as are false eye spots (34). For example, the longitudinal division of the wing disc into the anterior-posterior compartments associated with the regulators *engrailed* (32) and *hedgehog* (23), is directly reflected in WIPs. There is a distinct color shift indicating a transition line in membrane thickness

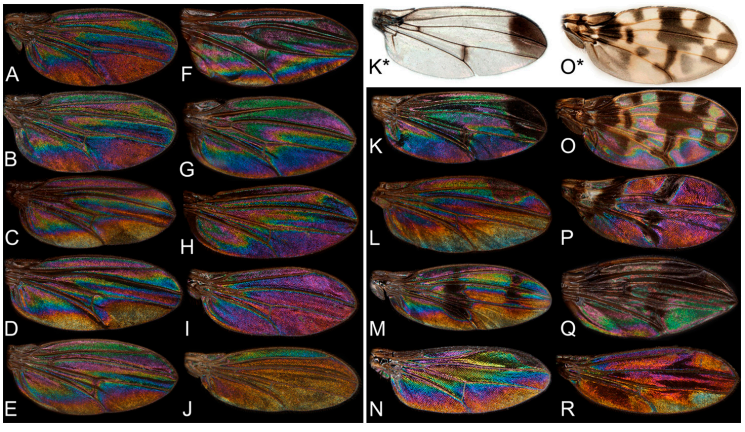

Fig. 4. WIP diversity across Drosophilidae. With wing lengths from 1.5 to 3.5 mm, these WIPs are mainly found within the three first Newton orders; i.e. membrane thickness up to about 600 nm (compare to Fig. 2*H*). The left half (*A–J*) shows wings without pigment patterns whereas the right half (*K–R*) shows wings with pigment patterns. (*A–B*) *Drosophila melanogaster* (Laboratory breed Canton-S, A = male, B = female). (*C–D*) *Drosophila obscura* (Sweden, C = male, D = female). (*E*) *Drosophila kuntzei* (female, Germany). (*F*) *Amiota magna* (female, Japan). (*G*) *Mycodrosophila gratiosa* (female, Japan). (*H*) *Sphaerogastrella javana* (male, Sri Lanka). (*I*) *Liodrosophila globosa* (male, Sri Lanka). (*J*) *Scaptomyza* sp. (male, Peru). (*K*) *Zygotricha* sp. (female, Ecuador, K* = on white background). *L*. *Drosophila pulchrella* (male, Japan, only the males have this preapical pigment spot). (*M*) *Chymomyza amoena* (male, Canada). (*N*) Drosophilidae indet. (male, Sierra Leone). (*O*) *Drosophila calloptera* (female, Peru, O* = on white background). (*P*) *Mycodrosophila* sp. (female, Peru). (*Q*) *Leucophenga digmasoma* (female, Borneo). (*R*) *Threocephala inornata* (male, Sri Lanka).

as observed in *Drosophila guttifera*, *Drosophila melanogaster* and several related species (Fig. 1*K*, 4 *A–E*, *G*, *L*, and *M*). In chalcidoids a proximal-apical division of the WIP is commonplace (Figs. 2 *D–F* and 3). In the case of "balloon wings" (Fig. 2*L*), where the dorsal and ventral wing membranes are unfused, there are clear differences between the WIPs of these two surfaces (Fig. 2*M*). The dorsal membrane is thicker and produces the actual interference pattern, whereas the ventral membrane displays a vague gradient. It appears that genetic control of the dorsal membrane has an active role in producing the WIP whereas the ventral membrane appears more passive, a parallel with the higher evolutionary rates of dorsal wing patterns found in butterflies (35).

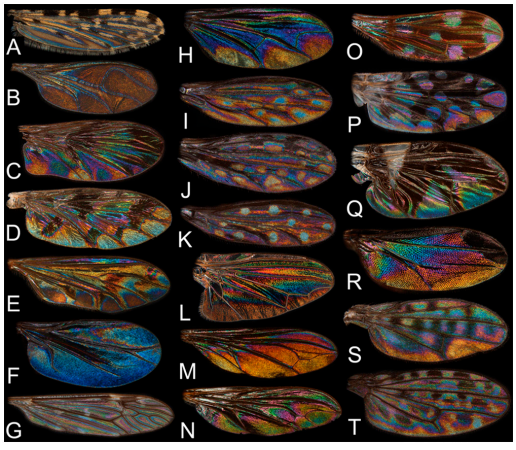

Fig. 5. WIP diversity across Diptera. The first row (*A–G*) displays lower Diptera ("Nematocera"), the second row (*H–N*) displays lower flies whereas the last row (*O–T*) displays higher flies (Acalyptrata). (*A*) Culicidae, *Anopheles melas* (female, Ghana). (*B*) Sciaridae, *Zygoneura* sp. (male, Japan). (*C*) Keroplatidae, *Macrocera fascipennis* (male, Sweden). (*D*) Keroplatidae, *Proceroplatus* sp. (male, Honduras). (*E*) Lygistorrhinidae, *Lygistorrhina pictipennis* (male, Japan, note that the M fork appears complete here despite the veins being gone except for apical parts of M1 and M2). (*F*) Scatopsidae, *Swammerdamella brevicorne* (female, Sweden). (*G*) Tipulidae, *Tipula confusa* (male, Sweden). (*H*) Dolichopodidae, *Condylostylus* sp. (female, Canada). (*I*) Empididae, *Dolichocephala guttata* (female, Sweden). (*J*) Empididae, *Dolichocephala irrorata* (female, Sweden). (*K*) Empididae, *Dolichocephala ocellata* (male, Sweden). (*L*) Platypezidae, *Paraplatypeza atra* (male, Sweden). (*M*) Pipunculidae, *Eudorylas obscurus* (male, Sweden). (*N*) Pipunculidae, *Nephrocerus scutellatus* (male, Sweden). (*O*) Diopsidae, *Teleopsis rubicunda* (male, Philippines). (*P*) Tephritidae, *Actinoptera discoidea* (male, Sweden). (*Q*) Tephritidae, *Rhagoletis pomonella* (male, USA). (*R*) Chloropidae, *Chloropsina* sp. (female, Malaysia). (*S*) Ephydridae, *Paralimna* sp. (female, Ghana). (*T*) Ephydridae, *Limnellia quadrata* (male, Sweden).

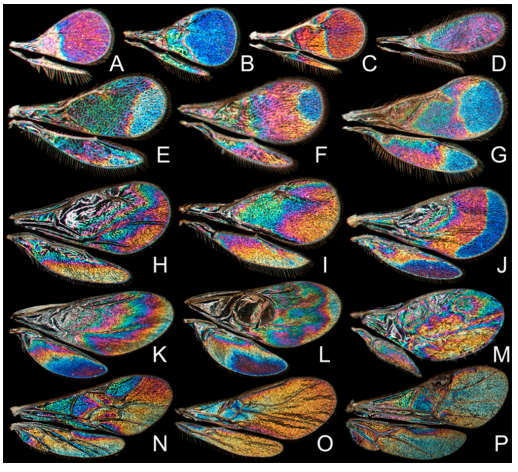

Fig. 6. WIP diversity in small Hymenoptera. (*A–C*) Trichogrammatidae, three unidentified species (females, Costa Rica). (*D*) Mymaridae (male, Sweden). (*E*) Aphelinidae (female, Malaysia). (*F*) Aphelinidae (female, Costa Rica). (*G*) Aphelinidae (female, Malaysia). (*H*) Pteromalidae, *Pteromalus* sp. (male, United States). (*I*) Eulophidae, *Omphale clypealis* (female, Spain). (*J*) Torymidae, *Idiomacromerus* sp. (male, Turkey). (*K*) Encyrtidae, *Cerchysius* sp. (female, Canada). (*L*) Encyrtidae, *Microterys* sp. (female, Greece). (*M*) Agaonidae (female, Brazil). (*N*) Ichneumonidae (female, Sweden). (*O*) Braconidae, Dacnusiini (female, Sweden). (*P*) Braconidae, *Meteorus* sp. (female, Sweden).

WIP Diversity and Stability. The majority of the more than 17,000 species of butterflies can be distinguished by their wing color patterns (16, 30), though it is also the case that many of these seemingly species-specific color patterns may be in common throughout complexes of visually "identical" sibling species (36). Our observations of WIPs suggest that species identification in many groups of Hymenoptera and Diptera is enhanced if WIPs are added to the set of taxonomic characters. These two orders are estimated to contain far more than twenty times the number of butterfly species (21, 37). This diversity remains unknown partly due to difficulties in distinguishing morphologically similar species (e.g., ref. 37), also known as "cryptic species" (which often means "not readily distinguishable by a large diurnal mammal with a microscope"). In a recent paper (38) we described cryptic species in the chalcidoid genus *Achrysocharoides* (wasp family Eulophidae). Five species, three of which were described as new, were initially separated by relying exclusively on distinctive male WIPs (Fig. 3) and subsequently confirmed as distinct species through finding additional differences in morphology and biology. Wings of chalcidoid wasps have long been regarded as poor in features because most species lack pigment patterns. WIPs as morphological characters will aid their identification and species discovery.

The fly family Drosophilidae ranks among the most studied organisms and displays excellent interspecific variation in WIPs (Fig. 4) and low intraspecific variation. When we compared WIPs from closely related *Drosophila* species, we found the overall pattern to be interspecifically similar but with distinct features for each species (Fig. 4 *A*–*E* and *L*). A superficial visual survey of Diptera (Fig. 5) and Hymenoptera (Fig. 6) wings encounters a diverse colorful array in all small wings (and to some degree in individual wing cells of large wings). There is a wide variety of kinds of WIPs from unicolored to elaborate patterns and spots. The claim that fly (e.g., ref. 2) and wasp wing patterns are no match for the incredible diversity of colorful butterfly wing patterns is obsolete.

WIPs, just as are other traits, are intraspecifically variable and phenotypically plastic. However, our preliminary impression is that they are largely uniform among conspecifics and often appear to be characteristic of a species, at least to the degree encountered in other insect color patterns. An evolutionary or environmentally induced change in wing size may affect the thickness of the membrane, thereby displacing the sequence of colors within the same WIP. The stable pattern may be more relevant to taxonomy and the insect than is the hue or color sequence. For example, the intraspecific variation of WIP in a sample ($n = 20$) from a laboratory bred Canton-S strain of *D. melanogaster* is small with a moderate size-dependent color displacement (Fig. 4 *A* and *B*). In this case, the wings of males had less variable WIPs than did those of females, despite the larger variation in size of male wings.

We have encountered sexual dimorphism in WIPs in species with completely transparent wings such as parasitic *Achrysocharoides* wasps and in those with pigment patterns (e.g., *Drosophila*, Fig. 4*L*). This dimorphism may either be a result of difference in size (usually large female, small male) affecting the hue but with the same pattern in both sexes or, the more indicative, with different patterns between the sexes (Fig. 3 *A*–*H*). The latter case, with species-specific and sexually dimorphic patterns, suggests that sexual selection is one of the driving forces for the evolution of these patterns. When the males and females of the same species have identical WIPs (Fig. 3 *I*–*L*), but differ in other external morphological characters, the WIPs can be used to match the sexes.

WIP Perspectives for Biodiversity Studies. WIPs are an additional and overlooked trait for identifying and discovering (especially cryptic) species, just as have been DNA barcodes (e.g., ref. 36). Two-dimensional patterns on a flat wing are technically straightforward to document and analyze with pattern recognition software tools (39) and couple well with wing morphometrics (40). For phylogenetic classifications, WIPs are promising unexplored traits that can be used to visually map wing topography and measure wing membrane thickness. WIPs may reflect different types of microstructural arrangements in the wing such as nearly flat or strongly corrugated membranes and attendant membrane gradients (Fig. 5 *A*, *S*, and *L*). Alternatively they may independently cross over venation patterns (Fig. 5 *M* and *R*). The strong demarcation of the vein system via narrow color transitions along vein margins (e.g., Fig. 5 *B* and *H*) has been unrecognized and offers a unique functional and phylogenetic perspective to wing venation; it may even indicate the location and extension of wing veins that have been lost during evolution (Fig. 5*E*).

Behavioural, ecological, morphological, and evolutionary studies of insects with small wings will benefit from the discovery of WIPs in that they probably function in intra- and interspecific signaling. If so, they may be one more of the functionally dependent traits that may block evolutionary changes being driven by quite unrelated selective forces, such as wing aerodynamics, speed of wing hardening following adult eclosion, wing weight and durability. There is a definite possibility that some of the variation in membrane thickness, corrugations, pigmentations and venation reticulations has its adaptive value partly or solely in the WIPs they produce. If WIPs are truly important in the biology of insects, rather than being a byproduct of other physical traits (as is the case with inanimate oil slicks), they may in turn be one of the driving evolutionary processes affecting the nature of wing venation reticulation, with all its seemingly nonsensical variation among insects (which is commonly attributed to need for wing strength).

Wing displays play a central role in visual courtship communications in several families of Diptera (3, 19, 32, 41–44) as well as many other insects, and have been suggested as one of the drivers of the initial evolution of the insect wing (45). However, all research to date on the evolution of Diptera wings and courtship has focused solely on pigment patterns—phrased by the authors as "evolution in black and white" (3, 6, 19). Butterflies, where females may prefer males with bright structural ornamentations, emphasizing intraspecific selection as the driving force (46), reveal one intriguing difference when compared with Hymenoptera and Diptera. Whereas only a few larger species of these two orders are known to have red eye receptors, such receptors are much more common in the Lepidoptera, especially among butterflies (22), as are red butterfly scales produced by multilayer interference or red pigments (9). The Newton series color sequence displayed in single layer WIPs excludes pure red and fits most small insects' trichromatic UV-blue-green color vision (22), including those with transparent wings. Among flies, attraction to blue and green light in the dark may be stronger than attraction to UV light and red light (47). These observations suggest that the biological significance of WIPs is for visual signaling, including intraspecific recognition by their bearers.

Some peculiar behavior involving wings in small species of Hymenoptera may be explained through WIP display. For instance, why do females of pollinating fig wasps hold their unpigmented wings straight up in the air (48), like billboards, when walking on the fig as they arrive? When the female enters the fig's fruit-like reproductive structure (syconium) through the very tight opening (ostiole), the wings break off. A drop of liquid is excreted from the end of the abdomen and glues the wings into a protruding and visible position. This may be a species-specific signal that the syconium is now occupied, and the WIPs of these wings may be a part of the signal. Newly eclosed 2–3 mm long tropical microgastrine braconid wasps (37) raise their seemingly pattern-free transparent wings and wave them when encountering a sib while walking in the rearing container. Again, the WIPs may be part of the signal.

WIPs appear to be cheap visual signals, though wing thickness, setae, or other traits that modify a WIP may have strategic as

well as materials costs. Unlike moths and butterflies, where color patterns are made with complex scales and pigments, the WIPs of transparent wasp and fly wings appear to be of low cost. For the receiver of the signal, developing and maintaining photoreceptor systems are believed to be very energy consuming and demonstrate clear trade-offs between energy consumption and performance (49, 50). Crepuscular to nocturnal insects use dim light (51) and have evolved attraction to dark or contrasting dark/white swarm markers (e.g., ref. 52). WIPs perceived by insects may be a cheap complement to the unavoidable cost of having a color-sensitive receiving system (49).

WIPs offer opportunities for evo-devo studies that connect wing biophysics and topography to morphogenetics and regulating evolution. Colorful species-specific WIPs are, in contrast with DNA barcodes from highly conserved genes (36), traits that may have major behavioural importance to the insects bearing them, as well as be serendipitous byproducts of other traits. Wasps and flies are very species-rich and small (53), and their extremely thin wings are therefore ideal for displaying WIPs. The WIP is potentially a major contribution to the toolbox for evolution of small insects with transparent wings and thus an important piece of the evolutionary puzzle.

Methods

To acquire precisely comparable data for wing thickness and taxonomy, observations of WIPs were standardised by arranging the wing horizontally against a black background and viewing it at close to perpendicular incident angles under white ring light. Photos of dry and flattened wings were then taken with a 5MP Nikon DS-L1 camera unit on Nikon stereomicroscopes (SMZ1000 and SMZ1500) fitted with ring lights. The camera unit was white-balanced for a white background before the wings were arranged against a black background and captured with the exposure compensation adjusted down by 1–2 stops. Image processing in Photoshop was restricted to cropping, adding up to 10% saturation, shading the background all black and in some cases neutralizing dust from the wing surface with the spot-healing brush tool. Except when indicated otherwise, all wings are of dry specimens from museum collections.

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Paper II

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Article

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Three new species of *Achrysocharoides* Girault (Hymenoptera: Eulophidae) parasitoids of *Phyllonorycter* spp. (Lepidoptera: Gracillariidae) on *Acer platanoides* and *Robinia pseudoacacia*

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Abstract

Three new species of *Achrysocharoides* are described, one from northern Europe, *A. platanoidae* **sp. nov.**, one from central Europe and the U.S.A., *A. robiniae* **sp. nov.**, and one from the U.S.A**.,** *A. robinicolus* **sp. nov.** The descriptions are based on material reared from microlepidopterans of the genus *Phyllonorycter* Hübner (Gracillariidae): *A. platanoidae* from *P. platanoidella* (Joannis) on *Acer platanoides*, and *A. robiniae* and *A. robinicolus* from *P. robiniella* (Clemens) on *Robinia pseudoacacia* (black locust). The new species are very similar to previously described species, *A. platanoidae* to *A. acerianus* (Askew), and *A. robiniae* and *A. robinicolus* to *A. gahani* (Miller), but they are shown here to differ from their sibling species both in biology and in external morphology. The host of *A. robiniae* and *A. robinicolus*, *P. robiniella*, is a serious pest on the black locust tree in Europe, and the descriptions with diagnoses of these two species, and their scientific names, introduced here will aid the biological control efforts of this pest.

Key words: taxonomy, Chalcidoidea, Entedoninae, leafminer parasitoids, Achrysocharoides acerianus, Achrysocharoides platanoidae, Achrysocharoides robiniae, Achrysocharoides robinicolus, Achrysocharoides gahani, Phyllonorycter robiniella, Phyllonorycter geniculella, Phyllonorycter platanoidella, black locust, Acer pseudoplatanus, biological control

Introduction

Achrysocharoides Girault (Chalcidoidea: Eulophidae: Entedoninae) was originally described based on an Australian species (Girault 1913a) but its main distribution is now known to be the northern hemisphere (Bouček & Askew 1968; Burks 1979; Kamijo 1990a, 1990b, 1991). Worldwide, *Achrysocharoides* comprises 54 described species. Including the new species described here, 22 species are now known from Europe and 20 from North America.

Species of *Achrysocharoides* are unusually host specific. Most species of Entedoninae are polyphagous with broad host ranges (Askew & Shaw 1979; Hansson 1985), but *Achrysocharoides* species select their hosts among a very limited number of host species. They are larval endoparasitoids of leaf mining moths of the family Gracillariidae (Lepidoptera), mainly of species in *Phyllonorycter* Hübner (Askew & Ruse 1974). Apart from choosing their hosts from a very narrow range of moth species, the hosts are usually selected from only a few and related plant genera (Lopez-Vaamonde *et al.* 2005).

Two of the *Achrysocharoides* species described here are parasitoids of *Phyllonorycter robiniella* (Clemens), a small moth native to North America that is monophagous on black locust, *Robinia pseudoacacia* (Šefrov 2002). The black locust is a legume tree native to central and eastern U.S.A. (Mabberley 1997) that was introduced into most European countries (Polunin 1969). The introductions started in the beginning of the 17th century (Stojanović & Marković 2005) and the tree was introduced as an ornamental, for its resistant wood and fragrant flowers (used for honey and perfumery) (Polunin 1969). It is an economically important plant especially in central Europe (Melika *et al.* 2006). Since its introduction in Europe this tree has been considered free from serious pests. However, in 1983 *P. robiniella* was discovered in Switzerland (Whitebread

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1990) and since then it has spread rapidly through most of Europe (Šefrová 2002). The moth larva constructs a blotch mine in the leaf and several larvae can be present in one leaf (Stojanović & Marković 2005), causing considerable damage that can cause premature leaf-fall. Because of the economic importance of black locust several investigations have been carried out to find natural enemies of *P. robiniella*. Lists of parasitoid complexes of the moth have been published from Hungary (Melika *et al.* 2006), Serbia (Stojanović & Marković 2005), and Switzerland (Girardoz *et al.* 2007). All these investigations have included *Achrysocharoides* species among the parasitoids on *P. robiniella*, and in one investigation (Melika *et al.* 2006) this genus was reported as dominant.

Material and methods

The majority of the specimens of *Achrysocharoides* from *Acer* spp. accounted for in this paper were collected in southern Sweden during the summer of 2007. Leaves with mines of *Phyllonorycter* spp. were collected from *Acer platanoides* and *A. pseudoplatanus* and processed in the lab. The areas of the leaves with mines were cut out from the leaf to prevent mold from forming, and thereafter were kept in polythene bags. When the imagines emerged from the mine they were killed and kept in 80% ethanol. The specimens were later dried using a critical point drier and mounted on cardboard rectangles as described by Noyes (1982). All imagines from *Acer* emerged during the summer they were collected. All material of *Achrysocharoides* from *P. robiniella* was borrowed from the collections listed below.

The SEM photos were made from uncoated specimens on their original cardboard mounting. This was possible to do in low vacuum mode on a JEOL JSM 5600LV SEM microscope. The colour photos were taken through a Nikon SMZ 1000 microscope with Nikon camera equipment DS-L1 & DS-5M. Each photo was made by merging several photos taken at different focus levels using the software Helicon Focus version 4.75.5 Pro.

The ratios are calculated based on measures from the holotype, and from a paratype with same label data as the holotype for the other sex.

The terminology used here follows Gibson *et al.* (1997).

Morphological abbreviations and acronyms

HE = height of eye; HW = height of forewing; LG = length of gaster; LM = length of marginal vein; LW = length of forewing, measured from base of marginal vein to apex of wing; MM = length of mesosoma; MS = malar space; OOL = distance between one posterior ocellus and eye; PM = length of postmarginal vein; POL = distance between posterior ocelli; POO = distance between posterior ocelli and occipital margin; ST = length of stigmal vein; WH = width of head; WM = width of mouth; WT = width of thorax. For illustrations of the morphological terms see www.neotropicaleulophidae.com.

BMNH = Natural History Museum, London, England; CH = collection of Christer Hansson, Lund, Sweden; CNC = Canadian National Collection of Insects, Arachnids and Nematodes, Ottawa; CAES = Connecticut Agricultural Experiment Station, New Haven, U.S.A.; GG = collection of Giselher Grabenweger; HNHM = Hungarian Natural History Museum, Budapest; LUZM = Lund University Zoological Museum, Sweden; NHMV = Natural History Museum, Vienna, Austria; PDL = Pest Diagnostic Laboratory, Plant Protection and Soil Conservation Directorate of County Vas, Hungary.

Results

Achrysocharoides Girault

Achrysocharoides Girault, 1913a:168. Type species *Chrysocharis sarcophaga* Girault, 1913b:99, by original designation.

Neoderostenus Girault, 1913a:144. Type species *Neoderostenus australiensis* Girault, 1913a:144, by original designation. Synonymized by Peck (1951:464).

Enaysma Delucchi, 1954:1. Type species *Enaysma zwoelferi* Delucchi, 1954, by original designation. Synonymized by Yoshimoto (1977:907).

Diagnosis. Frontal suture almost straight, in females often replaced by a transverse ridge; males with a more or less developed frontal cross-carina (i.e. with a carina just below toruli), especially well developed in males with a strongly transverse head; eyes usually densely pubescent; pronotal collar without or with a transverse carina; forewing with postmarginal vein about as long as stigmal vein; petiole short with posterior raised portion short, never longer than broad.

Identification. To separate *Achrysocharoides* from other Eulophidae genera the keys in the following publications can be used: Bouček (1988) (Australasia), Gibson *et al.* (1997) (Nearctic), Graham (1959) (Europe). The key to European genera has a partly outdated nomenclature, e.g. *Achrysocharoides* is there referred to as *Enaysma* — now a synonym under *Achrysocharoides*, but this is the only available key for Europe. To separate the species-groups of *Achrysocharoides* the key in Kamijo (1991) can be used, and in the same publication there are detailed diagnoses for each species-group — this is excluding the *crassinervis*-group which is diagnosed in Kamijo (1990b).

Species-groups. The subdivision of *Achrysocharoides* was initiated by Graham (1959) who divided the European species into two subgenera, *Enaysma* Delucchi and *Pentenaysma* Graham. These correspond with the two species-groups, *atys*- and *latreilleii*-groups, which Bryan (1980) introduced for the European species, thus abandoning the formal subdivision into subgenera. Yoshimoto (1977) divided the Nearctic species into two species-groups, the *gahani*- and *guizoti*-groups. Kamijo (1991) transferred some of the Nearctic species from the *guizoti*-group to either of the two newly erected *clypeatus*- and *titiani*-groups, and removed the remaining species in the *guizoti*-group to the *latreilleii*-group, thus terminating the *guizoti*-group. Kamijo (1990b) established the *crassinervis*-group for two species from Japan and one undescribed species from Nepal. Hence there are currently six species-groups in *Achrysocharoides*: *atys*-, *clypeatus*-, *crassinervis*-, *gahani*-, *latreilleii*-, and *titiani*-groups. The two species described here belong to the *gahani*-group (*A. robiniae* and *A. robinicolus*) and the *latreilleii*-group (*A. platanoidae*) respectively.

Descriptions

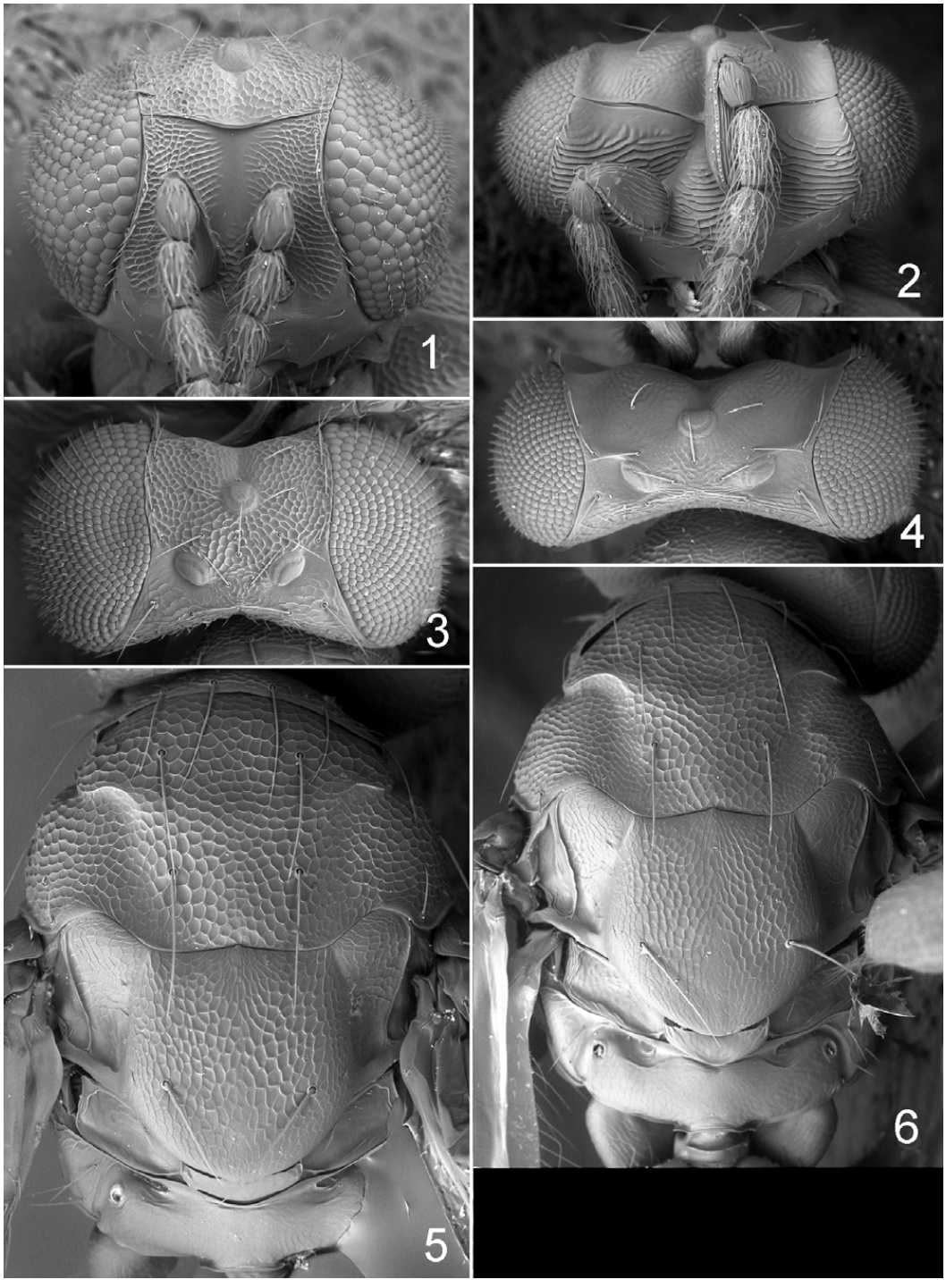
***Achrysocharoides platanoidae* sp. nov.**
(Figs 1–12, 49, 63–64)

Diagnosis. *Achrysocharoides platanoidae* is similar to *A. acerianus* (Askew) but females differ in having the pedicel predominantly white to yellowish-white with base infuscate to brown (Fig. 49) (predominantly to completely infuscate in *A. acerianus*, Fig. 50), the hind coxa white with base infuscate to golden-green (Fig. 11) (basal third to half metallic in *A. acerianus*, Fig. 23), and longer flagellomeres, e.g. flagellomeres 1–3 together 5.4X as long as wide (Fig. 63) as compared to 4.4X as long as wide in *A. acerianus* (Fig. 61). Males also differ in having longer flagellomeres, e.g. flagellomeres 1–4 together 9.4X as long as wide (Fig. 64) as compared to 8.5X as long as wide in *A. acerianus* (Fig. 62) and the hind coxa white with the base infuscate to golden-green (Fig. 12) (basal third to half metallic in *A. acerianus*, Fig. 24).

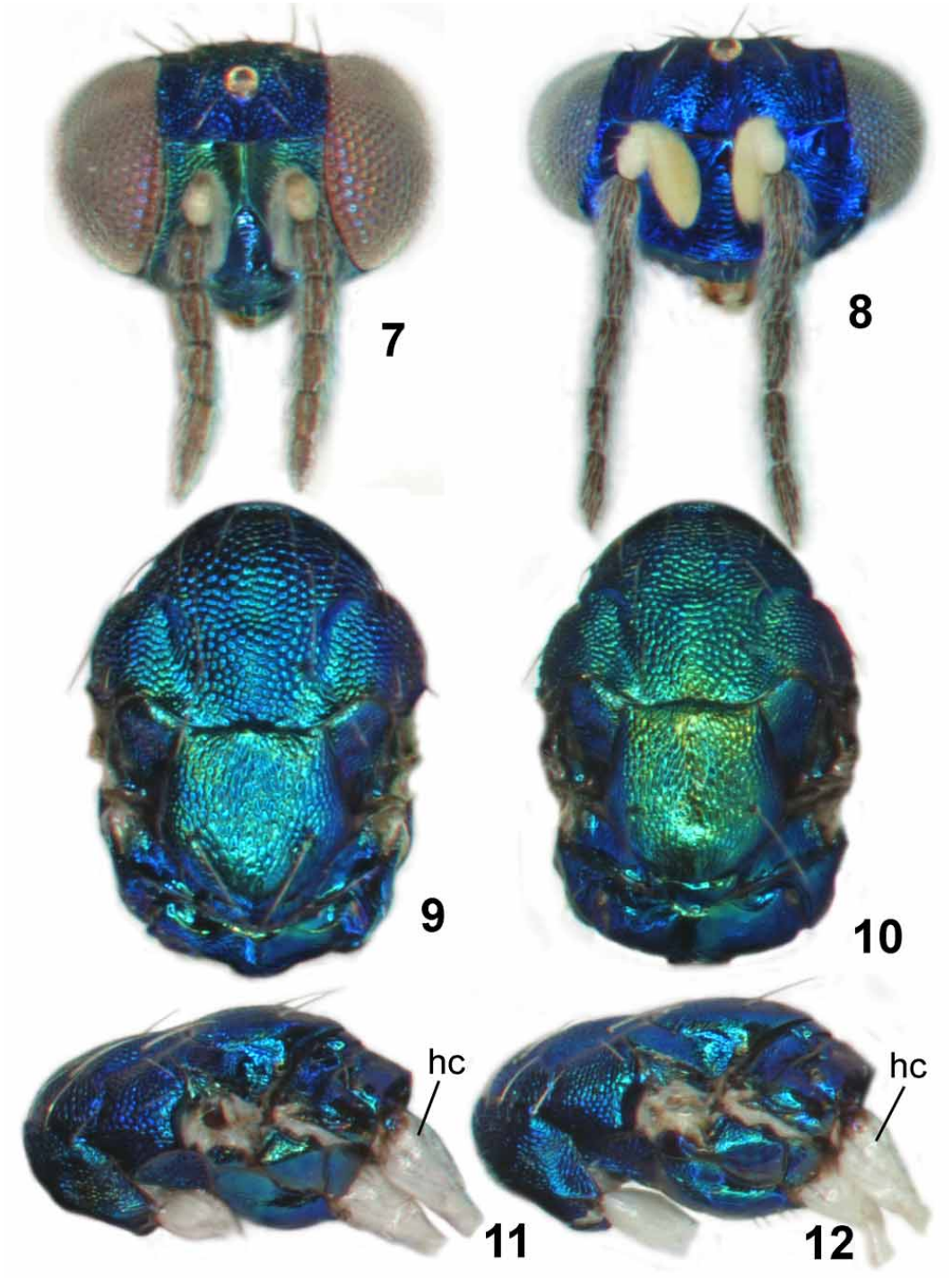
Description. FEMALE. Length 1.1–1.6 mm.

Scape white; pedicel white to yellowish-white with base infuscate to brown; flagellum dark brown. Frons below frontal suture golden-green with blue tinges, above frontal suture metallic blue. Vertex metallic bluish-green. Mesoscutum, scutellum and propodeum metallic bluish-green. Legs white, hind coxa with base infuscate to golden-green. Wings hyaline. Gaster metallic bluish-green.

Antenna as in Fig. 63. Frons with raised and strong reticulation, antennal scrobes smooth. Vertex inside ocellar triangle with raised and strong reticulation, outside ocellar triangle with raised and weak reticulation, partly smooth. Occipital margin rounded.



FIGURES 1–6. *Achrysocharoides platanoidae* **sp. nov.** 1. Head frontal, female. 2. Head frontal, male. 3. Vertex, female. 4. Vertex, male. 5. Thoracic dorsum, female. 6. Thoracic dorsum, male.



FIGURES 7–12. *Achrysocharoides platanoidae* **sp. nov.** 7. Head frontal, female. 8. Head frontal, male. 9. Thoracic dorsum, female. 10. Thoracic dorsum, male. 11. Mesosoma lateral, female. 12. Mesosoma lateral, male. Abbreviation: *hc* = hind coxa.

Mesoscutum with raised and strong reticulation; notauli as indistinct impressions in posterior 2/3. Scutellum with raised and strong reticulation, without scutellar pits. Dorsellum slightly convex, almost flat, and smooth. Propodeum smooth and shiny; propodeal callus with three setae. Forewing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 3.2/1.0/1.6; POL/OOL/POO = 5.7/2.3/1.0; WH/WT = 1.2; LW/LM/HW = 1.8/1.0/1.2; PM/ST = 0.8; MM/LG = 0.8–0.9.

MALE. Length 1.3–1.7 mm.

Scape and pedicel yellowish-white; flagellum dark brown. Frons below level of toruli golden-green, above level of toruli metallic blue. Vertex golden-green. Mesoscutum and scutellum golden-green or metallic bluish-green. Propodeum metallic bluish-green. Legs white, hind coxa with base infuscate to golden-green. Wings hyaline. Gaster with first tergite with anterior 1/4 golden-green, posterior 3/4 dark brown, anteromedially with a large white spot; remaining tergites 3/4 dark brown.

Antenna as in Fig. 64. Frons below level of toruli smooth and shiny, between level of toruli and frontal suture with strong and transverse striae, above frontal suture medially with raised and rather weak reticulation and close to eyes smooth. Vertex inside ocellar triangle with engraved and weak reticulation, outside ocellar triangle predominantly smooth. Occipital margin rounded.

Mesoscutum with raised and strong reticulation; notauli as indistinct impressions in posterior 2/3. Scutellum with raised and strong reticulation, without scutellar pits. Dorsellum slightly convex, almost flat, and smooth. Propodeum smooth and shiny; propodeal callus with three setae. Forewing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 1.8/1.0/1.1; POL/OOL/POO = 2.3/1.0/1.1; WH/WT = 1.3; LW/LM/HW = 1.9/1.0/1.3; PM/ST = 0.5; MM/LG = 0.9–1.1.

Distribution. Sweden and the United Kingdom.

Host. *Phyllonorycter platanoidella* (Joannis) (Lepidoptera: Gracillariidae) on *Acer platanoides*.

Material examined. Holotype female labeled SWEDEN: Skåne, Silvåkra, 55°41'N, 13°30'E, 26.vi.2007, C. Hansson & E. Shevtsova (LUZM). Paratypes: 17 females 5 males with same label data as holotype (BMNH, CH, LUZM, NHMV); 1 male from same locality as holotype but collected 17.x.1981 (CH); 1 male "SWEDEN: Skåne, Lake Kranke, Lottagården, 55°42'N, 13°29'E, 1.vii.2007, C. Hansson" (CH); 5 females "SWEDEN: Skåne, Torna Hällestad, 55°41'N, 13°25'E, 18.vii.1981, C. Hansson" (CH, LUZM); 5 females, 1 male from same locality as previous but collected 16.x.1981 (CH, BMNH); 7 females "SWEDEN: Skåne, Dalby, 9.vii.1981, C. Hansson" (CH, LUZM); 2 males "SWEDEN: Skåne, Höör, Jularp, 22.vii.1979, C. Hansson" (CH); 4 females 3 males "UNITED KINGDOM: Berkshire, Ascot, Silwood Park [no date], C.L. Vaamonde" (BMNH). All specimens are reared from *Phyllonorycter platanoidella* on *Acer platanoides*.

Identification. To include *A. platanoidae* in the latest key to European *Achrysocharoides* (Bryan 1980) the following additions should be made for females:

6. Hind coxa white with basal 1/3 to1/2 metallic 7
- Hind coxa white with only dorso-basal 1/5 metallic 7a
7. Scape white with inner-apical part pale brown; on *Acer* ..... *acerianus* (Askew)
- Entire scape white or yellowish-white; on *Quercus* ..... *latreilleii* (Curtis)
7a. Pedicel white to yellowish-white with base infuscate to brown (Fig. 49) ..... *platanoidae* **sp. nov.**
- Pedicel dark brown as flagellomeres ..... *butus* (Walker)

and for males:

10. Scutellar pits absent and mesoscutum with strong reticulation (Figs 6, 18) 10a
- Scutellar pits usually present (see fig. 1 in Bryan 1980), but if absent then mesoscutum with weak reticulation ... 11
10a. Flagellomeres 1–4 together 9.4X as long as wide (Fig. 64); hind coxa with dorso-basal 1/5 infuscate to metallic (Fig. 12) ..... *platanoidae* **sp. nov.**
- Flagellomeres 1–4 together 8.5X as long as wide (Fig. 62); hind coxa with basal 1/3 to 1/2 metallic (Fig. 24) ..... *acerianus* (Askew)

Etymology. Named after the host plant, *Acer platanoides*, of its lepidopteran host.

Remarks. Askew & Ruse (1974) mention some additional material under the treatment of *A. acerianus*, two males and eight females from *Phyllonorycter acerifoliella* (Zeller) on *Acer campestre*. These specimens were not included in the description of *A. acerianus*, and hence not included in the type material, but were nevertheless regarded as conspecific with *A. acerianus*. This material differed from "typical" *A. acerianus* in having completely pale hind coxae and slightly longer funicle segments. We were not able to examine these specimens but in view of the diagnostic characters given above, it is possible that they belong to *A. platanoidae*, or to a new species close to *A. platanoidae.*

The species cited as *A. acerianus* in Lopez-Vaamonde *et al.* (2005) is actually *A. platanoidae*, and the gene sequences accounted for in that paper, and deposited in GeneBank, concern *A. platanoidae* not *A. acerianus*.

***Achrysocharoides acerianus* (Askew)**
(Figs 13–24, 50, 61–62)

Enaysma aceriana Askew *in* Askew & Ruse, 1974:264. Holotype female in BMNH, examined.
Achrysocharoides acerianus (Askew); Bouček & Graham (1978:232).

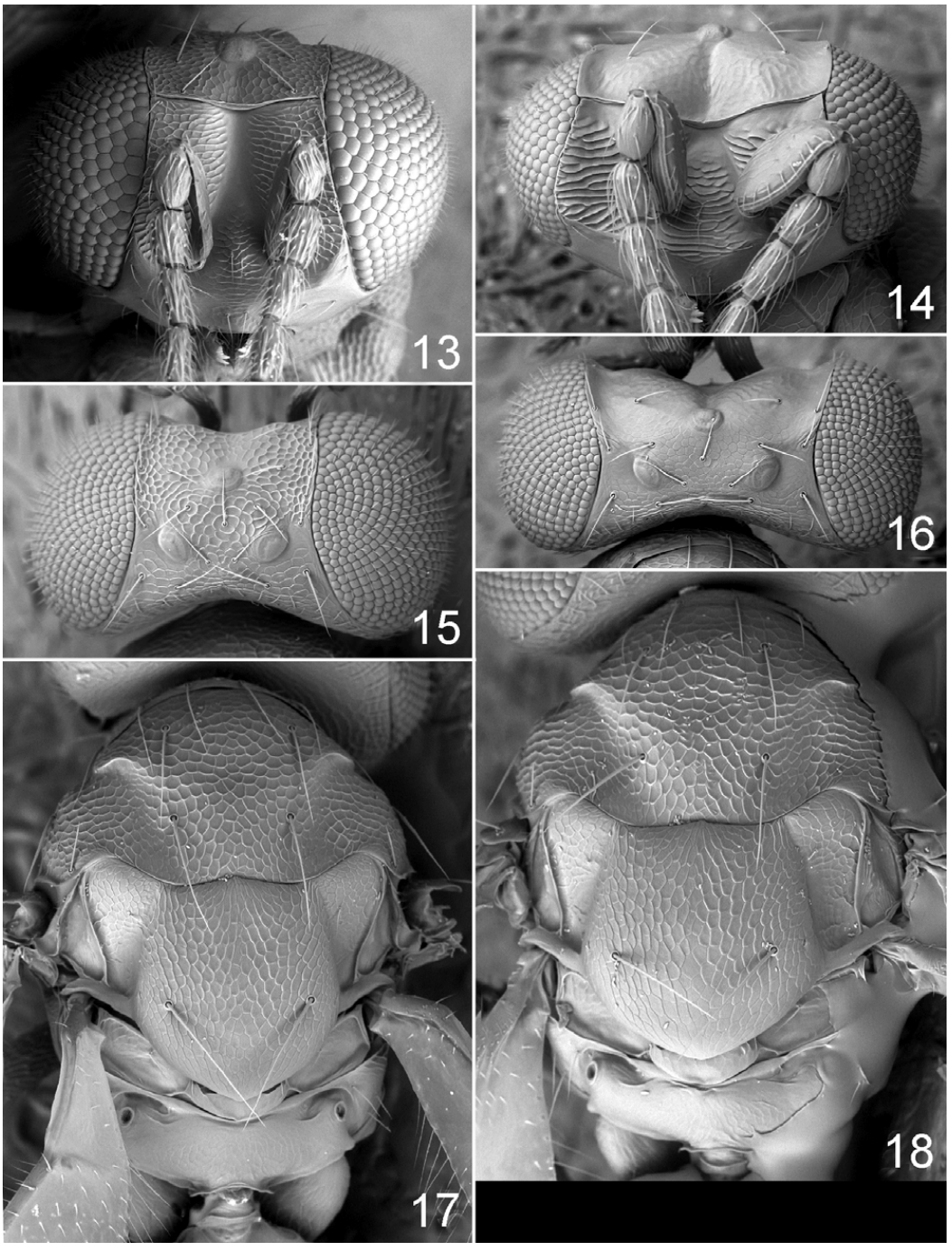
Hosts. *Phyllonorycter geniculella* (Ragonot) (Lepidoptera: Gracillariidae) on *Acer pseudoplatanus*.

Material examined. SWEDEN: Skåne, 155 females and 54 males from *Phyllonorycter geniculella* on *Acer pseudoplatanus* (CH).

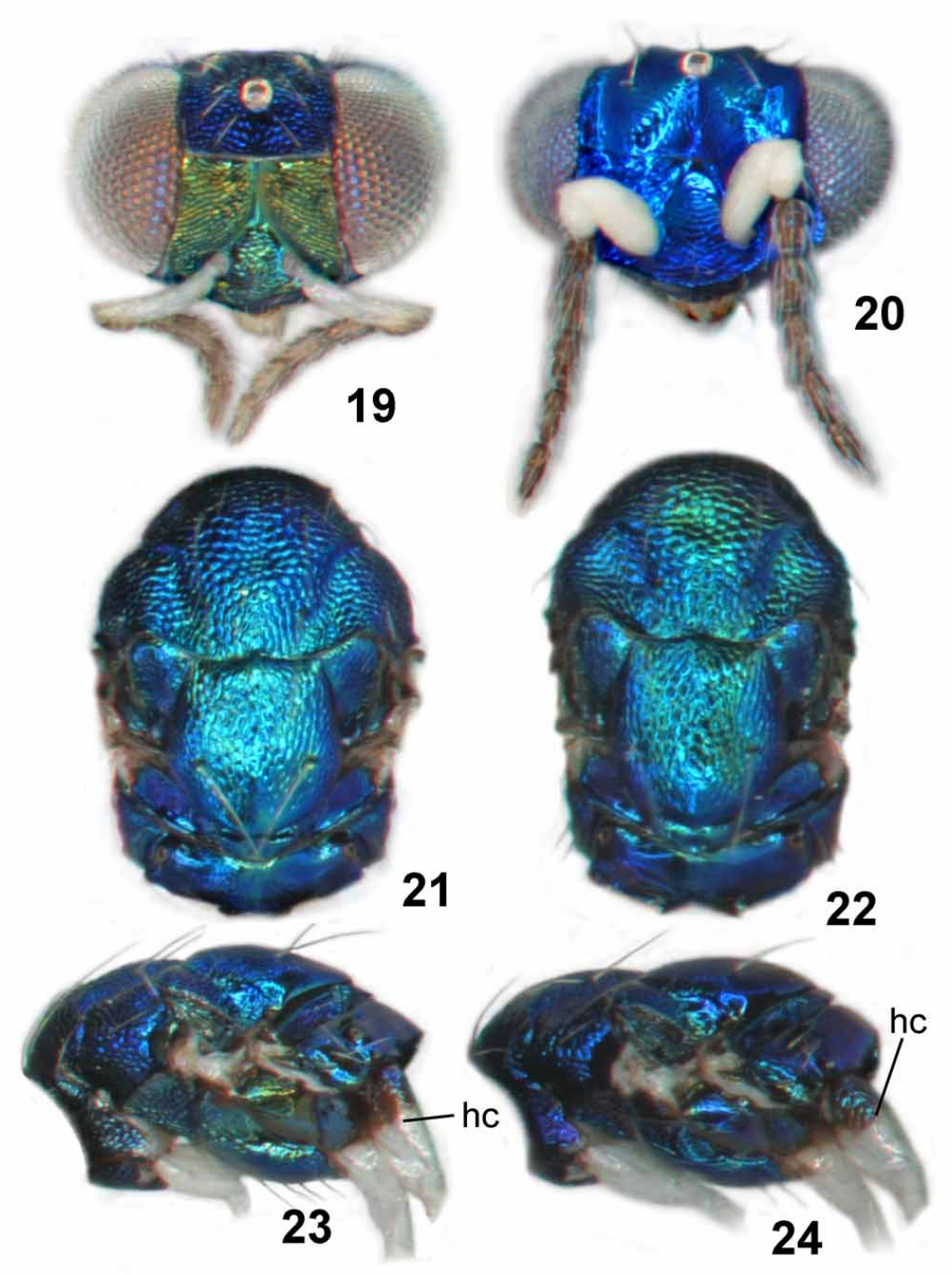
Remarks. The species cited as *Achrysocharoides sp.* from *Acer pseudoplatanus* in Lopez-Vaamonde *et al.* (2005) is *A. acerianus*, and the gene sequences accounted for in that paper, and deposited in GeneBank, are for *A. acerianus*.

***Achrysocharoides robiniae* sp. nov.**
(Figs 25–36, 51–52, 56, 59, 65–66)

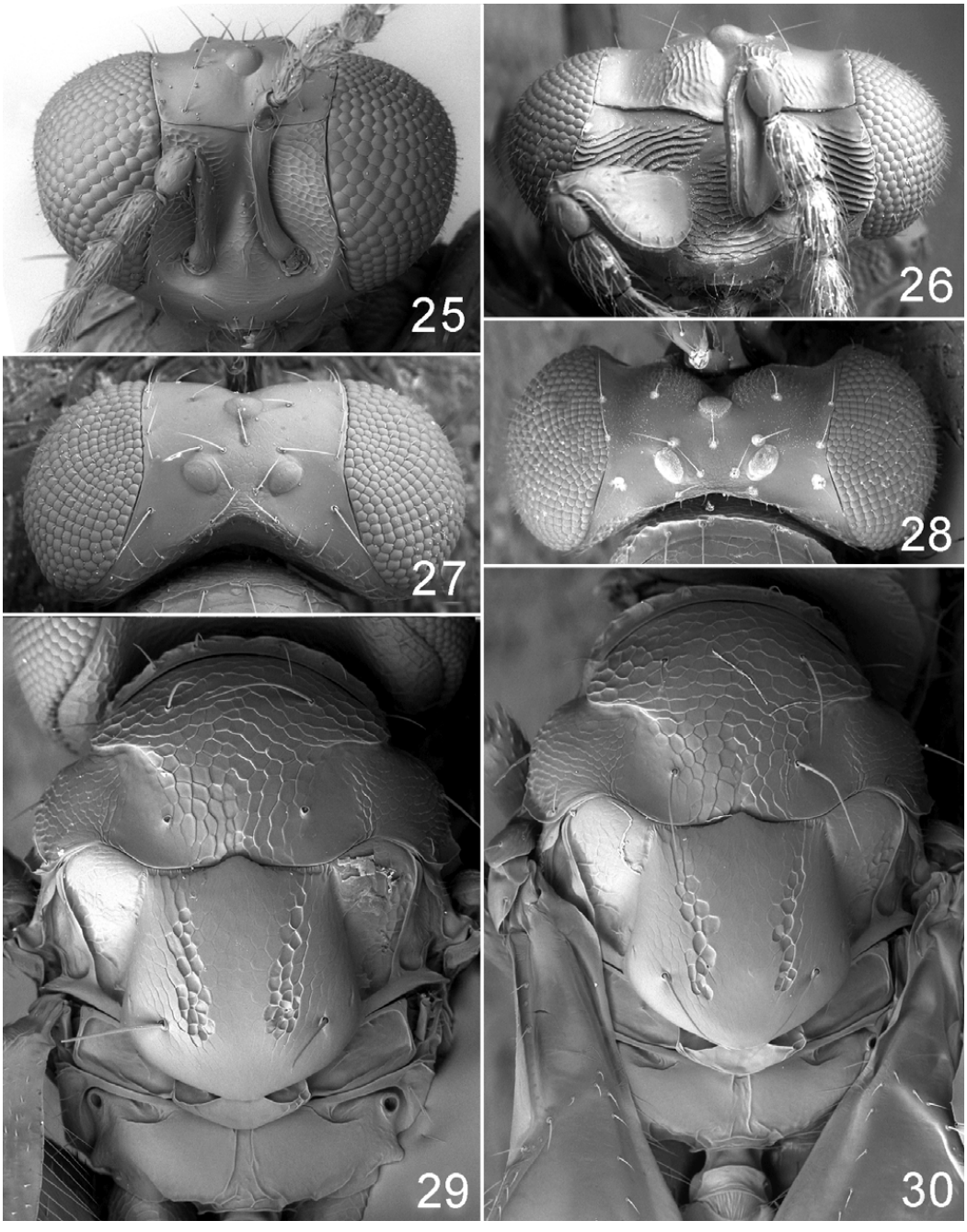
Diagnosis. *Achrysocharoides robiniae* belongs to the *gahani*-group sensu Kamijo (1991), i.e. with the pronotal collar sharply margined (Figs 29–30), the occipital margin carinate (Figs 27–28), the propodeum with submedian carinae that diverge posteriorly (Figs 29–30), and the male scape widest at base (Figs 32, 66). These features differentiate *A. robiniae* from all known European species of *Achrysocharoides*. This group also includes *A. gahani* (Miller), *A. reticulatus* Yoshimoto, *A. villosus* Kamijo, and the new species *A. robinicolus* described below, from North America, and *A. littoralis* Kamijo from Japan. *Achrysocharoides robiniae* differs from all but *A. gahani* and *A. robinicolus* in having the following combination of characters: scutellum more or less smooth with rows of punctate-reticulate pits on each side (Figs 29–30) (*A. littoralis* and *A. villosus* with scutellum completely reticulate without pits), and forewing rounded (*A. reticulatus* with forewing truncate). We are currently unable to distinguish females of *A. gahani* and *A. robiniae* from each other with certainty, but males do differ morphologically. Males of *A. robiniae* and *A. robinicolus* have the frons below the frontal suture bright blue (Figs 32, 44, 51–52), whereas males of *A. gahani* have this part bright green (Figs 53–54), males of *A. robiniae* and *A. robinicolus* have the white anteromedian spot on gaster with posterior margin straight (spot is shaped like a pentagon or a triangle respectively) and extending over tergites 1 and 2 (Figs 56–57, 59–60), whereas males of *A. gahani* have posterior margin of this spot rounded (spot is oval-shaped), extending over tergites 1–3 (Figs 55, 58). The host information is also an important diagnostic tool to separate *A. robiniae* and *A. robinicolus* from *A. gahani*, as accounted for below in the discussion. *Achrysocharoides robiniae* is very similar to *A. robinicolus* but differs in having hind coxae completely white in both sexes (Figs 35–36) (coxae completely white also in *A. gahani*, but base brown to metallic in both sexes of *A. robinicolus*, Figs 47–48), a wider male scape (Fig. 66) which is 1.8X as long as wide (holotype) (3.0X as long as wide in holotype of *A. robinicolus*, Fig. 68), white anteromedian spot in male gaster shaped like a pentagon (Figs 56, 59) (shaped like a triangle in *A. robinicolus*, Figs 57, 60).



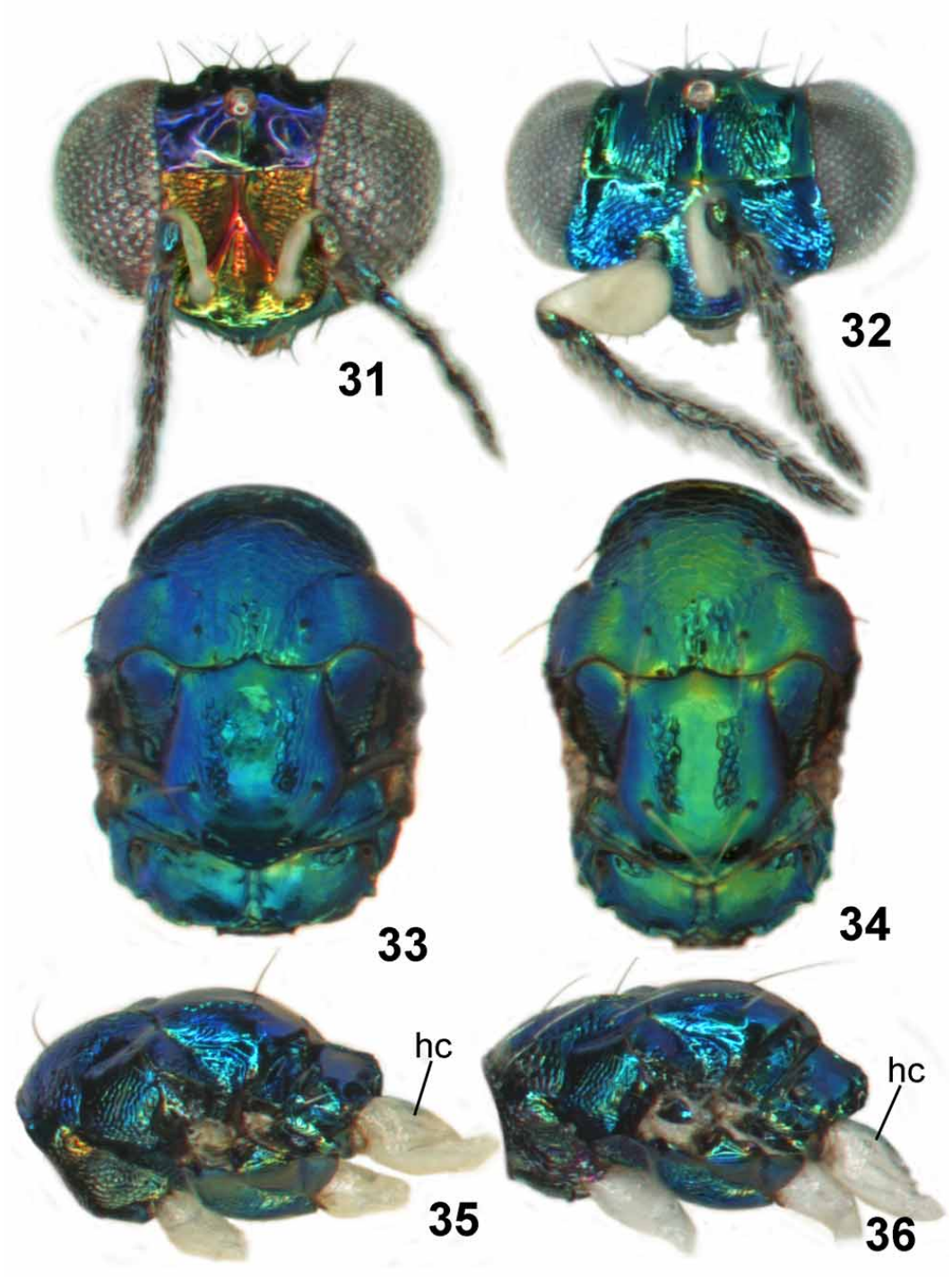
FIGURES 13–18. *Achrysocharoides acerianus* (Askew). 13. Head frontal, female. 14. Head frontal, male. 15. Vertex, female. 16. Vertex, male. 17. Thoracic dorsum, female. 18. Thoracic dorsum, male.



FIGURES 19–24. *Achrysocharoides acerianus* (Askew). 19. Head frontal, female. 20. Head frontal, male. 21. Thoracic dorsum, female. 22. Thoracic dorsum, male. 23. Mesosoma lateral, female. 24. Mesosoma lateral, male. Abbreviation: *hc* = hind coxa.



FIGURES 25–30. *Achrysocharoides robiniae* **sp. nov.** 25. Head frontal, female. 26. Head frontal, male. 27. Vertex, female. 28. Vertex, male. 29. Thoracic dorsum, female. 30. Thoracic dorsum, male.



FIGURES 31–36. *Achrysocharoides robiniae* **sp. nov.** 31. Head frontal, female. 32. Head frontal, male. 33. Thoracic dorsum, female. 34. Thoracic dorsum, male. 35. Mesosoma lateral, female. 36. Mesosoma lateral, male. Abbreviation: *hc* = hind coxa.

Description. FEMALE. Length 1.0–1.4 mm.

Scape white; pedicel and flagellum dark brown with metallic blue shine, pedicel with ventral side pale. Frons below level of toruli golden-green, between level of toruli up to frontal suture golden-green to golden-red, antennal scrobes golden-red, above frontal suture metallic bluish-purple. Vertex inside ocellar triangle golden-red, outside ocellar triangle golden-green with blue tinges. Mesoscutum and scutellum metallic bluish-green. Propodeum golden-green. Legs white. Wings hyaline. Gaster with first two tergites metallic green, remaining tergites dark brown with metallic tinges.

Antenna as in Fig. 65. Frons below level of toruli smooth and shiny, between level of toruli and frontal suture with raised and strong reticulation with antennal scrobes smooth, above frontal suture smooth and shiny. Vertex inside ocellar triangle with engraved and very weak reticulation, outside ocellar triangle smooth and shiny. Occipital margin with a sharp edge behind ocellar triangle.

Pronotal collar with a sharp carina. Mesoscutum with midlobe with raised and strong reticulation, sidelobes with fine and weak reticulation; notauli as smooth impressions in posterior 2/3. Scutellum smooth and shiny with rows of punctate-reticulate pits on each side. Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny with two submedian carinae which are more or less parallel and diverging posteriorly; propodeal callus with 3–4 setae. Forewing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 4.2/1.0/2.0; POL/OOL/POO = 1.6/1.4/1.0; WH/WT = 1.3; LW/LM/HW = 1.7/1.0/1.0; PM/ST = 1.1; MM/LG = 1.0.

MALE. Length 0.9–1.4 mm.

Scape yellowish-white; pedicel dark brown with ventral side white; flagellum dark brown with golden-green shine. Frons below level of toruli golden-green or golden-red, above level of toruli metallic blue. Vertex inside ocellar triangle golden-red, outside metallic blue. Mesoscutum and scutellum golden-green with blue tinges. Propodeum golden-green with red tinges. Legs white. Wings hyaline. Gaster with first tergite metallic green, anteromedially with a white spot shaped like a pentagon that extends over tergites 1 and 2; remaining tergites dark brown with metallic tinges.

Antenna as in Fig. 66. Frons below level of toruli smooth and shiny, between level of toruli and frontal suture with strong and transverse striae, above frontal suture medially with raised and rather weak reticulation and close to eyes smooth. Vertex inside ocellar triangle with engraved and very weak reticulation, outside ocellar triangle smooth and shiny. Occipital margin with a sharp edge.

Pronotal collar with a sharp carina. Mesoscutum with midlobe with raised and strong reticulation, sidelobes with fine and weak reticulation; notauli as smooth impressions in posterior 2/3. Scutellum smooth and shiny with rows of punctate-reticulate pits on each side. Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny with two submedian carinae which more or less parallel and diverging posteriorly; propodeal callus with 3–4 setae. Forewing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 2.1/1.0/1.3; POL/OOL/POO = 3.3/2.5/1.0; WH/WT = 1.3; LW/LM/HW = 1.6/1.0/1.0; PM/ST = 1.0; MM/LG = 1.2–1.6.

Distribution. Austria, Germany, Hungary, Italy, U.S.A.

Host. *Phyllonorycter robiniella* (Clemens) (Lepidoptera: Gracillariidae) on *Robinia pseudoacacia*. In three previous investigations of the parasitoids associated with *P. robiniella* two different species of *Achrysocharoides* were reported. Stojanović & Marković (2005) and Melika *et al.* (2006) reported *A. cilla* (Walker) as a parasitoid of *P. robiniella*, and Navone (2003) and Girardoz *et al.* (2007) reported *A. gahani* from the same host, although Navone was not adamant in his identification and left the possibility open for alternate interpretations. We examined material used in Navone (2003) and Melika *et al.* (2006) and this material belongs to *A. robiniae*. So very likely neither *A. cilla*, nor *A. gahani* are parasitoids on *P. robiniella*.

Material examined. Holotype male labeled "HUNGARY: Pest Co., Gödöllö, 14.viii.2003, Balács Klára", "Robinia pseudoacacia", "ex Phyllonorycter robiniella, em. 1.ix.2003" (HNHM); 2 females with same label data as holotype (HNHM); 9 females 6 males" HUNGARY: Vas Co., Meszlen, 6.viii.2002, leg. I. Mikó", "Ex. Phyllonorycter robiniella" (BMNH, CH, PDL); 6 females 5 males "HUNGARY: Vas Co., Köszeg, Als-erd,

23.vi.2002, leg, Zs. Pénzes", "Ex. Phyllonorycter robiniella" (BMNH, CH, PDL); 2 females 5 males" AUSTRIA: Vienna, Laaer Berg, 48°10'N, 16°24'E, 20.vi.2007", "Phyllonorycter robiniella, G. Grabenweger" (CH, GG, NHMV); 1 female 2 males "GERMANY: Berlin, Wönnichstrasse, 5.ix.2005", "Phyllonorycter robiniella on Robinia pseudoacacia, G. Grabenweger" (GG, NHMV); 1 female and 1 male "ITALY: Torino, Pianezza, em. 21-27.iv.2003, P. Navone", "Host: Phyllonorycter robiniella" (Clemens) (CNC); 1 female "U.S.A.: Connecticut, Hartford Co., Farmington, near jct. State Road 4 and River Road, 18.x. 2002, C.T. Maier", "Host: Phyllonorycter robiniella" (CNC); 2 females 2 males from same locality and host as previous but collected 26.vi.2002 (CAES, USNM); 1 male "U.S.A.: New Hampshire, Cheshire Co., Town of Hinsdale, 1.5 km S jct. State Highways 63 and 119, 17.x. 2002, C.T. Maier", "Host: Phyllonorycter robiniella" (CNC); 2 females 3 males from same locality and host as previous but collected 28.vi.2002 (CAES, CH).

Identification. To include *A. robiniae* in the latest key to European *Achrysocharoides* (Bryan 1980) the following addition should be made:

Start with

1a. Pronotal collar sharply margined (Figs 29–30); propodeum with submedian carinae (Figs 29–30) ..*robiniae* **sp. nov.**
- Pronotal collar without sharp margin (e.g. Figs 5–6); propodeum without submedian carinae (e.g. Figs 5–6) 1

To include *A. robiniae* and *A. robinicolus* in the latest key to Nearctic *Achrysocharoides* (Kamijo 1991) the following addition should be made:

In the key to the species of the *gahani*-group (starts on page 27) the second alternative in couplet 2 should run to 3 instead of *gahani*

3. Male frons below frontal suture bright green (Figs 53–54); male gaster with white spot oval-shaped and reaching over tergites 1–3 (Figs 55, 58) . ..... *gahani* (Miller)
- Male frons below frontal suture bright blue (Figs 32, 44, 51–52); male gaster with white spot triangular or shaped like a pentagon and reaching over tergites 1–2 (Figs 56–57, 59–60) 4
4. Both sexes with hind coxae completely white (Figs 35–36); male scape 1.8X as long as wide (Fig. 66); male gaster with white spot shaped like a pentagon (Figs 56, 59) ..... *robiniae* **sp. nov.**
- Both sexes with hind coxae with base (up to 1/3) brown (Figs 47–48); male scape 3.0X as long as wide (Fig. 68); male gaster with white spot shaped like a triangle (Figs 57, 60) ..... *robinicolus* **sp. nov.**

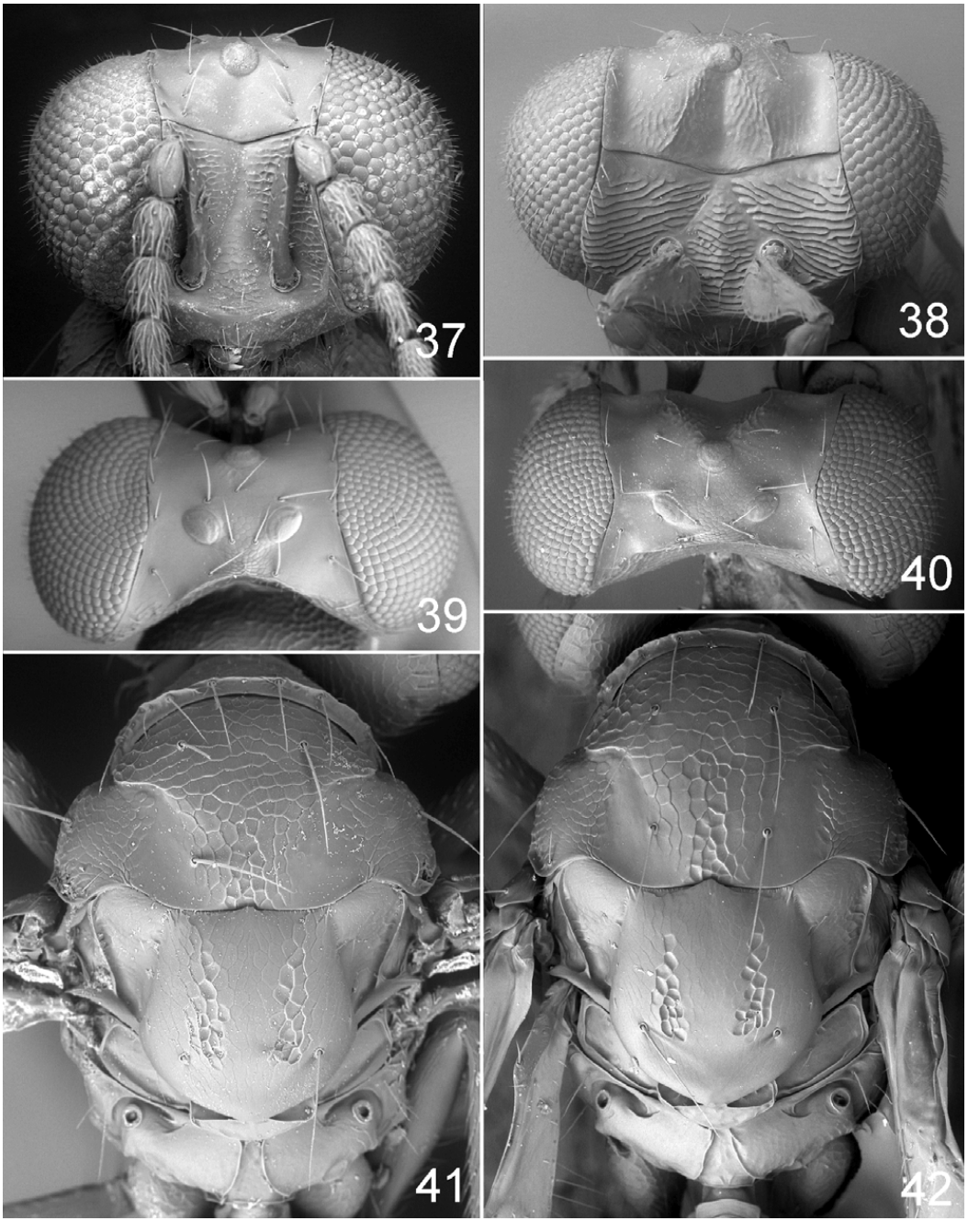
Etymology. Named after the host plant, *Robinia pseudoacacia*, of its lepidopteran host.

***Achrysocharoides robinicolus* sp. nov.**
(Figs 37–48, 57, 60, 67–68)

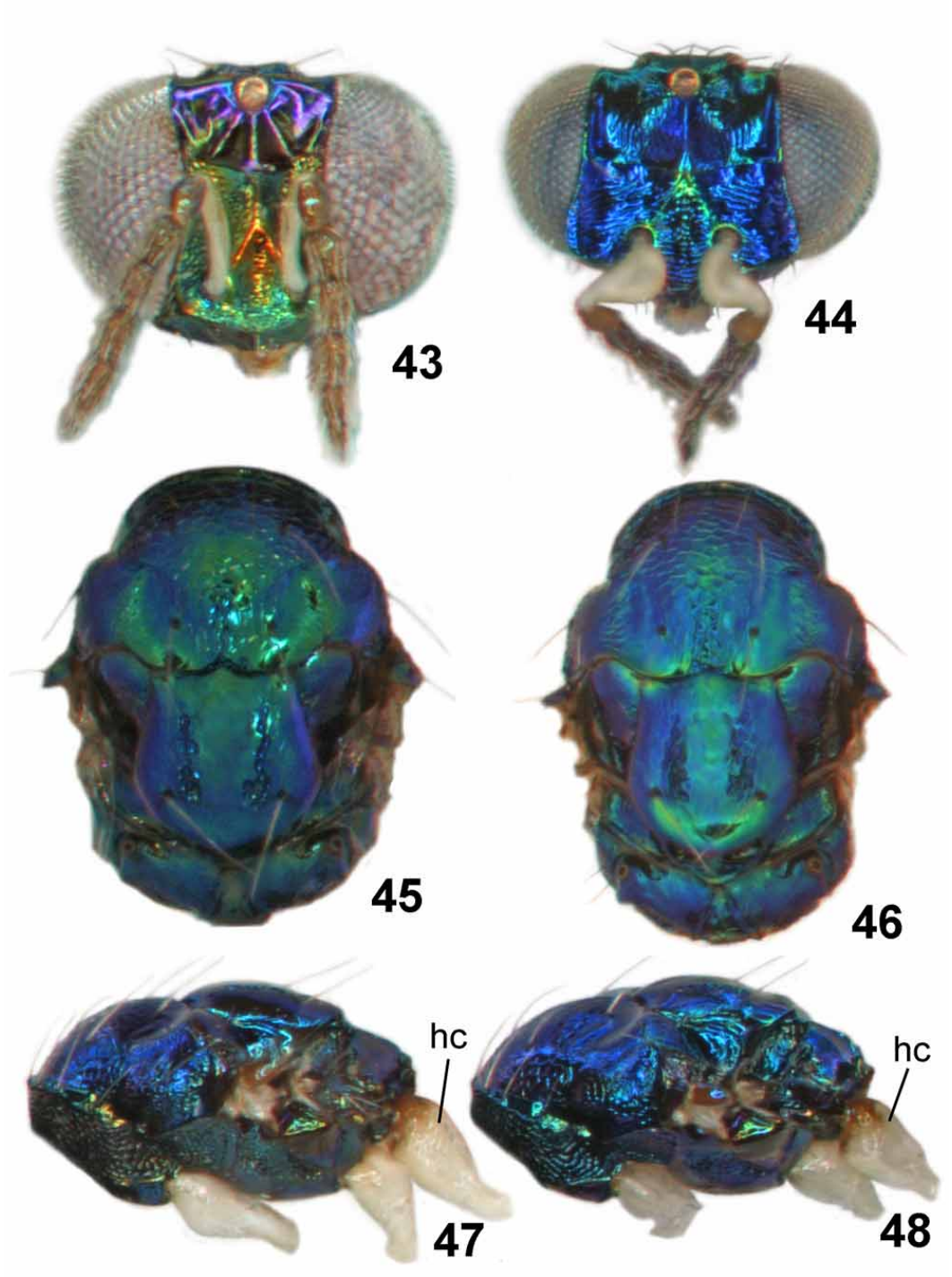
Diagnosis. *Achrysocharoides robinicolus* is very similar to *A. robiniae* but differs in having a narrower male scape (Figs 44, 68), which is 3.0X as long as wide (holotype) (1.8X as long as wide in holotype of *A. robiniae*, Figs 32, 66), anteromedian white spot in male gaster triangular and small, reaching to posterior margin of 1st or 2nd tergites (Figs 57, 60) (shaped as a pentagon in *A. robiniae*, Figs 56, 59), hind coxae in both sexes with base brown to metallic (Figs 47–48) (completely white in both sexes of *A. robiniae*, Figs 35–36).

Description. FEMALE. Length 1.2–1.4 mm.

Scape white with apical 1/3 infuscate; pedicel and flagellum brown with weak metallic tinges. Frons below frontal suture golden-green to golden-red, antennal scrobes golden-red, above frontal suture metallic bluish-purple with metallic green tinges. Vertex inside ocellar triangle golden-red, outside ocellar triangle golden-green. Mesoscutum and scutellum metallic bluish-green. Propodeum metallic blue with green tinges. Legs white, hind coxa with base brown to metallic. Wings hyaline. Gaster with first two tergites golden-green, remaining tergites dark brown with metallic green tinges.

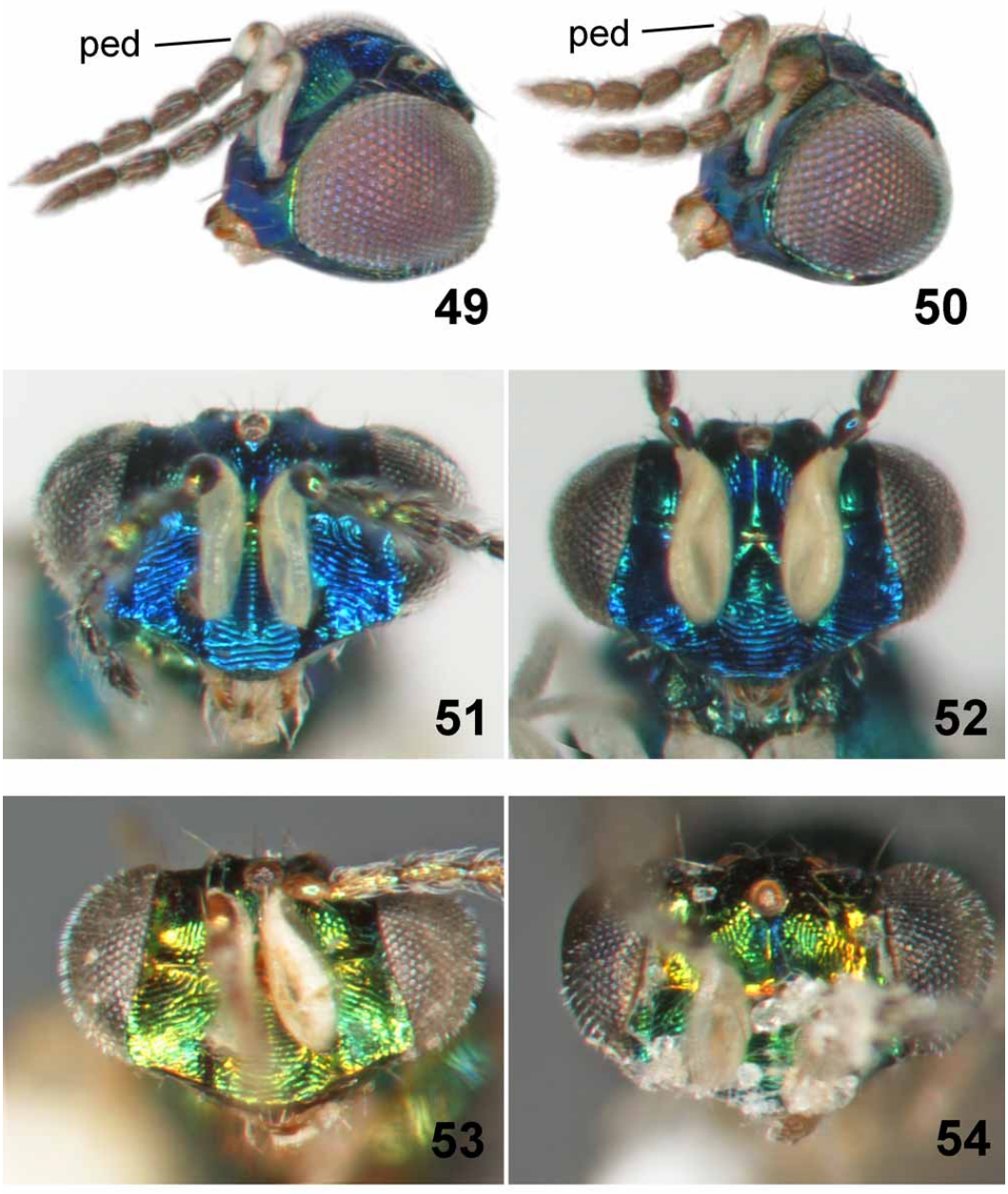


FIGURES 37–42. *Achrysocharoides robinicolus* **sp. nov.** 37. Head frontal, female. 38. Head frontal, male. 39. Vertex, female. 40. Vertex, male. 41. Thoracic dorsum, female. 42. Thoracic dorsum, male.

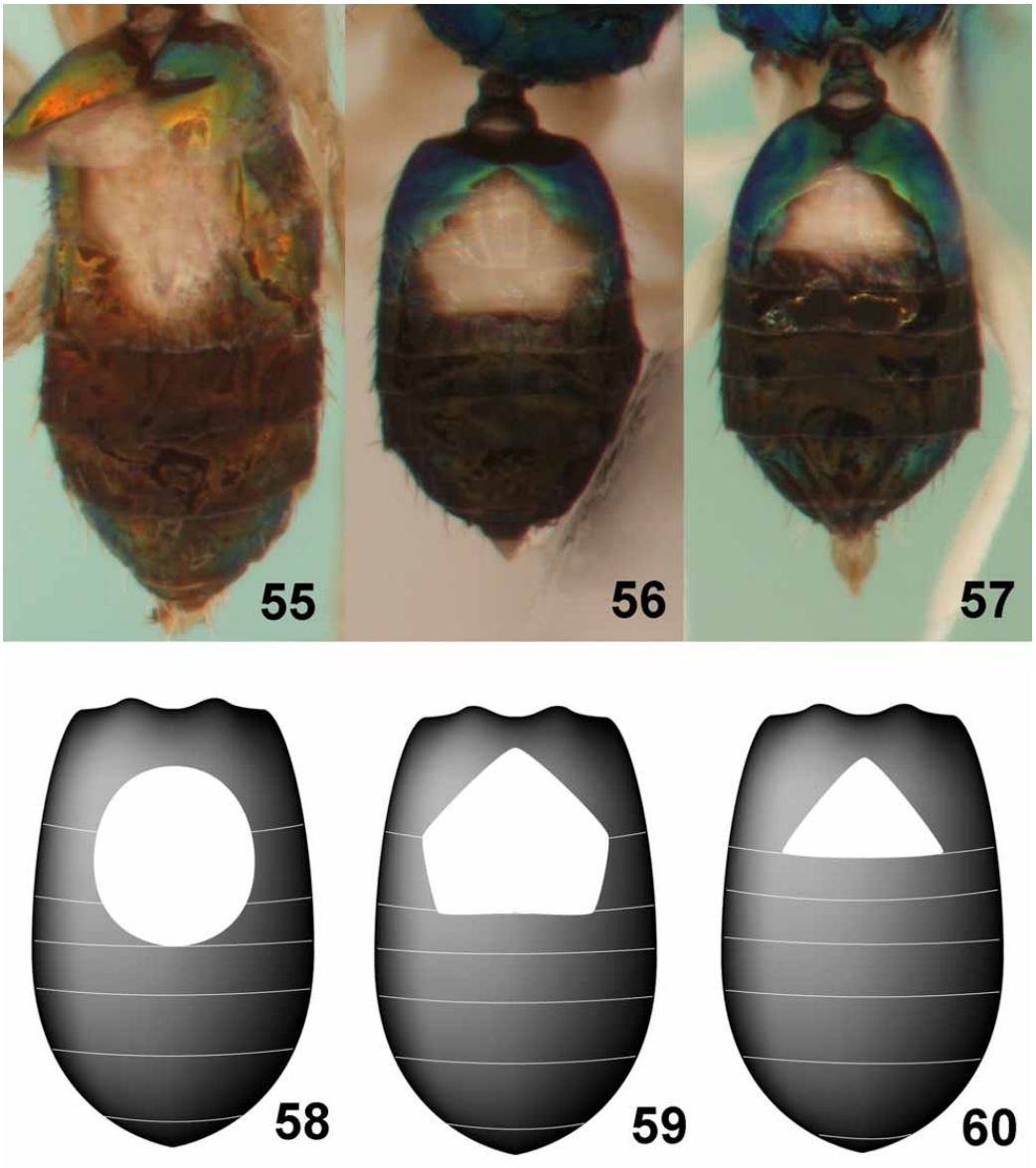


FIGURES 43–48. *Achrysocharoides robinicolus* **sp. nov.** 43. Head frontal, female. 44. Head frontal, male. 45. Thoracic dorsum, female. 46. Thoracic dorsum, male. 47. Mesosoma lateral, female. 48. Mesosoma lateral, male. Abbreviation: *hc* = hind coxa.

Antenna as in Fig. 67. Frons below level of toruli smooth and shiny, between level of toruli and frontal suture with raised and strong reticulation with antennal scrobes smooth, above frontal suture smooth and shiny. Vertex inside ocellar triangle with engraved and very weak reticulation, outside ocellar triangle smooth and shiny. Occipital margin with a sharp edge behind ocellar triangle.

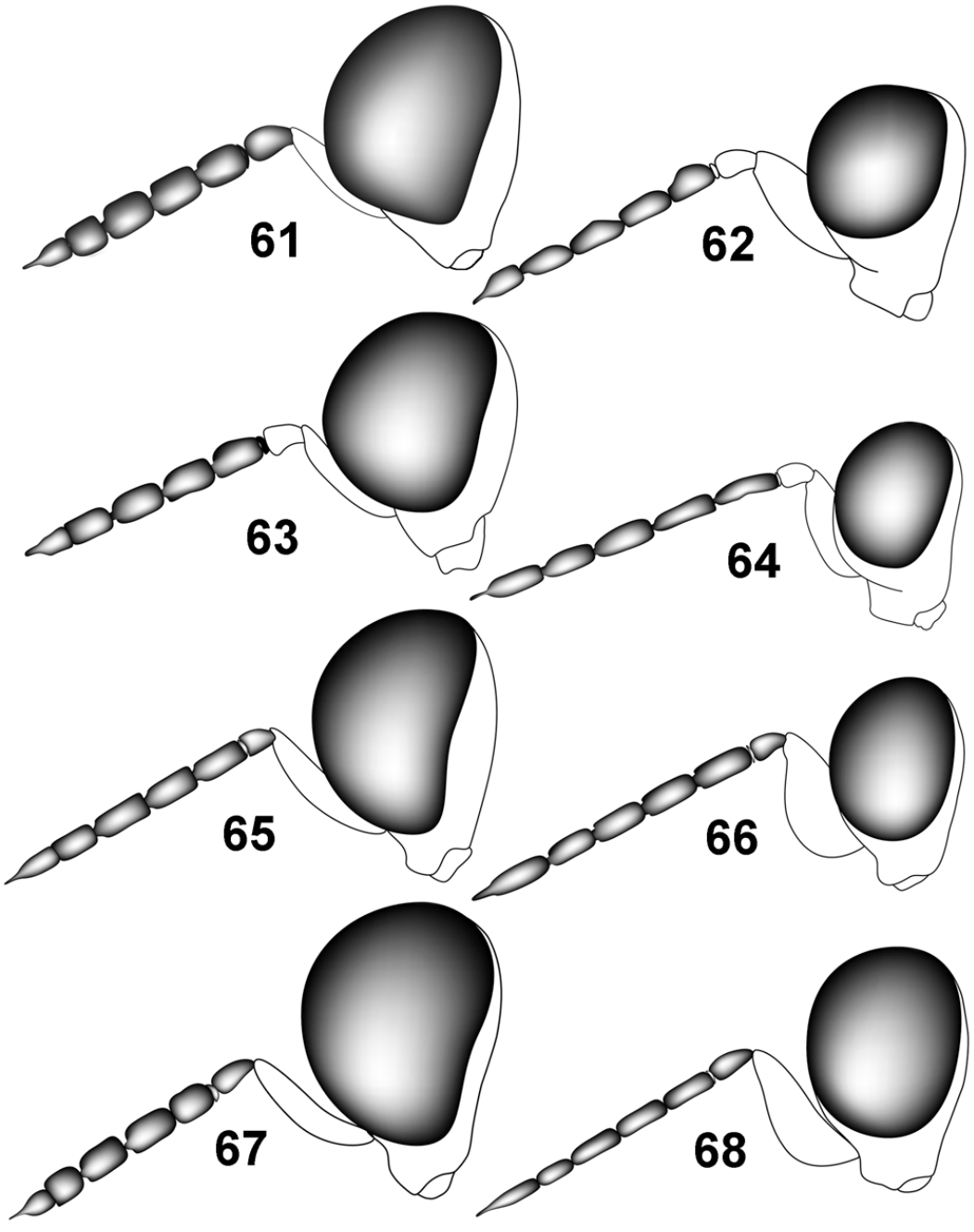


FIGURES 49–54. 49–50. Head and antenna lateral, female. 49. *Achrysocharoides platanoidae* **sp. nov.** 50. *A. acerianus* (Askew). 51–54. Head frontal, male. 51. *A. robiniae* **sp. nov.**, holotype. 52. *A. robiniae*, paratype from U.S.A., New Hampshire. 53. *A. gahani* (Miller), paratype from Canada, Ottawa, on *Tilia americana*. 54. *A. gahani*, non-type specimen from Canada, Ottawa, on *Tilia americana*. Abbreviation: *ped* = pedicel.



FIGURES 55–60. Gaster dorsal, male. 55. *Achrysocharoides gahani* (Miller), paratype from Canada, Ottawa, on *Tilia americana*. 56. *A. robiniae* **sp. nov.**, paratype from Hinsdale, New Hampshire. 57. *A. robinicolus* **sp. nov.**, paratype from Highland Falls, New York, 58. *A. gahani*, schematic drawing. 59. *A. robiniae*, schematic drawing. 60. *A. robinicolus*, schematic drawing

Pronotal collar with a sharp carina. Mesoscutum with midlobe with raised and strong reticulation, sidelobes with fine and weak reticulation; notauli as smooth impressions in posterior 2/3. Scutellum with rows of punctate-reticulate pits on each side, remaining surfaces with very weak and engraved reticulation to smooth. Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny with two submedian carinae that diverge towards posterior margin of propodeum; propodeal callus with three setae. Forewing speculum closed below. Petiole conical without shoulders.



FIGURES 61–68. Head and antenna lateral. 61. *Achrysocharoides acerianus* (Askew), female. 62. *A. acerianus*, male. 63. *A. platanoidae* **sp. nov.**, female. 64. *A. platanoidae*, male. 65. *A. robiniae* **sp. nov.**, female. 66. *A. robiniae*, male. 67. *A. robinicolus* **sp. nov.**, female. 68. *A. robinicolus*, male.

Ratios. HE/MS/WM = 3.9/1.0/1.7; POL/OOL/POO = 1.4/1.0/1.0; WH/WT = 1.2; LW/LM/HW = 1.7/1.0/1.1; PM/ST = 1.0; MM/LG = 1.1–1.2.

MALE. Length 1.3–1.4 mm.

Scape white; pedicel brown with metallic tinges and with ventral side white; flagellum dark brown with golden-green tinges. Frons below level of toruli golden-green or golden-red, above level of toruli metallic blue. Vertex inside ocellar triangle golden-red, outside ocellar triangle metallic bluish-green. Mesoscutum and scutellum metallic blue with green tinges. Propodeum metallic blue with green tinges. Legs white, hind coxae with base brown to metallic. Wings hyaline. Gaster with tergites 1, 2, 6, 7 golden-green, 3–5 dark brown with metallic tinges, anteromedially with a triangular white spot that extends over first tergite, sometimes reaching posterior margin of 2nd tergite.

Antenna as in Fig. 68. Frons below level of toruli smooth and shiny, between level of toruli and frontal suture with strong and transverse striae, above frontal suture medially with raised and rather weak reticulation and close to eyes smooth. Vertex inside ocellar triangle with engraved and very weak reticulation, outside ocellar triangle smooth and shiny. Occipital margin with a sharp edge behind ocellar triangle.

Pronotal collar with a sharp carina. Mesoscutum with midlobe with raised and strong reticulation, sidelobes with fine and weak reticulation; notauli as smooth impressions in posterior 2/3. Scutellum with rows of punctate-reticulate pits on each side, remaining surfaces with very weak and engraved reticulation to smooth. Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny with two submedian carinae that diverge towards posterior margin of propodeum; propodeal callus with three setae. Forewing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 2.5/1.0/1.2; POL/OOL/POO = 3.2/1.8/1.0; WH/WT = 1.3; LW/LM/HW = 1.7/1.0/1.1; PM/ST = 1.1; MM/LG = 1.1–1.2.

Distribution. U.S.A.

Host. *Phyllonorycter robiniella* (Clemens) (Lepidoptera: Gracillariidae) on *Robinia pseudoacacia*.

Material examined. Holotype male labeled "U.S.A.: New York, Orange Co., Town of Highlands, Highland Falls, along US highway 9W near Catholic Cemetery, 6.vi.2002, C.T. Maier", "Tentiform mine of Phyllonorycter robiniella collected on Robinia pseudoacacia, emerged in laboratory within 3 weeks" (CNC); 2 females 2 males with same label data as holotype (CAES, USNM); 1 female from same locality and host as holotype but collected 6.vi.2001 (CNC); 1 female 1 male from same locality and same host as holotype but collected 30.viii.2002 (CNC).

Identification. See above under *A. robiniae*.

Etymology. Named after the host plant, *Robinia pseudoacacia*, of its lepidopteran host.

***Achrysocharoides gahani* (Miller)**
(Figs 53–54, 55, 58)

Enaysma gahani Miller, 1962:1041. Holotype female in CNC, not examined.
Achrysocharoides gahani (Miller) (Yoshimoto 1977:928).

Hosts. The type material of *A. gahani* was reared from *Phyllonorycter* spp. on *Fagus grandifolia*, *Tilia americana* and *Quercus alba* (Miller 1962). Kamijo (1991) transferred the material from *Quercus alba* to *A. reticulatus* Yoshimoto. Apart from these records *A. gahani* has also been reported from *Phyllonorycter* sp. on *Rhus toxicodendron* (Kamijo 1991), *P. blancardella* (Fabricius) on *Malus* sp. (Maier 1984), *P. crataegella* (Clemens) on *Prunus pensylvanica* (Maier 1988) and *P. propinquella* (Braun) on *Prunus serotina* (Maier 1988). We have examined 4 female paratypes of *A. gahani* from *Q. alba* and we agree with Kamijo that these do not belong to this species. We have also been able to examine 3 females and 7 males from *P. propinquella* on *P. serotina* and 5 females and 1 male from *P. crataegella* on *P. pensylvanica* reared by Chris T. Maier (specimens in CAES and CNC). These specimens belong to species-group *clypeatus*, i.e. they do not have a transverse carina on pronotum or submedian carinae on propodeum — characteristics of the *gahani*-group, and are hence not *A. gahani*. Furthermore, we have seen a single female from *Phyllonorycter* sp. on *Rhus*

radicans (in CNC) identified as *A. gahani* by Kamijo. This female is similar to paratypes of *A. gahani* from *Fagus* and *Tilia*, but females of *Achrysocharoides* are difficult to separate and we would like to see a male from *Rhus* before confirming microlepidopterans on *Rhus* as hosts for *A. gahani*. We have not been able to examine any material from *P. blancardella* on *Malus* and we can not state anything regarding the validity of this record. However, in view of our findings regarding *Achrysocharoides* from *Phyllonorycter* spp. on *Prunus* spp. this record needs confirmation.

Material examined. Type material: 10 female paratypes (2 from *Fagus*, 4 from *Quercus*, 4 from *Tilia*) and 2 male paratypes (1 from *Fagus* and 1 from *Tilia*) (CNC).

Discussion

The establishment of *A. platanoidae*, *A. robiniae* and *A. robinicolus* further emphasizes the host/host plant specificity of *Achrysocharoides* species described previously (e.g. Lopez-Vaamonde *et al*. 2005). *Achrysocharoides platanoidae* and *A. acerianus* both have an obligate association with *Phyllonorycter* spp. on the plant genus *Acer*, *A. platanoidae* with *P. platanoidella* on *A. platanoides* (and possibly *A. campestre* — see above under remarks for *A. platanoidae*) and *A. acerianus* with *P. geniculella* on *A. pseudoplatanus*. Previously *A. acerianus* has been recorded also from *Acer platanoides*, but these records are either confirmed misidentifications (Hansson 1983, Lopez-Vaamonde *et al*. 2005), or (Bryan 1980) need confirmation. *Achrysocharoides robiniae* and *A. robinicolus* are both exclusively associated with *P. robiniella* on *Robinia pseudoacacia*. As potential biological control agents against *P. robiniella*, a serious pest on the economically important black locust tree in Europe, it is essential to supply tools for the identification of *Achrysocharoides* species from *Robinia*, which previous misidentifications clearly demonstrate. Using the information in this article: the diagnoses, the well-illustrated descriptions with biological information, and the alterations to already existing keys, it is now possible to unambiguously identify the species of *Achrysocharoides* parasitizing *Phyllonorycter robiniella* on *Robinia*.

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Paper III

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Species recognition through wing interference patterns (WIPs) in *Achrysocharoides* Girault (Hymenoptera, Eulophidae) including two new species

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Abstract

Wing interference patterns (WIPs) are shown to be an important tool for species recognition in the genus *Achrysocharoides* Girault (Hymenoptera: Eulophidae). This is demonstrated by combining information from two previously published papers, comprising two cases of cryptic species, and by new material including the description of two new species, *A. maieri* and *A. serotinae* from North America. The cryptic species were initially separated through their distinct male WIPs. Subsequent analyses of the external morphology uncovered additional morphological differences supporting the original findings through WIPs, and biological data further strengthened the identity of these species. The new species described here also differ in their WIPs but the WIPs are similar in both sexes. Thus they provide a strong link between male and female and demonstrate that WIPs can also be useful for species recognition when the sexes are otherwise difficult to associate. Both new species are from Connecticut, USA, and were reared from *Phyllonorycter propinquinella* (Braun) (Lepidoptera: Gracillariidae) on black cherry (*Prunus serotina*); *A. maieri* has also been reared from *Ph. nr crataegella* on pin cherry (*P. pensylvanica*). To facilitate the identification of the new species they are included in a previously published key to North American species of *Achrysocharoides*. As a supplement to colourful WIPs we also demonstrate that grey scale images of uncoated wings from scanning electron microscopy can be used for visualization of the thickness distribution pattern in wing membranes.

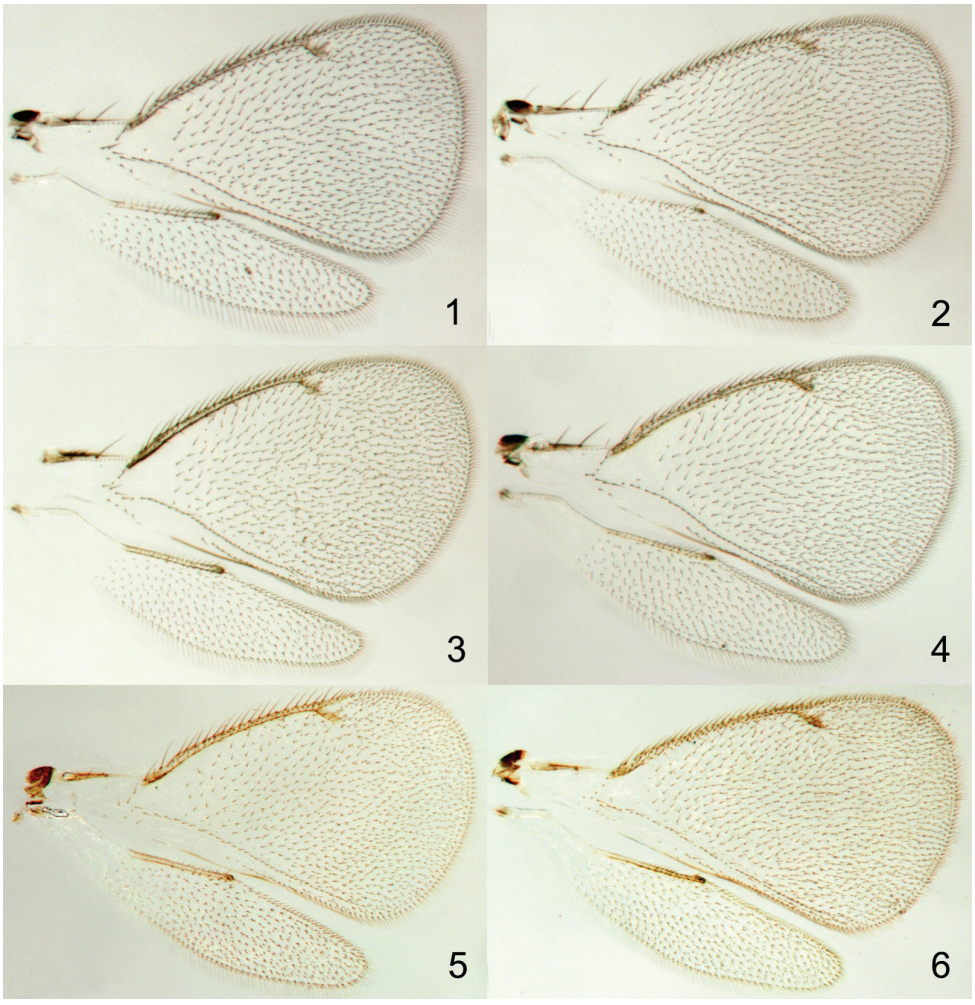
Keywords
taxonomy, cryptic species, structural colours, sexual dimorphism, wing membrane thickness, Chalcidoidea, Entedoninae, leafminer parasitoids, *Achrysocharoides acerianus, Achrysocharoides platanoidae, Achrysocharoides robiniae, Achrysocharoides robinicolus, Achrysocharoides butus, Achrysocharoides latreilleii, Achrysocharoides albiscapus, Achrysocharoides maieri*, *Achrysocharoides serotinae*, *Phyllonorycter propinquinella*, *Phyllonorycter nr crataegella*, *Prunus serotina*, *Prunus pensylvanica*

Introduction

Species of *Achrysocharoides* Girault (Hymenoptera: Eulophidae) are small parasitic wasps with transparent non-pigmented wings (Figs 1–6, 9). The short postmarginal vein in the fore wing is characteristic for the genus and the shape of the fore wing can be used to distinguish males of some species, but otherwise wings have been disregarded as non-informative neutral entities in this genus (e.g. Askew and Ruse 1974; Kamijo 1991). Recently wings in this group were discovered to display patterns with stable structural colours (Fig. 7), comparable to other insect groups with colourful wings such as butterflies (Shevtsova et al. 2011). These wing interference patterns (WIPs) become visible when transparent insect wings are seen against a dark background, and are most distinctive in small species with exceptionally thin wing membranes.

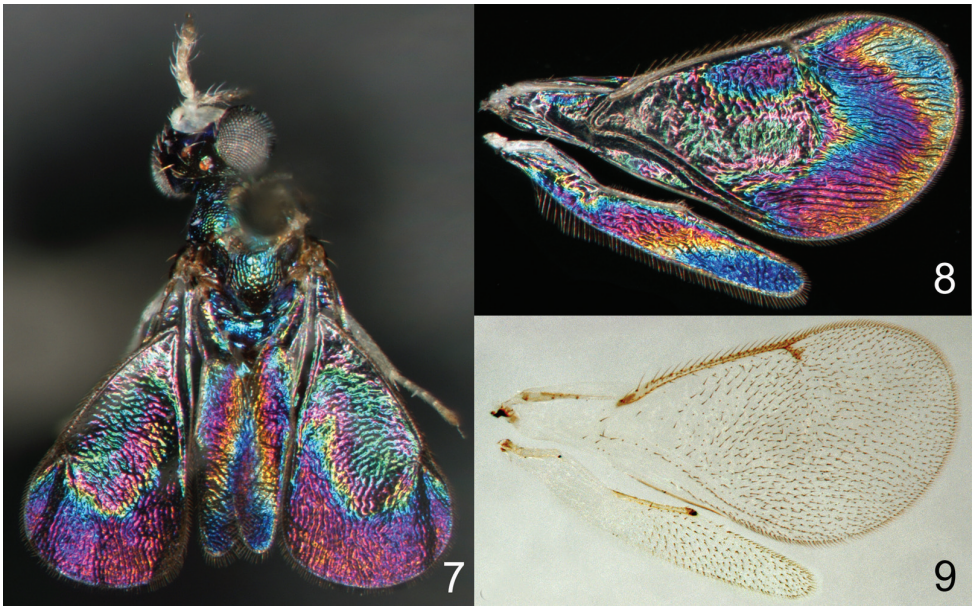
WIPs as a morphological character are so new that very little is known about the significance of these patterns for their bearers or for entomologists studying them, although they have already proven useful for generic-level classification in Eulophidae (Hansson 2011). The application of WIPs as a species character was first used in a study including two cases of cryptic species in *Achrysocharoides* (Hansson and Shevtsova 2010), where the initial species separation was based solely on male WIPs. However, data showing the usefulness of WIPs were withheld pending the publication of Shevtsova et al. (2011) where a general background to these patterns was outlined. In order to expand the knowledge of WIP diversity and to prove the usefulness of these patterns for studies at the species level it is important to link the information from these two publications. To further enhance this knowledge we also describe two new *Achrysocharoides* species with distinct WIPs.

The two new species of *Achrysocharoides* described here are from North America and the genus was initially recorded from this region by Miller (1962), as the genus *Enaysma* Delucchi, including six new species from Canada which were placed in the same subgenus (*Pentenaysma* Graham). Yoshimoto (1977) synonymized *Enaysma* with *Achrysocharoides*, and added nine species (six newly described) to the six described by Miller. He also separated the 15 species into two newly created species groups, thus abandoning the division into subgenera. The latest comprehensive treatment of North American *Achrysocharoides* is by Kamijo (1991), who treated 18 species, including four new species and one new synonym, separated into five



Figures 1–6. *Achrysocharoides* spp., transparent wings: **1** *A. acerianus* (Askew), male **2** Ditto, female **3** *A. platanoidae* Hansson & Shevtsova, male **4** Ditto, female **5** *A. butus* (Walker), male **6** Ditto, female. Wings on Figs **1–4** from Sweden, Skåne, 2010 **5–6** from Wales, 1976.

species groups, two of which were newly created. Hansson and Shevtsova (2010) added two new species to the North American fauna, increasing the total to 20 species. With the two new species described here this total is now 22, equal to the number of species in Europe. Worldwide, including the two new species described here, 56 species of *Achrysocharoides* are known. The majority (ten) of the remaining species are from Japan (Kamijo 1990a, b), thus establishing the main distribution of *Achrysocharoides* as the northern hemisphere.

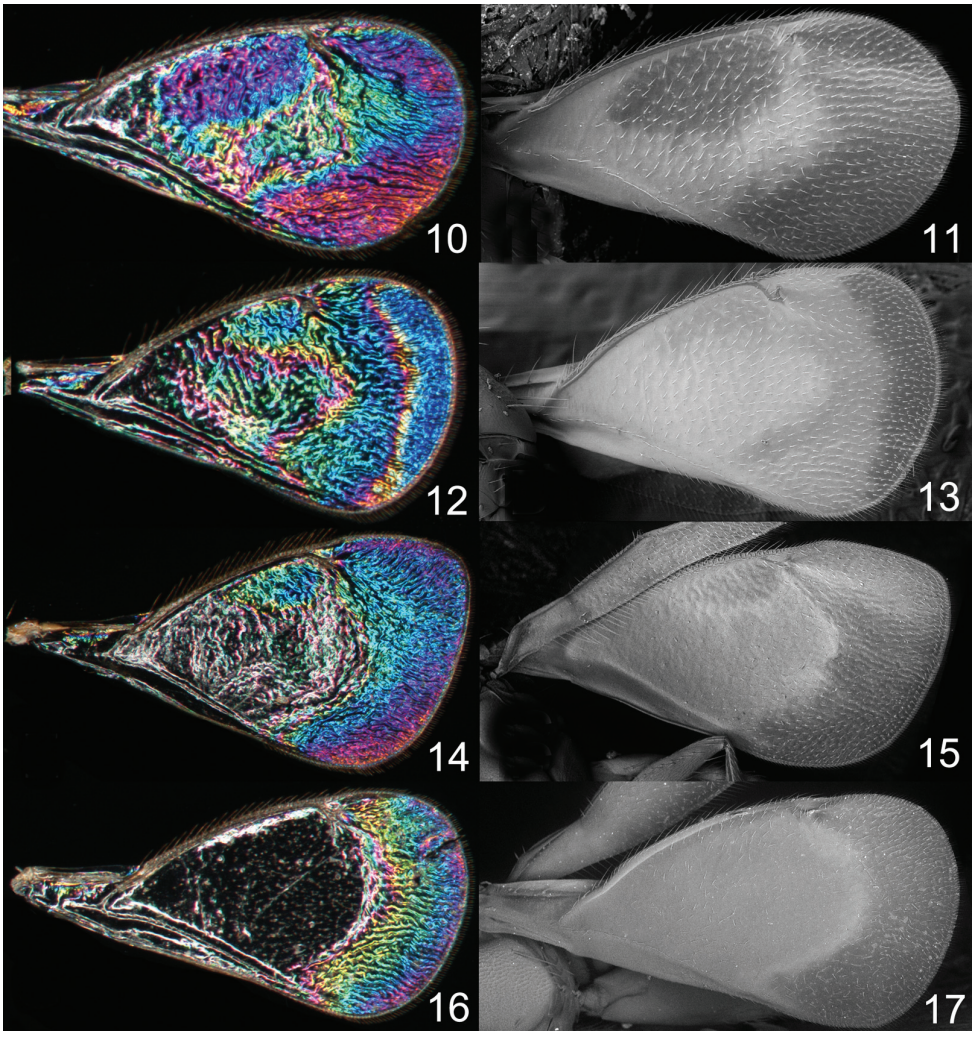


Figures 7–9. *Achrysocharoides* spp.: **7** *A. zwoelferi* (Delucchi), male, from Sweden, Blekinge, 1956 **8** Undescribed species from USA, Arizona, 1982, male, wing interference pattern (WIP) **9** The same wings as in Fig. **8** in transparent mode.

Material and methods

The observation and documentation of WIPs do not require a special light source and can be done on any dry specimen with intact wings arranged against a dark background. However, to make the illustrations comparable all photos in this paper as well as in Shevtsova et al. (2011) are of wings removed from the specimens and horizontally arranged, and with the same magnification (6×). To achieve this, the wings are flattened between a glass slide and a glass cover slip on top of the wings. The underside of the glass slide is stained with a drop of black ink to make the background pitch black and homogeneous (this was proposed by J. Kjærandsen). In a few cases where the wings could not be properly flattened the slide was slightly tilted so that the pattern in a non-flattened area, e.g. in a wrinkle, became visible and could be documented. This area was then manually combined in Adobe Photoshop with the initial horizontal photo of the wing, thus showing the complete pattern. A Nikon SMZ1000 stereomicroscope and 5MP Nikon DS-L1 camera were used to take photos of the wings at different focus levels, and Helicon Focus Pro version 4.75 software was used to merge them into a single image. WIPs are usually too shiny for the camera to balance brightness automatically and therefore the brightness was individually adjusted in Adobe Photoshop. Subsequent editing included cleaning and cropping of the photo. After the fore and hind wings were documented they were glued back to the card with the original specimen, which retained the second pair of wings for future observations – structural colours disappear on glued or slide mounted wings (Figs 1–6). The images of transparent wings in this paper are from temporary slide

preparations with wings mounted in a water-soluble clear gel. The scanning electron microscopy (SEM) images (Figs 11, 13, 15, 17, 66–71, 82–87) are from uncoated specimens on their original card mountings. The photos were taken in low vacuum mode via a backscattered electron detector on a JEOL® JSM 5600LV microscope.



Figures 10–17. *Achrysocharoides* spp., males, wing interference patterns (WIPs) to the left, scanning electron micrographs from uncoated wings to the right: **10–11** *A. robiniae* Hansson & Shevtsova **12–13** *A. butus* (Walker) **14–15** *A. latreilleii* (Curtis) **16–17** *A. albiscapus* (Delucchi).

Morphological abbreviations and acronyms

HE = height of eye; HW = height of fore wing; LG = length of gaster; LM = length of marginal vein; LW = length of fore wing, measured from base of marginal vein to apex of wing; MM = length of mesosoma; MS = malar space; OOL = distance between one

posterior ocellus and eye; PM = length of postmarginal vein; POL = distance between posterior ocelli; POO = distance between posterior ocelli and occipital margin; ST = length of stigmal vein; WH = width of head; WM = width of mouth; WT = width of thorax. For illustrations of the morphological terms see http://www.neotropicaleulophidae.com/.

Collection acronyms, for the deposition of type material: BMNH = Natural History Museum, London, England; CAES = Connecticut Agricultural Experiment Station, New Haven, U.S.A; CNC = Canadian National Collection of Insects, Ottawa, Canada.

Results and discussion

The paper by Hansson and Shevtsova (2010) included two cryptic *Achrysocharoides* species from *Acer, A. platanoidae* Hansson & Shevtsova from *Acer platanoides* and *A. acerianus* (Askew) from *A. pseudoplatanus,* and two cryptic species from *Robinia pseudoacacia, A. robiniae* and *A. robinicolus*, both described in that paper. The transparent wings in these four species are very similar and identical between males and females (Figs 1–4). Nevertheless the initial differences distinguishing these cryptic species were found in the wing morphology through distinct WIPs, which visualize uneven thickness of the wing membrane through different interference colours (Shevtsova et al. 2011).

In both cryptic cases only one of the species displays a distinct species specific WIP, and in males only, while conspecific females and both sexes of the other cryptic species have similar WIPs. In the two *Achrysocharoides* species associated with *Acer* only males of *A. platanoidae* have a distinctive WIP with an eye-catching blue spot in the upper-apical corner of the fore wing (Figs 18–21). The female WIP of *A. platanoidae* displays no such spot (Figs 22–23) and is very similar to *A. acerianus*, which has the same WIP in both sexes (Figs 24–27). In the two other cryptic species, associated with *Robinia pseudoacacia,* only males of *A. robiniae* display a very characteristic WIP with a large ovate spot below the marginal vein. The male WIP also has an extended and usually green triangular area in the medio-apical part of the fore wing (Figs 28–33). In the female WIP the triangular area is usually less pronounced than in males and the submarginal ovate spot is significantly smaller (Figs 34–35). As the female does not display the characteristic features in these patterns as distinctly as the male, it can be confused with the female of *A. robinicolus*, which has the same WIP in both sexes (Figs 36, 37).

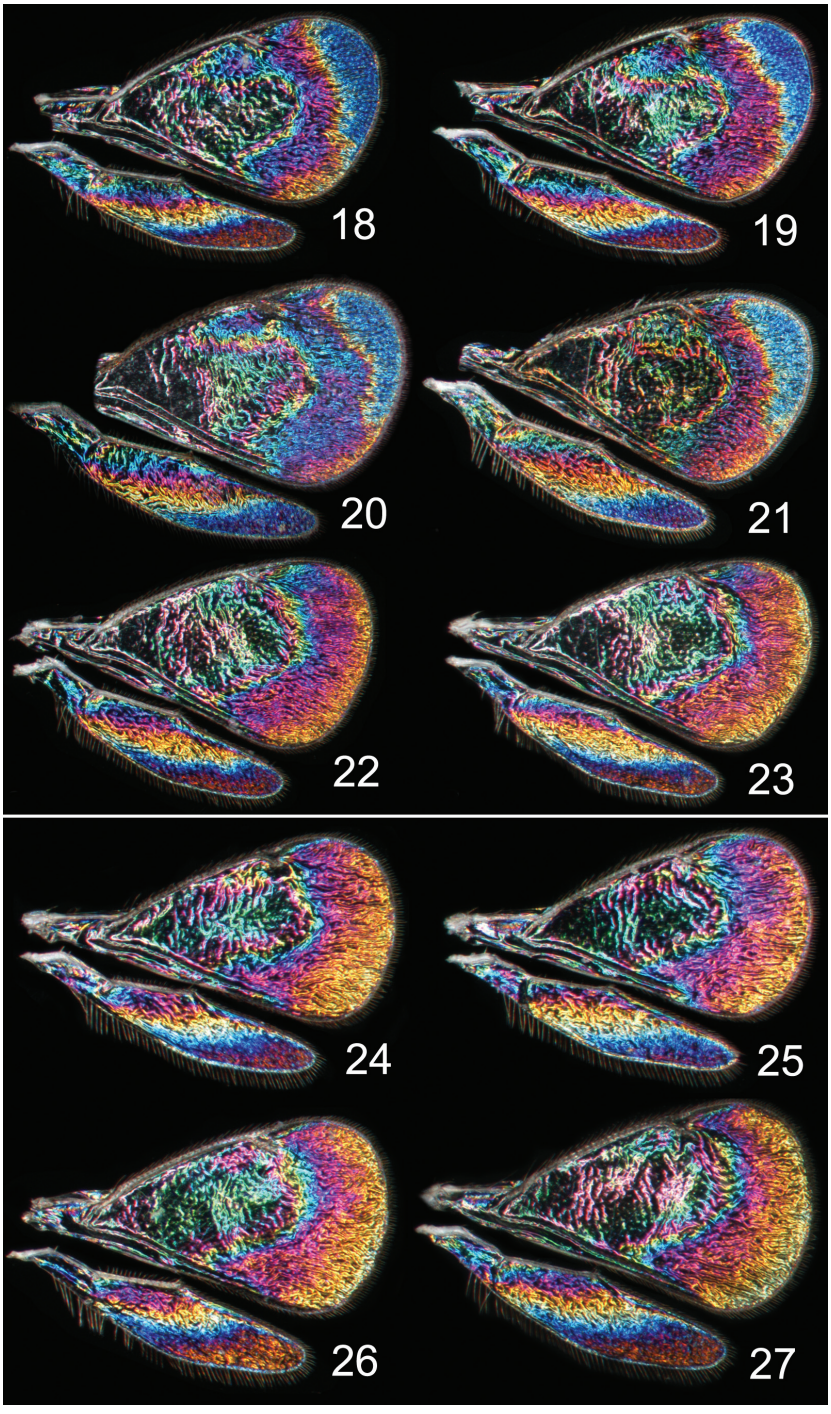
The two North American species described here, *A. maieri* and *A. serotinae*, are known only from, and are probably confined to, *Phyllonorycter* species on *Prunus*. Males can be distinguished through easy-to-see differences in the external morphology, e.g. the shape of the head (Figs 57, 67, 73, 83) but females are not so distinct and display less divergent characters (Figs 56, 66, 72, 82). Even though the wings of *A. maieri* and *A. serotinae* appear very similar in transparent mode (similar to Figs 1–6) the WIPs in these species are distinct and specific. Apart from being useful in the discrimination of the females, in this case WIPs are also useful for the association of otherwise

dimorphic males and females of the same species. The external morphology in these species exhibits a pronounced sexual dimorphism and as they share the same host it is not obvious which females and males are conspecific. However, there is one important character they have in common – WIPs, which are identical in both sexes but different between the species. *Achrysocharoides maieri* has a WIP with wide coloured cross bands on the fore wing (Figs 64–65), and *A. serotinae* has a quite featureless almost unicoloured WIP (Figs 80–81).

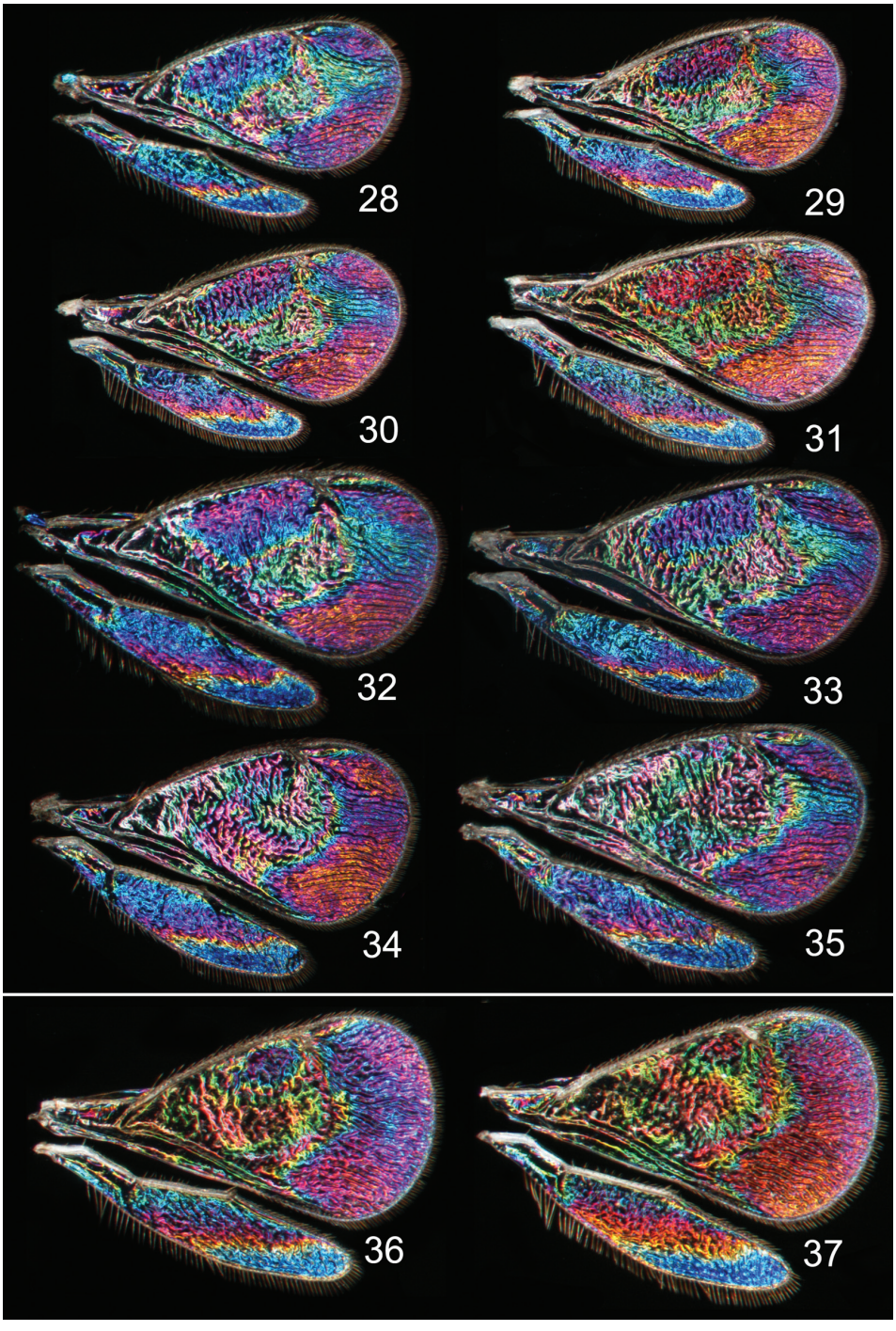
Additional examples of *Achrysocharoides* species with distinct and sexually dimorphic WIPs are *A. butus* (Walker) (Figs 38-43) and *A. latreilleii* (Curtis) (Figs 46-49) where characteristic and specific WIPs, again, are confined to males. Female WIPs of these two species are similar (Figs 44–45, 50–51), and as in females of *A. platanoidae* and *A. acerianus* (Figs 22–23, 26–27), and *A. robiniae* and *A. robinicolus* (Figs 34–35, 37), WIPs are not useful for species recognition. The WIP of male *A. butus* is similar to that of male *A. platanoidae* because the apical margin of the fore wing has a blue spot in both species. However, in *A. butus* this spot is prolonged and reaches along a major part of the apical margin (Figs 38–43) whereas in *A. platanoidae* the spot is short and confined to the upper-apical corner of the fore wing (Figs 18–21). The male of *A. latreilleii* is distinct not only in the truncate shape of the fore wing but also in its WIP (Figs 46–49). The basal 2/3 of the fore wing is the thickest part of the wing membrane and due to its micromorphology reflects very weak interference colours (Shevtsova et al. 2011). The apical part of the fore wing, and a small submarginal spot located in the corner between marginal and stigmal veins, are brightly coloured. The potential of WIPs as a character for separating species can be further demonstrated through two species where only male WIPs are known. *Achrysocharoides albiscapus* (Delucchi) has a WIP similar to that of *A. latreilleii*, but differs in having the basal 2/3 of the fore wing completely transparent without colour reflections and no submarginal colour spot (Figs 52–55). The shape of the fore wing is also different between males of these two species. The other species is undescribed, from Arizona, USA (specimen in CNC), and we have only seen a single male. This specimen has a distinctive WIP which emphasizes very unusual shapes of both fore and hind wings. The WIP includes a blue spot in the upper-apical corner of the fore wing (Fig. 8), comparable to *A. platanoidae* (Figs 18–21) but with the blue spot smaller and differently shaped.

Similar to other morphological characters there is a certain intraspecific variation in WIPs (Figs 18–55), but the species specific traits nevertheless remain clearly recognizable and are reliable for species separation. The intraspecific variation in WIPs can be divided into two types, variation in colour and in shape of patterns. Variation in colour is basically size-dependent – the thickness of the wing membrane usually varies with the size of the specimen and there is a general shift of the hues in WIPs from larger to smaller specimens. Variation in the shapes of pattern outlines of conspecific WIPs is not apparently size dependent but reflects individual differences between specimens - the overall pattern nevertheless remains the same.

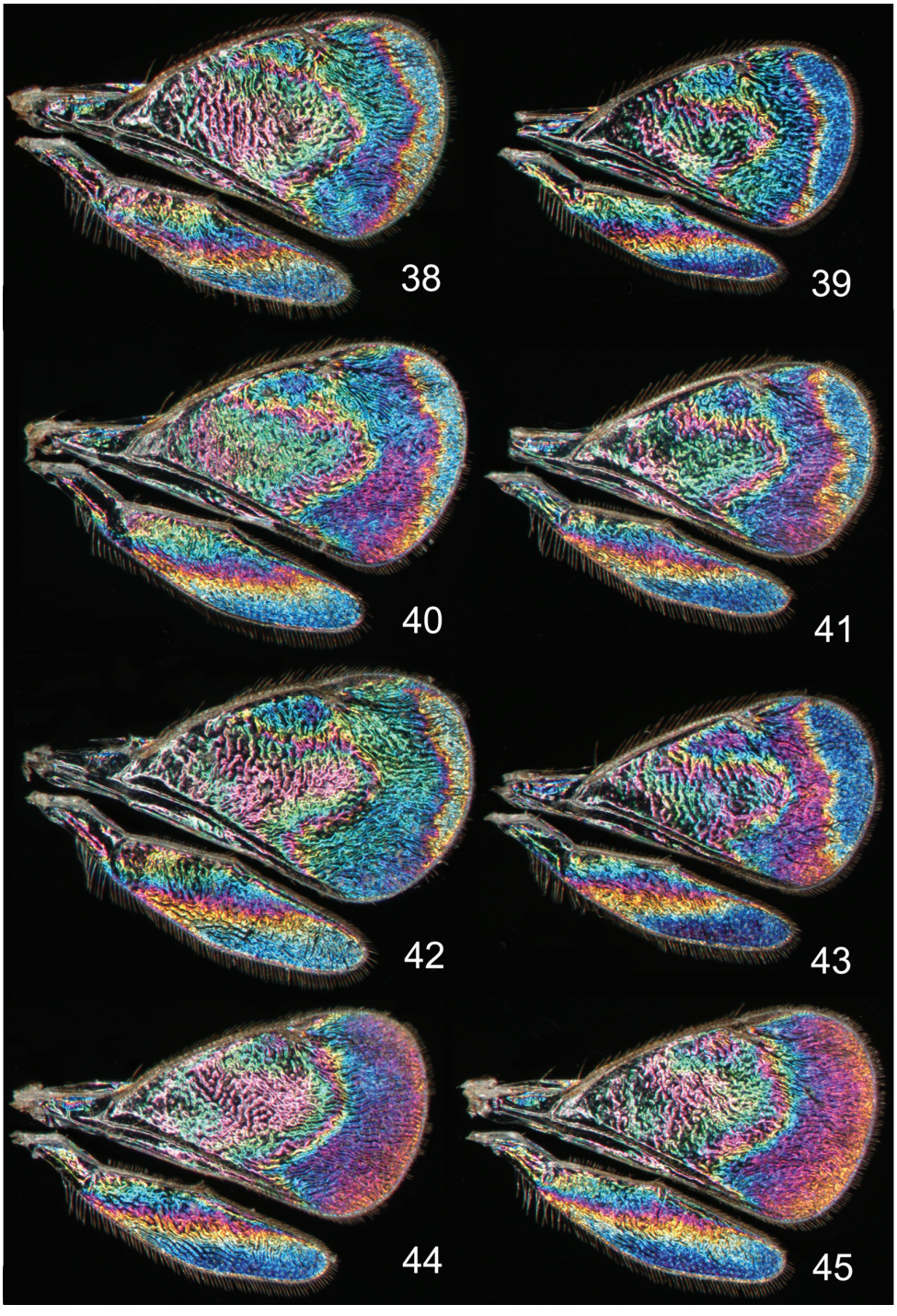
Wing interference patterns are due to structural organization patterns of the wing membrane where areas of different thickness reflect certain interference colours (Shevt-



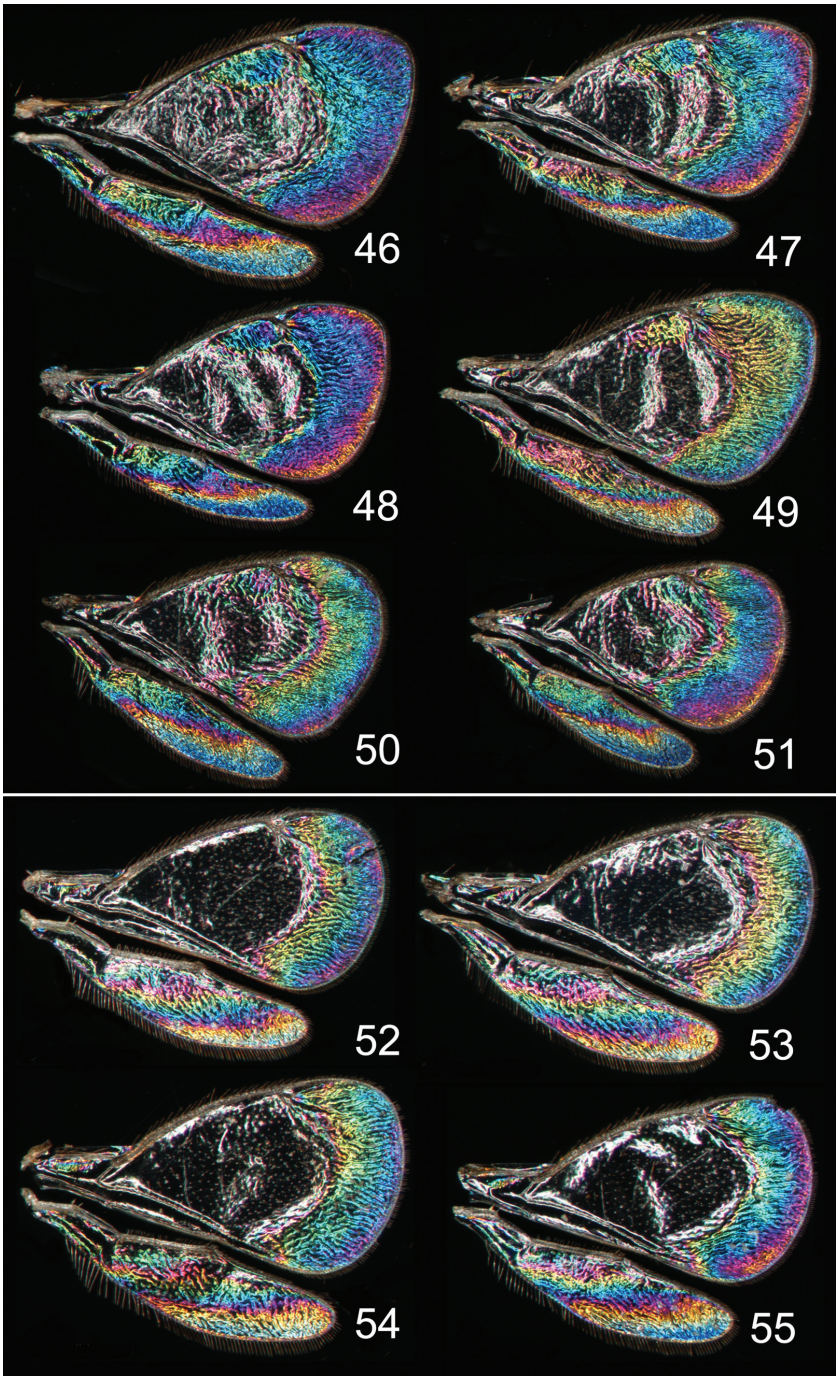
Figures 18–27. *Achrysocharoides* spp., wing interference patterns (WIPs): **18–23** *A. platanoidae* Hansson & Shevtsova **18–21** Males **22–23** Females **24–27** *A. acerianus* (Askew) **24–25** Males **26–27** Females. All wings from specimens from Sweden, Skåne, 2010.



Figures 28–37. *Achrysocharoides* spp., wing interference patterns (WIPs): **28–35** *A. robiniae* Hansson & Shevtsova **28–33** Males **34–35** Females **36–37** *A. robinicolus* Hansson & Shevtsova **36** Male **37** Female. Wings on Figs **28–31, 34–37** from USA, Connecticut, 2002 **32, 33**, from Hungary, Vas Co., 2002.



Figures 38–45. *Achrysocharoides butus* (Walker), wing interference patterns (WIPs): **38–43** Males **44–45** Females. Wings on Figs **38, 40–45** from Wales, 1976 **39** from Sweden, Skåne, 2010.



Figures 46–55. *Achrysocharoides* spp., wing interference patterns (WIPs): **46–51** *A. latreilleii* (Curtis) **46–49** Males **50–51** Females **52–55** *A. albiscapus* (Delucchi), males. Wings on Figs **46, 47, 49–51** from England, Surrey 1986–2004 **48** from Sweden, Skåne, 2010; **52, 53, 55** from Greece, Crete, 1997 **54** from France, 1984.

sova et al. 2011). We have found that the uneven thickness of the wing membrane also can be demonstrated and authenticated through the contrast in grey scale SEM images of uncoated wings. The SEM images created through back-scattered electrons (BSEs) visualize specific patterns on wings (Figs 11, 13, 15, 17). These patterns fully correspond to the approximate mapping of the wing thickness based on WIPs where the thickness of the wing membrane at any point can be estimated by the reflected interference colour (Shevtsova et al. 2011). The thickness gradient as seen through grey scale gradients in SEM images is due to specific properties of BSEs which have the escape depth of up to hundreds of nanometers (Egerton 2005). This means that the signal comes from a sample depth in the range comparable to membrane thickness in wings producing bright WIPs, i.e. 100–600 nm. In uncoated wings the primary (incident) electrons are scattered inside the membrane and reflected as BSEs to the back-scatter detector. In thick areas of the membrane the amount of BSEs is large, resulting in a strong signal, while thin areas of the membrane produce fewer BSEs and a weaker signal, thus displaying light and dark grey hues respectively. If the wings are coated with platinum or gold, the resulting picture is completely different due to secondary electrons (SEs) which are generated only within a very small distance below the surface as the escape depth of SEs is less than two nanometers (Egerton 2005), thus displaying the surface of the specimen rather than the underlying structure. In Shevtsova *et al*. (2011) secondary electron images were used to illustrate the microstructures of the wing surface, such as the ridges of membrane corrugations with rows of setae.

The clarification of the two cryptic species on *Acer* spp. (Hansson and Shevtsova 2010, Shevtsova et al. 2011) requires a correction of the molecular information deposited in Genbank. At the time of the publication of Lopez-Vaamonde et al. (2005) the identity of the *Achrysocharoides* species associated with *Acer* spp. was not clear, and "Achrysocharoides acerianus ex Acer platanoides" and "Achrysocharoides sp. ex Acer pseudoplatanus" in Lopez-Vaamonde et al. (2005) are *A. platanoidae* and *A. acerianus* respectively, which is confirmed here with new molecular analyses compared to data of "Achrysocharoides sp." and "A. acerianus" in Genbank. Our new sequences include CO1, 18S, 28S and will be deposited in Genbank.

Species descriptions

***Achrysocharoides maieri* sp. n.**
urn:lsid:zoobank.org:act:2E20B2E3-557F-413E-8729-D9ADD646FE3C
http://species-id.net/wiki/Achrysocharoides_maieri
Figures 56–71

Material. HOLOTYPE male (CNC) glued to a card, labeled "U.S.A.: Connecticut, New Haven Co., New Hamden, Lockwood Farm, 1.viii.1980, C.T. Maier", "Tentiform mine of Phyllonorycter propinquinella on Prunus serotina, emerged in laboratory within 3 weeks". PARATYPES: 1 female 3 males with same label data as holotype

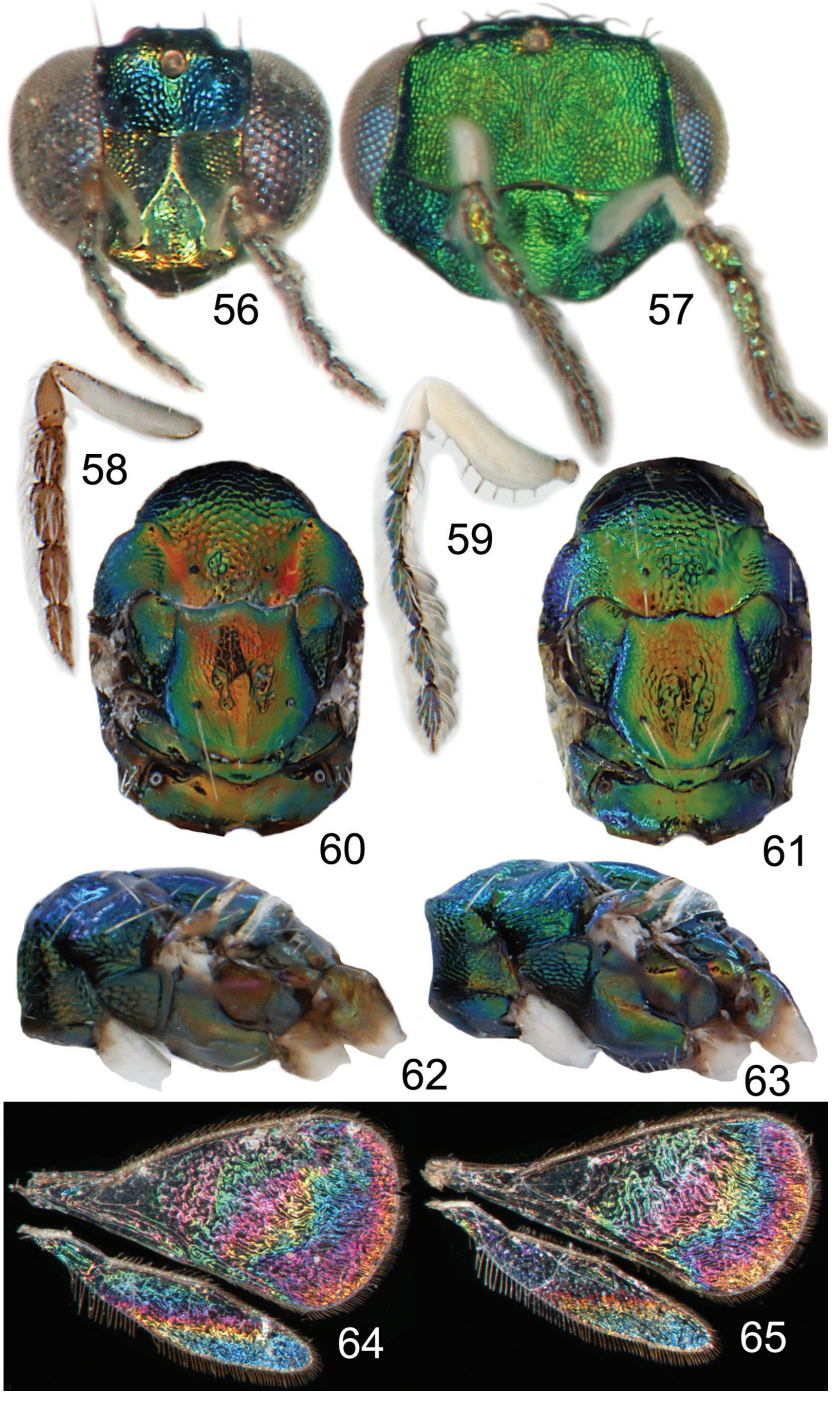
(BMNH, CAES, CNC)); 1 male labeled "U.S.A.: Connecticut, Tolland Co., Willington, 21.x.1981, Chris T. Maier", "Mines of Phyllonorycter propinquinella on black cherry, Prunus serotina, on 21.x.1981, chilled outdoors, parasitoid emerged in laboratory in April 1982" (CNC); 3 females "U.S.A.: Connecticut, New Haven Co., North Haven, 1.vii.1981, C.T. Maier", "Tentiform mine of Phyllonorycter nr crataegella on Prunus pensylvanica, emerged in laboratory within 3 weeks" (BMNH, CNC); 2 females 1 male from same locality and same host as previous but collected 2.vi.1986 (BMNH).

Diagnosis. Both sexes: fore wing WIP with several distinct wide colourful cross-bands traversing the wing (Figs 64, 65), fore coxa white, hind coxa except apex golden green (Figs 62, 63); male: scape widest just below median part, with a single sparse row of setae along ventral margin (Fig. 59), antennal scrobes join frontal suture wide apart (Figs 67), vertex with long forward pointing setae (Fig. 69) – setae about as long as distance between posterior ocelli, upper frons without setae (Fig. 67), frons very large and wide (Fig. 67) - at its widest part 0.8X as wide as width of head; female: scape predominantly white and widest medially, with a single row of setae along ventral margin, propodeum smooth (Fig. 70), frons above frontal suture with raised and strong reticulation (Fig. 66).

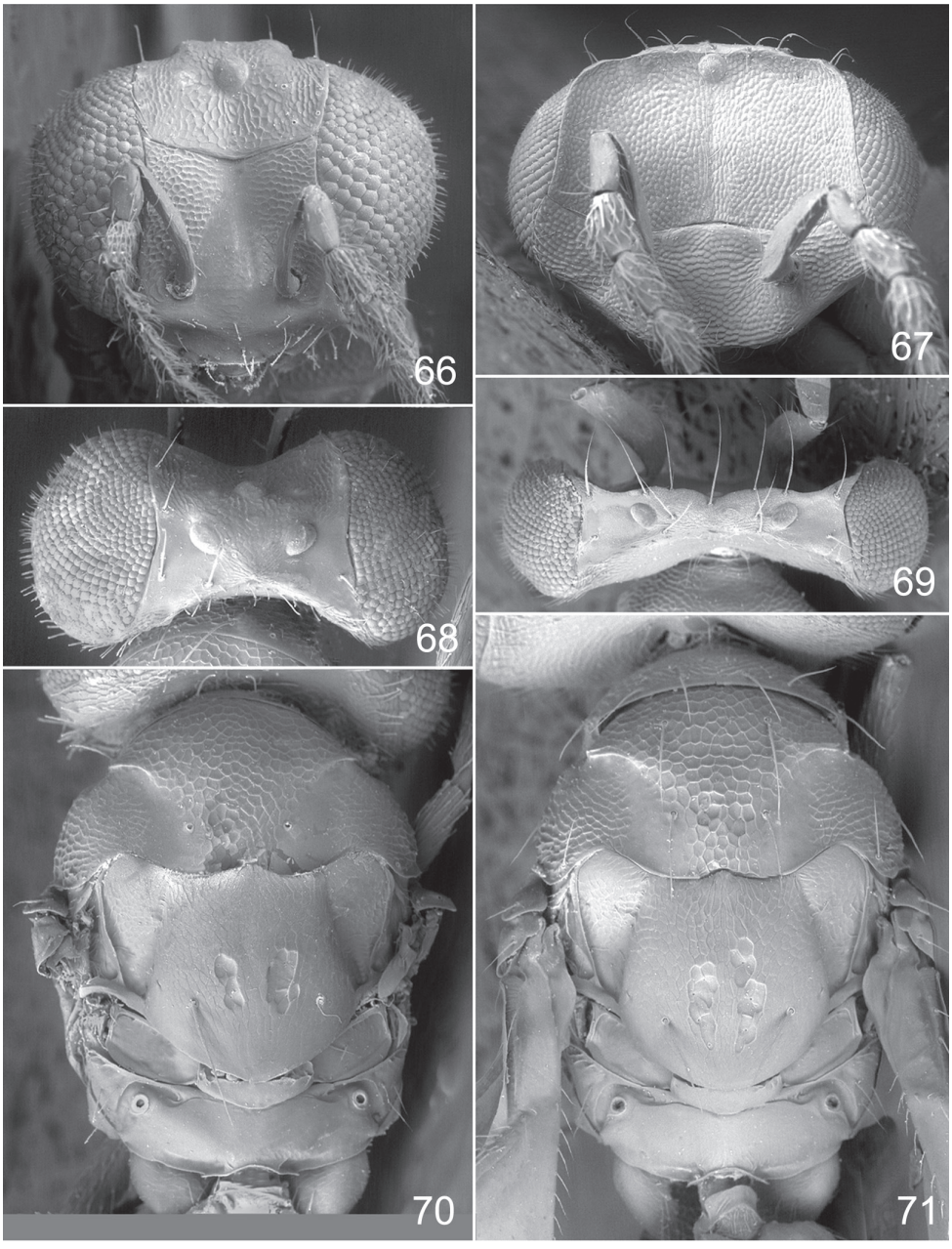
Description. *Female*. Length 1.1–1.5 mm. Scape white with inner apical tip infuscate; pedicel pale brown; flagellum dark brown (Fig. 58). Frons below frontal suture golden green to golden red, above frontal suture bluish green metallic (Fig. 56). Vertex inside ocellar triangle golden red, outside ocellar triangle golden green. Mesoscutum golden green with golden red areas – especially so in smooth posterior notaular depressions, to completely golden green (Fig. 60). Scutellum golden red with sides and posterior margin bluish green metallic, to completely golden green (Fig. 60). Propodeum golden red to golden green (Fig. 60). Fore coxa white, mid coxa dark brown with apical 1/3 white to completely dark brown, hind coxa golden green (Fig. 62); femora, tibiae and tarsi on all legs white. Wings without pigmented areas; WIP in fore wing with several distinct wide colourful cross-bands traversing the wing (Fig. 64). Gaster with first two tergites golden green, remaining tergites golden purple with green metallic tinges.

Antenna as in Fig. 58. Frons below level of toruli smooth and shiny (Fig. 66), between level of toruli and frontal suture with raised and strong reticulation lateral to antennal scrobes, between antennal scrobes with very weak reticulation, above frontal suture with raised and strong reticulation. Vertex inside ocellar triangle with engraved and weak reticulation, outside ocellar triangle smooth and shiny (Fig. 68). Occipital margin rounded.

Pronotal collar without transverse carina (Fig. 70). Mesoscutum with raised and strong reticulation (Fig. 70), meshes of reticulation smaller on sidelobes than on midlobe, midlobe with pits (i.e. with very strong reticulation) posteromedially; notauli as smooth impressions in posterior 2/3. Scutellum with very weak reticulation and shiny, smooth along posterior margin, with 3–4 pits medially on either side of imaginary median longitudinal line (Fig. 70). Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny (Fig. 70); propodeal callus with three setae. Fore wing speculum closed below. Petiole conical without shoulders.



Figures 56–65. *Achrysocharoides maieri* sp. nov.: **56** Head frontal, female **57** Ditto, male **58** Antenna lateral, female **59** Ditto, male **60** Mesosoma dorsal, female **61** Ditto, male **62** Mesosoma lateral, female **63** Ditto, male **64** Wing interference pattern (WIP), female **65** Ditto, male.



Figures 66–71. *Achrysocharoides maieri* sp. n.: **66** Head frontal, female **67** Ditto, male **68** Vertex, female **69** Ditto, male **70** Mesosoma dorsal, female. **71** Ditto, male.

Ratios. HE/MS/WM = 5.0/1.0/2.3; POL/OOL/POO = 2.6/1.1/1.0; WH/WT = 1.2; LW/LM/HW = 1.6/1.0/1.0; PM/ST = 1.0; MM/LG = 0.8–0.9.

Male. Length 1.4–1.5 mm. Scape and pedicel white; flagellum dark brown with golden green tinges (Fig. 59). Frons green metallic (Fig. 57). Vertex inside ocellar tri-

angle golden red, outside ocellar triangle golden green. Mesoscutum golden green with posterior 1/3 of notaular depressions golden red (Fig. 61). Scutellum golden red with sides bluish green metallic (Fig. 61). Propodeum golden green (Fig. 61). Fore coxa white, mid coxa dark brown with apical 1/3 white to completely dark brown, hind coxa golden green with apical half white (Fig. 63); femora, tibiae and tarsi on all legs white. Wings without pigmented areas; WIP very similar to that of the female (Fig. 65). Gaster with tergites 1–2 golden green with a large white spot medially, remaining tergites dark brown with purple metallic tinges.

Antenna as in Fig. 59, i.e. scape widest just below middle. Frons with engraved and strong reticulation (Fig. 67); antennal scrobes reaching frontal suture wide apart; transverse ridge straight medially. Vertex inside ocellar triangle with engraved and very weak reticulation, outside ocellar triangle smooth and shiny (Fig. 69); anterior part with a row of seven long and proclinate setae. Occipital margin rounded.

Pronotal collar without transverse carina (Fig. 71). Mesoscutum with raised and strong reticulation (Fig. 71), meshes of reticulation smaller on sidelobes than on midlobe, midlobe with pits (i.e. with very strong reticulation) posteromedially; notauli as smooth impressions in posterior 2/3. Scutellum very weak reticulation and shiny, smooth along posterior margin, with 3–4 pits medially on either side of imaginary median longitudinal line (Fig. 71). Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny (Fig. 71); propodeal callus with three setae. Fore wing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 2.3/1.0/1.3; POL/OOL/POO = 14.4/6.4/1.0; WH/WT = 1.4; LW/LM/HW = 1.5/1.0/1.0; PM/ST = 1.0; MM/LG = 1.0.

Etymology. Named after Dr. Chris T. Maier, Entomologist at the Connecticut Agricultural Experiment Station, who collected all material of the two new species described here.

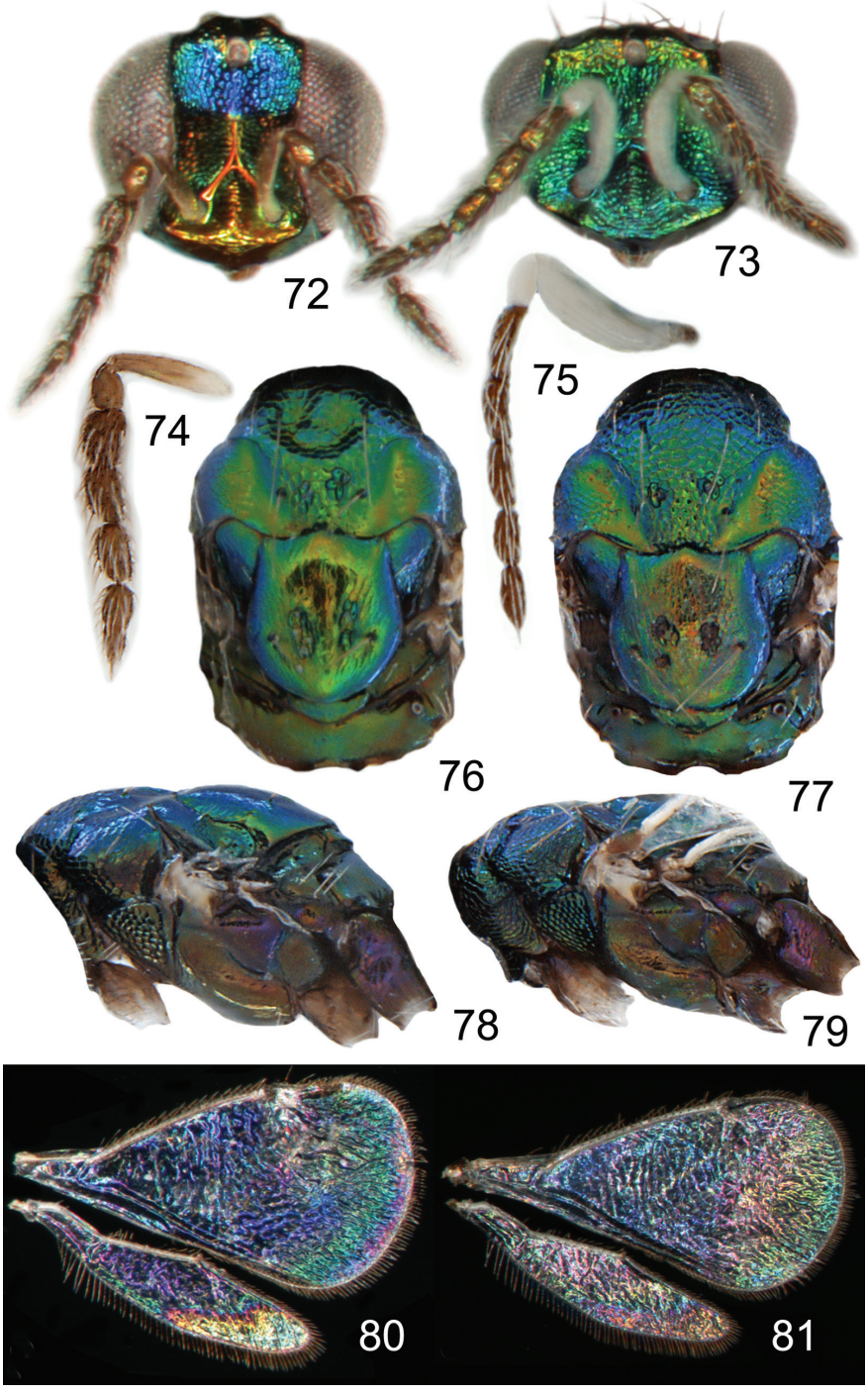
Distribution. U.S.A. (Connecticut).

Hosts. *Phyllonorycter propinquinella* (Braun) (Lepidoptera: Gracillariidae) on black cherry (*Prunus serotina*), and *Phyllonorycter nr crataegella* on pin cherry (*Prunus pensylvanica*).

Achrysocharoides serotinae sp.n.

urn:lsid:zoobank.org:act:C0EA95FF-793E-46BF-AF38-E300F345AB48
http://species-id.net/wiki/Achrysocharoides_serotinae
Figures 72–87

Material. HOLOTYPE male (CNC) glued to a card, labelled "U.S.A.: Connecticut, New Haven Co., North Haven, 30.ix.1981, Chris T. Maier", "Adult parasitoid lab-reared from tentiform mine of *Phyllonorycter propinquinella* collected on black cherry, *Prunus serotina* on 30.ix.1981". PARATYPES: 1 male with same label data as holotype (CNC); 2 females labeled "U.S.A.: Connecticut, Tolland Co., Union, 23.vi.1981, Chris T. Maier", "Adult parasitoid lab-reared from tentiform mine of *Phyllonorycter propinquinella* collected on black cherry, *Prunus serotina* on 23.vi.1981" (CNC).



Figures 72–81. *Achrysocharoides serotinae* sp. n.: **72** Head frontal, female **73** Ditto, male **74** Antenna lateral, female **75** Ditto, male **76** Mesosoma dorsal, female **77** Ditto, male **78** Mesosoma lateral, female **79** Ditto, male **80** Wing interference pattern (WIP), female **81** Ditto, male.

Diagnosis. Both sexes: fore wing WIP almost unicoloured, gradually changing hue from purple to green towards the margin, without any distinct details such as lines or spots (Figs 80, 81), fore coxa predominantly dark brown, hind coxa golden green (Figs 78, 79); male: scape with about same width throughout, with a single sparse row of setae along ventral margin, antennal scrobes joining on frontal suture (Fig. 73, 83), vertex with long forward pointing setae (Fig. 85) – setae at most as long as distance between posterior ocelli, upper frons without setae (Fig. 83); female: scape pale brown and widest medially, with a single row of setae along ventral margin (Fig. 74), propodeum smooth (Fig. 86), frons above frontal suture with raised and strong reticulation (Fig. 82).

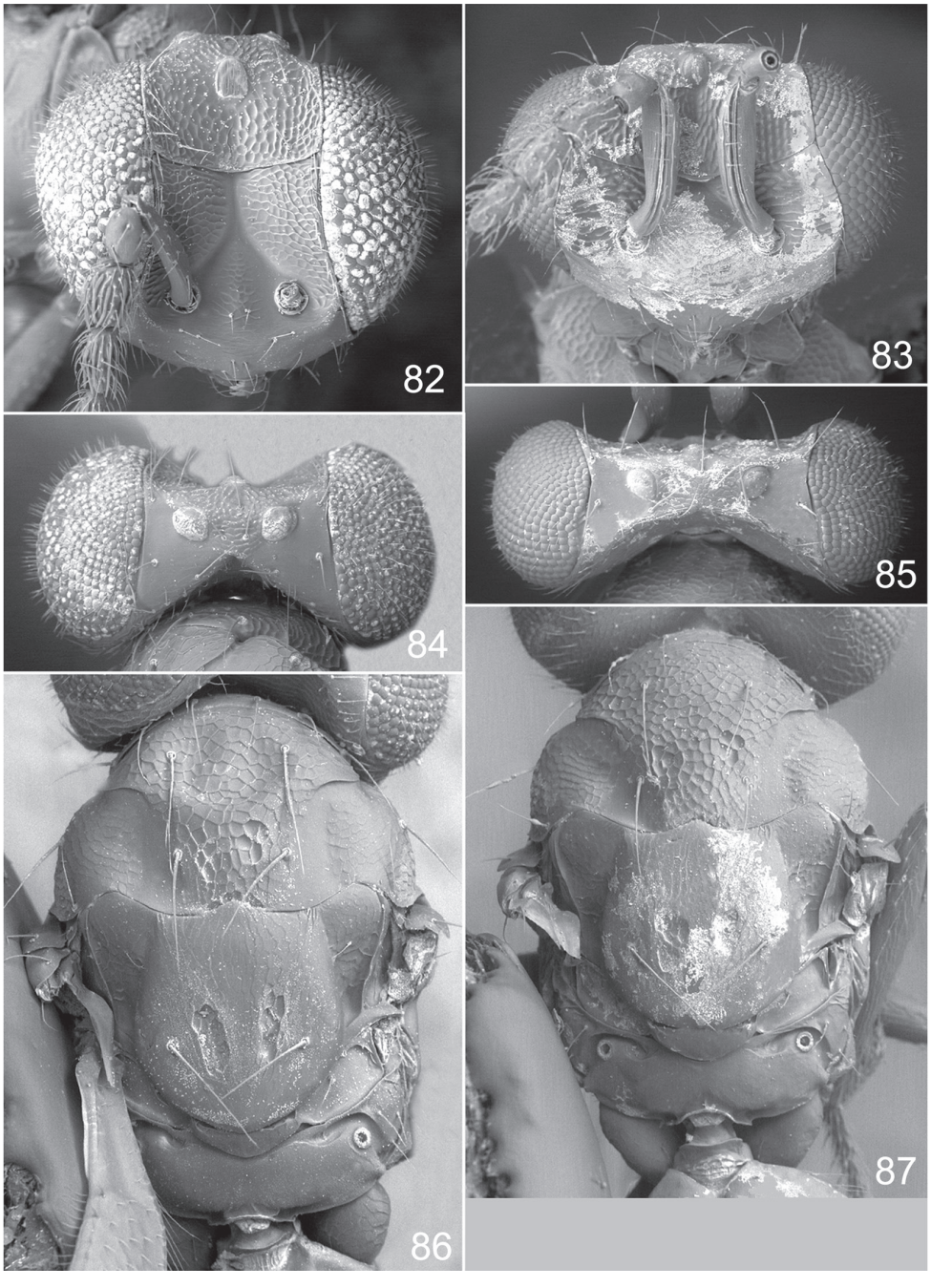
Description. *Female*. Length 1.2–1.3 mm. Scape and pedicel pale brown; flagellum dark brown (Fig. 74). Frons below frontal suture golden red, above frontal suture bluish green metallic (Fig. 72). Vertex inside ocellar triangle golden red, outside ocellar triangle golden green. Mesoscutum green metallic with blue metallic tinges, smooth parts of notaular depression golden green (Fig. 74). Scutellum golden green with sides and posterior margin bluish green metallic (Fig. 74). Propodeum golden green with blue metallic tinges (Fig. 74). Fore coxa dark brown with apical 1/3 white, mid coxa dark brown, hind coxa purple metallic (Fig. 78); femora, tibiae and tarsi on all legs white. Wings without pigmented areas; WIP in fore wing almost unicoloured, gradually changing hue from blue to green towards the margin when the membrane becomes gradually thinner (Fig. 80). Gaster with first two tergites golden green, remaining tergites golden purple with green metallic tinges.

Antenna as in Fig. 74. Frons below level of toruli smooth and shiny (Fig. 82), between level of toruli and frontal suture with raised and strong reticulation with antennal scrobes smooth, above frontal suture with raised and strong reticulation. Vertex inside ocellar triangle with engraved and weak reticulation, outside ocellar triangle smooth and shiny (Fig. 84). Occipital margin rounded.

Pronotal collar without transverse carina (Fig. 86). Mesoscutum with raised and strong reticulation (Fig. 86), meshes of reticulation smaller on sidelobes than on midlobe, midlobe with singular pits (i.e. with very strong reticulation) posteromedially; notauli as smooth impressions in posterior 2/3. Scutellum with very weak reticulation and shiny, smooth along posterior margin, with 2–4 pits medially on either side of imaginary median longitudinal line (Fig. 86). Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny (Fig. 86); propodeal callus with three setae. Fore wing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 3.7/1.0/1.6; POL/OOL/POO = 1.7/1.0/1.0; WH/WT = 1.2; LW/LM/HW = 1.6/1.0/1.0; PM/ST = 1.0; MM/LG = 0.8–0.9.

Male. Length 1.4 mm. Scape and pedicel white; flagellum dark brown (Fig. 75). Frons green metallic (Fig. 73B). Vertex inside ocellar triangle golden red, outside ocellar triangle golden green. Mesoscutum golden green with anterior part blue (Fig. 77). Scutellum golden green with golden red tinges and with lateral parts blue (Fig. 77).



Figures 82–87. *Achrysocharoides serotinae* sp. n.: **82** Head frontal, female **83** Ditto, male **84** Vertex, female **85** Ditto, male **86** Mesosoma dorsal, female **87** Ditto, male.

Propodeum golden green with golden red tinges (Fig. 77). Fore coxa dark brown with apical 1/3 white, mid coxa dark brown, hind coxa purple metallic (Fig. 79); femora, tibiae and tarsi on all legs white. Wings without pigmented areas; WIP very similar to that of the female (Fig. 81). Gaster with tergites 1–2 dark brown with golden green tinges, remaining tergites dark brown with weak metallic tinges, over tergites 1–3 with a large median white spot.

Antenna as in Fig. 75, i.e. scape with about same width throughout. Frons with raised and strong reticulation, some parts with transverse striation (Fig. 83); antennal scrobes joining on frontal suture; transverse ridge evenly curved. Vertex inside ocellar triangle with engraved and very weak reticulation (Fig. 85), outside ocellar triangle smooth and shiny; anterior part with a row of 3–5 long and forward directed setae. Occipital margin rounded.

Pronotal collar without transverse carina (Fig. 87). Mesoscutum with raised and strong reticulation (Fig. 87), meshes of reticulation smaller on sidelobes than on midlobe, midlobe with pits (i.e. with very strong reticulation) posteromedially; notauli as smooth impressions in posterior 2/3. Scutellum with weak reticulation, smooth along posterior and lateral margins, with 2–5 pits medially on either side of imaginary median longitudinal line (Fig. 87). Dorsellum flat and smooth, anterolaterally with two foveae. Propodeum smooth and shiny (Fig. 87); propodeal callus with three setae. Fore wing speculum closed below. Petiole conical without shoulders.

Ratios. HE/MS/WM = 2.5/1.0/1.3; POL/OOL/POO = 4.6/1.8/1.0; WH/WT = 1.1; LW/LM/HW = 1.6/1.0/1.0; PM/ST = 1.0; MM/LG = 0.9.

Etymology. Named after black cherry (*Prunus serotina*), the host plant.

Distribution. U.S.A. (Connecticut).

Host. *Phyllonorycter propinquinella* (Braun) (Lepidoptera: Gracillariidae) on black cherry (*Prunus serotina*).

Identification of the new species

In the most recent key to North American *Achrysocharoides* by Kamijo (1991) the two newly described species both key out to the *clypeatus* group. To include them in the key to species of this group the following changes can be made:

Females of both species run to couplet 3, alternative 2 (where *A. arienascapus* falls out). The second alternative is changed to lead to 3a instead of *A. arienascapus* and then:

3a Fore coxa and scape predominantly brown (Figs 74, 78) ... ***A. serotinae* sp. n.**

– Fore coxa and scape white **3b**

3b Entire frons above frontal suture with raised and strong reticulation (Fig. 66); scutellum with very weak and superficial reticulation (Fig. 70) ... ***A. maieri* sp. n.**

– Frons strongly reticulate medially and weakly reticulate laterally; scutellum with strong reticulation ..... ***A. arienascapus* (Miller)**

Males run to couplet 4:

4 Frons above frontal suture with many short and scattered setae (see fig. 5 in Kamijo (1991)); scape with long dense setae ventrally (see fig. 5 in Kamijo (1991)) ..... ***A. hirtiscapus* (Miller)**

– Frons above frontal suture bare (Figs 67, 83); scape with a few short setae along ventral edge (Figs 59, 75) **5a**

5a Vertex with long setae about as long as distance between posterior ocelli (Figs 69, 85) **5b**

– Vertex with long setae at least as long as width of vertex (see fig. 7 in Kamijo (1991)) **5**

5b Scape widest close to base (Fig. 59); fore coxa white (Fig. 63) .... ***A. maieri* sp. n.**

– Scape with about same width throughout (Fig. 75); fore coxa predominantly brown (Fig. 79) ..... ***A. serotinae* sp. n.**

Acknowledgements

Our foremost thanks are due to ArtDatabanken who through the Swedish Taxonomy Initiative funds the PhD project of Ekaterina Shevtsova. Thanks also to Chris T. Maier (at CAES) who generously sent us his material of *Achrysocharoides*, and to Gary A.P. Gibson and John T. Huber (both at CNC) for the loan of material. The help of Andrew Polaszek (BMNH) with obtaining molecular data for *Achrysocharoides acerianus* and *A. platanoidae* is highly appreciated. We thank the microscopy unit at the Biology Department, Lund University, for the use of their SEM. We also thank two anonymous reviewers for their useful suggestions.

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Paper IV

Do Wing Interference Patterns (WIPs) in Minute Parasitoid Wasps Show Reproductive Character Displacement?

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Abstract

Wing interference patterns (WIPs) are a recently discovered type of visual signal found in very small insects with what at first sight appear to be transparent wings. We hypothesised that these patterns may be used in intraspecific recognition and also show reproductive character displacement. Tests of this hypothesis are difficult because typically little is known about the detailed biology of minute insects, though an exception is tiny parasitic wasps in the genus Achrysocharoides Girault. We asked whether sympatric species of this genus, that may encounter each other while mating, are more likely to show divergent WIPs than allopatric species. While a formal test of the hypothesis was not possible our results show significant evidence in support of the prediction.

Key Words: Sympatric, allopatric, leaf miners, parasitoids, *Phyllonorycter, Achrysocharoides.*

Introduction

It is nearly always disadvantageous for an animal to mate with an individual belonging to a different species and a wide variety of different signal types are used as behavioural isolating mechanisms. In some cases, the signals produced by two related species are more different when they co-occur in the same geographical region or microhabitat compared to where each species occurs alone and such reproductive character displacement (Brown & Wilson, 1956, Pfennig & Pfennig, 2009, Servedio & Noor, 2003, Butlin, 1995) provides strong evidence that a particular signal is involved in species recognition.

The first signals to be associated with reproductive isolation were, understandably, those in the visual and auditory frequencies readily detected by man. But as our technological ability to detect other signalling modalities has developed many examples of species recognition mechanisms involving visual and auditory frequencies out of the human range have been discovered, as well as different types of signals including volatile and soluble chemicals, sonar, vibration and electrical pulses (Espmark et al., 2000).

A novel type of visual signal was recently discovered in very small species of

insects: wing interference patterns (WIPs) (Shevtsova et al., 2011). The wings of tiny insects appear transparent and featureless unless they are viewed against a dark background where they show colourful patterns. These structural colours are caused by the interference of refracted and reflected light in very thin wing membranes. Variation in wing thickness and structure gives rise to different patterns that can be species and sometimes also sex specific (Shevtsova & Hansson, 2011).

We explore here whether WIPs may be used as species recognition mechanisms by looking for evidence of reproductive character displacement. The motivation for this study was the observation that species of the chalcidoid wasp genus *Achrysocharoides* that attacked the same host insects on the same species of plant in North America had particularly divergent WIPs compared with species that would encounter no other congeneric wasp on their host's food plant (Hansson & Shevtsova, 2010, Shevtsova & Hansson, 2011). This genus is much better known in Europe compared with North America and we survey all known European species and ask whether species that share hosts on the same food plant are more likely to have WIPs that diverge from the ancestral state.

Methods

Biology of the system

Achrysocharoides (Hymenoptera, Chalcidoidea, Eulophidae, Entedoninae) are minute, metallic parasitoid wasps with a wing length of ~1.5mm. European species are almost exclusively parasitoids of micromoths in the genus *Phyllonorycter* Hübner (Lepidoptera, Gracillariidae) (Lopez-Vaamonde et al., 2005) which form distinctive "tentiform" mines in the leaves of deciduous trees and a few herb species (Lopez-Vaamonde et al., 2003). The first instar larvae of the host feed initially within the upper or lower epidermal cells while later instars feed extracellularly and lay down a layer of silk on the surface of the mine which, as it dries, puckers the leaf so that it becomes tent-shaped (Davis, 1987). Different species are consistent in whether they form upper- or under-surface mines with the latter habit being the most frequent. *Achrysocharoides* species, like most Entedoninae, are koinobiont endoparasitoids – that is they feed internally and can suspend development as first instar larvae while their host grows large enough to support them (Askew & Ruse, 1974). However, they have highly unusually progeny and sex allocation behaviours which have been the subject of several studies by evolutionary biologists (Askew & Ruse, 1974, Bryan, 1980a, West et al., 1999). Females of some species oviposit small clutches of 1-4 male and female eggs into each host while others produce equal-sized clutches composed only of males or females. A third strategy is to produce gregarious clutches of females but for males to develop alone. One species is thelytokous and produces males only on very rare occasions (Bryan, 1980a).

The majority of species of *Phyllonorycter* attack plants belonging to a single or a few closely related genera of plants though there are a few cases of moths being restricted to host plant species rather than genera (Lopez-Vaamonde et al., 2003). Frequently more than one and sometimes up to about ten species of *Phyllonorycter* are found attacking the same plant. *Achrysocharoides* species are largely confined to individual host plant genera, or groups of closely related genera, and attack all the *Phyllonorycter* species found on the same group of host plants that form mines on the same surface of the leaf (Askew & Ruse, 1974). Most species of *Achrysocharoides* have two generations a year like their hosts. The parasitoids pupate in their hosts' mines, the first generation emerging from

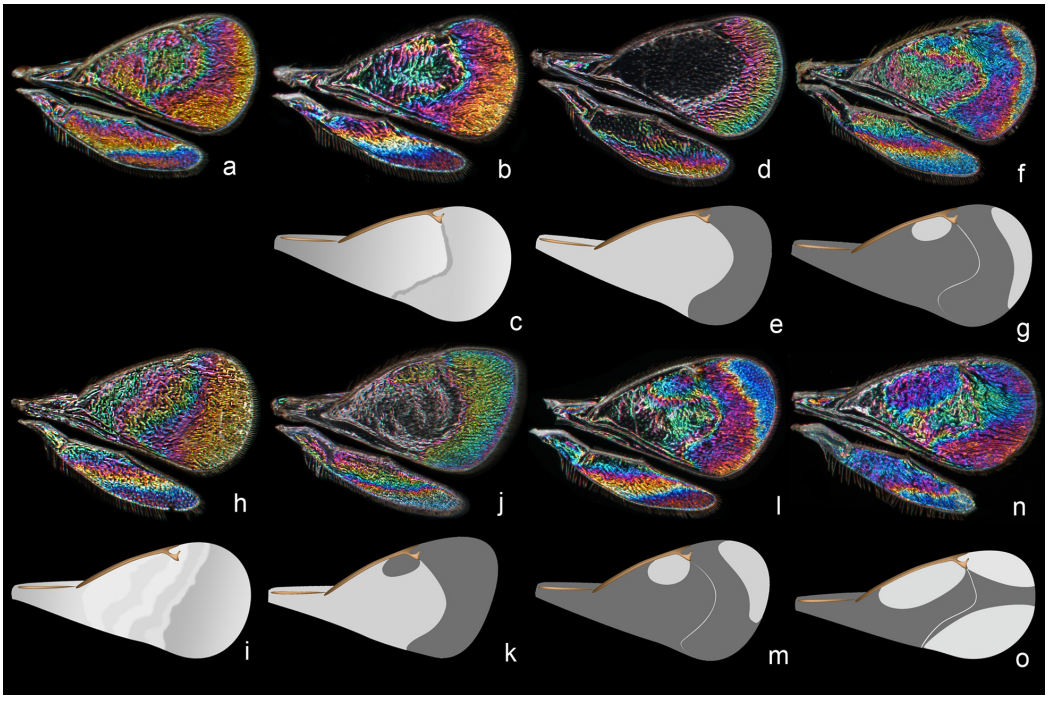

Figure 1. Wing interference patterns (WIPs) in males. (a–c) Ancestral patterns, (d–o) Derived patterns. Grey-scale illustrations are included to highlight the patterns, with pale areas indicating the distinctive parts. (**a**) *Chrysocharis prodice* (Walker). (**b–c**) *Achrysocharoides acerianus* (Askew). (**d–e**) *A. albiscapus* (Delucchi). (**f–g**) *A. butus* (Walker). (**h–i**) *A. cruentus* Hansson. (**j–k**) *A. latreilleii* (Curtis). (**l–m**) *A. platanoidae* Hansson & Shevtsova. (**n–o**) *A. robiniae* Hansson & Shevtsova.

leaves attached to the tree while the second generation in spring emerge from mines in leaf litter. The precise location of mating is not known but is likely to be on or in the near vicinity of the host plant.

WIPs of European Achrysocharoides

The ancestral WIP of *Achrysocharoides* (Figs 1b–c) can be inferred by examining the WIPs of closely related genera such as *Chrysocharis* Förster (Fig. 1a). In it a major feature is a narrow coloured band curving from the stigmal vein to the posterior wing margin. The band forms a border that splits the wing pattern into a basal part with some indistinct curved bands, and an apical part which is usually of one colour only. A variety of derived patterns are shown by a minority of species (Figs 1d–o), occasionally in both sexes but often only in the male.

There are currently 21 valid species of *Achrysocharoides* in Europe which are listed in Table 1 along with the plant species with which they are associated, whether they attack upper or lower surface miners, and whether they have ancestral or derived WIPs. To assess the validity of several poorer known species we had to examine type material and some resulting nomenclatural changes and taxonomic notes will be published in the taxonomic literature. We placed the 21 species into 16 ecological groups (Table 2) based on whether they attacked the same hosts on the same host plant though also placed in its own group the upper-surface mine specialist *Achrysocharoides suprafolius* (Askew) which attacks a relatively polyphagous upper-surface *Phyllonorycter* species. Of the 16 ecological groups 12 contained

single species and four contained more than one species. The groups could also be distinguished by whether they all had the ancestral WIP (12 groups) or if one or more species differed from the ancestral pattern (four groups).

Results

If WIPs are used in species recognition we would expect an association of derived WIPs and situations where multiple *Achrysocharoides* search for hosts on the same food plants. The data (Table 3a) do show such a trend though it is not significant (Fisher's Exact Test, $P = 0.18$).

The presence of the trend prompted us to look at the ecological groups that differed from the predicted pattern. The biology suggested that two groups should be reclassified. First, *Achrysocharoides robiniae* Hansson & Shevtsova is anomalously a species with a derived wing pattern that alone searches for its host on *Robinia pseudoacacia* L. But the parasitoid, host and food plant are all non-natives in Europe and introduced from North America where it is known that a second species of *Achrysocharoides* (*A. robinicolus* Hansson & Shevtsova) searches for the same host on the same tree species. We reclassified this ecological group as containing multiple parasitoid species. Second, the biology of *Achrysocharoides parva* (Delucchi) and *A. tavellae* Navone which both feed on *Quercus ilex* L. has recently been studied by Navone (2006) who has shown that they have different phenologies and the adults are not present together at the same time of year. In such a case one would not expect species recognition signals to evolve and hence we have reclassified this group as containing single species (to be conservative we have not erected two separate ecological groups for this pair of species). Analysing the reclassified data (Table 3b) the association between derived WIPs and multiple species on the same food plant is significant ($P = 0.026$).

We note two objections to this analysis. First, one of the ecological groups contains the thelytokous (asexual) *Achrysocharoides carpini* Bryan on *Carpinus* L.. The reason for the loss of sexual reproduction in this species is unknown as is whether it has occurred recently due, for example, to the presence of an endosymbiont such as *Wolbachia* Hertig. Clearly an asexual species does not currently need species recognition signals. The association is still significant ($P = 0.032$) if this ecological group is omitted. Second, it could be argued that ecological groups containing more than one species of wasp are more likely to have at least one species that differs from the ancestral WIP and that this might explain the association. However, if the analysis is repeated with the basic unit being the species rather than the ecological group (Table 3c) a significant association is found ($P = 0.028$; or omitting *A. carpini,* $P = 0.036$).

There are two final ecological groups (Table 2b) that do not conform to the prediction. One is *Achrysocharoides platanoidae* Hansson & Shevtsova which alone is present on *Acer platanoides* L. and has a derived WIP. It is known from molecular data (Shevtsova & Hansson, 2011) that this species is extremely similar to *A. acerianus* (Askew) which is found alone on *Acer pseudoplatanus* L.. It is unusual to find *Achrysocharoides* specialised at the host plant species level rather than the host plant genus level and conceivably this pair of species once searched together on *Acer* spp. The second anomaly is the pair of species *Achrysocharoides cilla* (Walker) and *A. buekkensis* (Erdös) on *Fagus sylvatica* L. and we note only that *A. buekkensis* is relatively poorly characterised and further research might reveal ecological differences between it and *A. cilla*.

Discussion

Our analysis of the distribution of WIPs in European parasitoid wasps of the genus *Achrysocharoides* provides evidence that this novel signal system is involved in reproductive isolation and species recognition. We do not think our study provides conclusive proof, but argue that the evidence is strong enough to warrant further research, both on these wasps and also on other insects showing variation in WIPs.

There are three main criticisms of our analysis. First, the initial statistical test showed a trend rather than a significant association. The latter was only found after *a posteriori* examination of the biology of the different ecological groups that did not accord with the prediction. Though the biology of *Achrysocharoides* is rather well known compared to the majority of microhymenoptera there is still much that is poorly understood. We have presented here the analysis as it was conducted and interpret the results as providing strong evidence for the role of WIP in species recognition rather than a formal test of the hypothesis. The latter might be performed, for example, on *Achrysocharoides* in other geographical regions as their biology becomes better known.

Second, we have treated ecological groups or wasp species as statistically independent data points without taking into account the phylogenetic relationships between them. Some molecular studies have been done on the genus but material was not available on all species for us to build a full phylogeny. Understanding the full evolutionary relationships between the species in this genus will be valuable not only for studying the evolution of WIPs but also in interpreting the interesting diversity of reproductive strategies in this group.

Finally, this study has only looked at wing patterns and has not studied experimentally mating behaviour in this genus. There is some information available on mating behaviour in a few species of *Achrysocharoides* (Bryan, 1980b) but only on species belonging to single-species ecological groups and collected without reference to the possible role of WIPs. Comparative studies of mating behaviour, as well as data on where mating occurs in the wild, would be valuable in further exploring the signalling role of WIPs hypothesised here.

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Achrysocharoides species	Host plant[1]	Mine position	WIP Ancestral[2]
acerianus (Askew)	*Acer pseudoplatanus*	underside	yes
albiscapus (Delucchi)	*Quercus* (deciduous)	underside	no
altilis (Delucchi)	*Populus nigra*	underside	yes
atys (Walker)	Woody Rosaceae	underside	yes
buekkensis (Erdös)	*Fagus sylvatica*	underside	yes
butus (Walker)	*Quercus* (deciduous)	underside	no
carpini Bryan	*Carpinus betulus*	upperside	yes
cilla (Walker)	*Fagus sylvatica*	underside	yes
cruentus Hansson	Woody Rosaceae	underside	no
insignitellae(Erdös) / *pannonica* (Erdös)[3]	*Medicago* & *Trifolium*	underside	yes
latreilleii (Curtis)	*Quercus* (deciduous)	underside	no
nigricoxae (Delucchi)	*Lathyrus* & *Vicia*	underside	yes
niveipes (Thomson)	*Betula*	underside	yes
parva (Delucchi)	*Quercus* (evergreen)	Both	yes
platanoidae Hansson & Shevtsova	*Acer platanoides*	underside	no
robiniae Hansson & Shevtsova	*Robinia pseudoacacia*	underside	no
scaposa (Erdös)	*Populus canescens* & *P. alba*	underside	yes
splendens (Delucchi)	*Corylus* & *Alnus*	underside	yes
suprafolius (Askew)	*Betula* and woody Rosaceae	upperside	yes
tavellae Navone	*Quercus* (evergreen)	upperside	yes
zwoelferi (Delucchi)	*Salix*	underside	yes

Table 1. List of European species of *Achrysocharoides* with their host plants, mine position and wing interference pattern (WIP).

[1] Some very rare host records omitted

[2] In both sexes

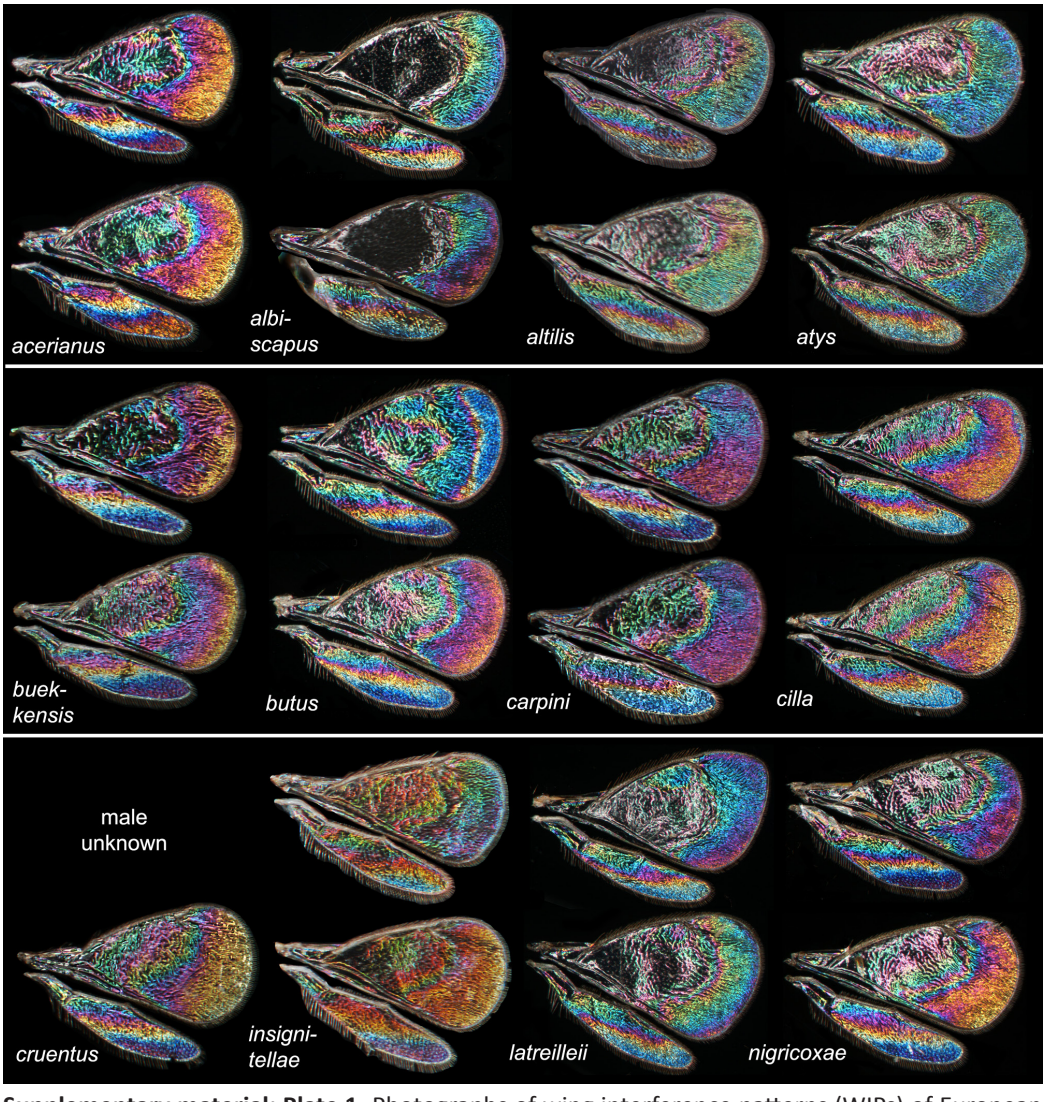
[3] Single species: taxonomic status to be resolved by Hansson (in prep.)

Group	Number of species in group	All retain ancestral WIP (Yes/No)
A. suprafolius	1	Y
Acer pseudoplatanus	1	Y
Acer platanoides	1	N
Betula	1	Y
Carpinus betulus	1	Y
Corylus & Alnus	1	Y
Crataegus etc.	2	N
Fagus sylvatica	2	Y
Lathyrus etc.	1	Y
Medicago etc.	1	Y
Populus nigra	1	Y
Populus canescens & alba	1	Y
Quercus (deciduous)	3	N
Quercus (evergreen)	2	Y
Robinia pseudoacacia	1	N
Salix	1	Y

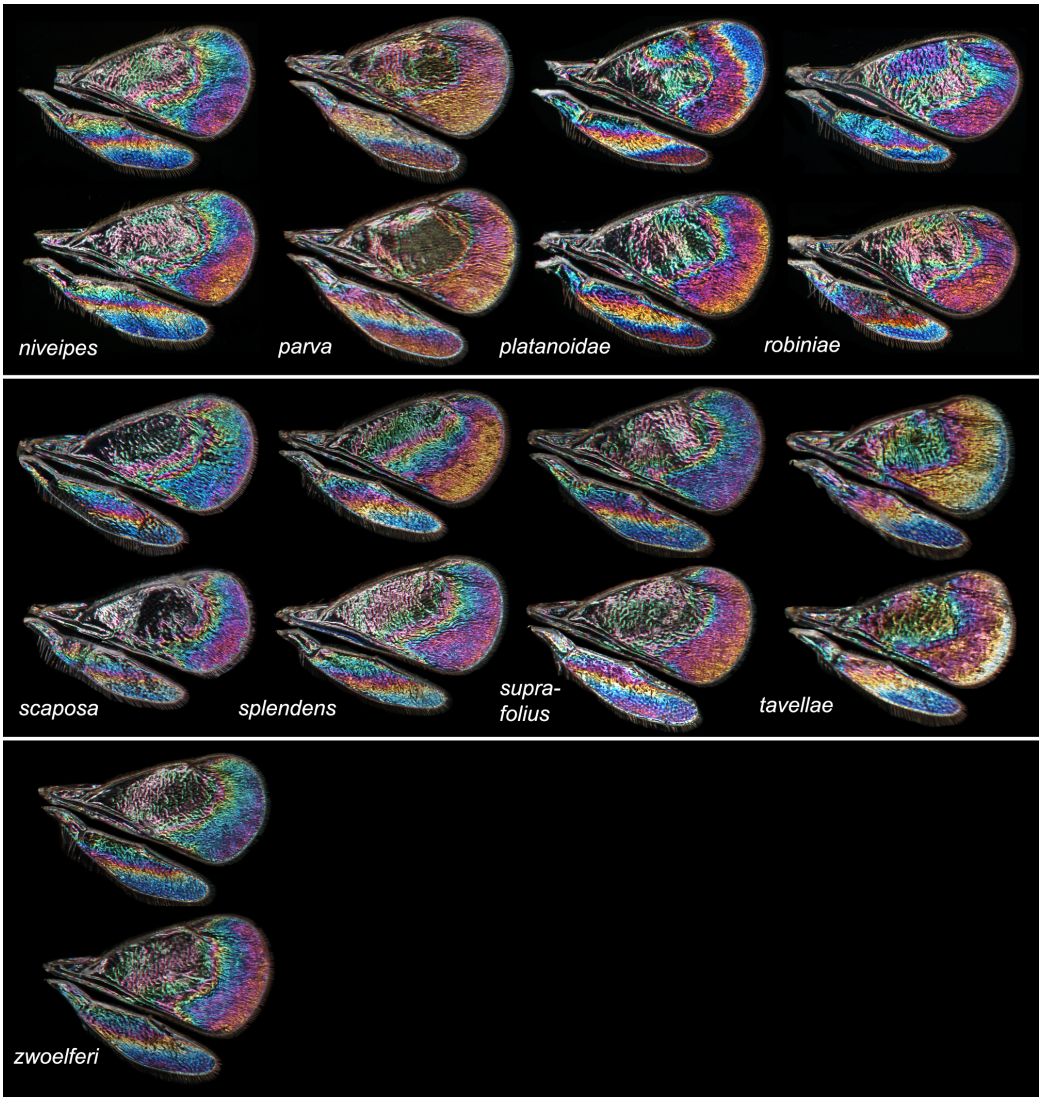
Table 2. Classification of European *Achrysocharoides* into groups based on host plants.

a) Initial tally	All ancestral WIP	All not ancestral WIP
Multiple species in group	2	2
Single species in group	10	2
b) Refined tally	All ancestral WIP	All not ancestral WIP
Multiple species in group	1	3
Single species in group	11	1
c) Species level tally	Ancestral WIP	Derived WIP
Multiple species in group	4	5
Single species in group	11	1

Table 3. Different tallies (see text) of WIPs in multiple and single species groups.



Supplementary material: Plate 1. Photographs of wing interference patterns (WIPs) of European *Achrysocharoides* Girault spp.; male wings above and female wings below.



Supplementary material: Plate 2. Photographs of wing interference patterns (WIPs) of European *Achrysocharoides* Girault spp.; male wings above and female wings below.

Paper V

Evolution in two newly introduced character sets in European *Omphale* Haliday (Hymenoptera: Chalcidoidea: Eulophidae: Entedoninae)

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Abstract

The evolution of wing interference patterns (WIPs) and male genitalia in European species of the genus *Omphale* Haliday is analyzed and discussed. The framework for the discussion is a phylogenetic analysis of 23 species based on all available morphological characters, including features in WIPs and male genitalia. WIPs in *Omphale* differ significantly in diversity from the only other comprehensively studied group this far, genus *Achrysocharoides* Girault (Eulophidae). Consequently this study contributes to the rapidly expanding knowledge of WIP diversity in Hymenoptera. *Omphale* WIPs are classified into four categories, and the derived states are shown to either having evolved just once, or having evolved independently several times. WIPs are thus less useful for phylogenetic analyses in *Omphale*. However, within species-groups WIPs are important for species identification, and since they are similar in both sexes they are also useful in linking conspecific males and females. Genus *Omphale* is defined through a single autapomorphy, the enlarged volsellar setae in male genitalia. Otherwise the male genitalia exhibit an extensive morphological diversity in *Omphale*, including both species-specific and group-specific characters. The analysis of male genitalia includes several new apomorphies which are very useful for the classification of European *Omphale*. Reasons for the unequal rate of divergent evolution of WIPs and male genitalia in *Omphale* are speculated upon. Allopatric distribution and sexual selection are suggested to be important factors for this.

Key words

Morphological characters, wing interference patterns, WIP, male genitalia, phallobase, aedeagus, phylogenetic analysis, TNT phylogeny program, sexual selection, lock-and-key, allopatry, sympatry, host specificity, runaway evolution

In a forthcoming revision by the authors of this paper European *Omphale* Haliday will be comprehensively revised for the first time. Previously this fauna has been studied only on a more limited scale, mainly by Graham (1959, 1963) who included northwestern Europe with a focus on the British fauna. Singular *Omphale* species have been described from other parts of Europe, mainly from Hungary (Erdös 1954, Szelényi 1978), but these species have never been included in a European context. Species of *Omphale* are relatively plain, with few externo-morphological characters, which perhaps is one reason why they have never been studied comprehensively in

Europe. However, taxonomic studies on the New World *Omphale* (Hansson 1996b, 1997, 2004) included new characters from male genitalia, findings that never have been applied to the European species. In addition a new character system, wing interference patterns (WIPs), present in minute winged insects have been discovered (Shevtsova et al. 2011). These new characters enhance the possibilities to ascertain species identities and relationships of European *Omphale*. The poor knowledge of this genus in Europe is further demonstrated by the fact that several new species will be described (referred to as sp1, sp2 etc. in this publication) in the forthcoming revision. The new species are mainly from Sweden and the United Kingdom, two of the most well investigated countries in the World as far as the fauna of Hymenoptera is concerned.

Wing interference patterns (WIPs) occur in transparent wings with a very thin membrane, i.e. mainly in small insects. WIPs appear when the wings are viewed against a dark background and visualize uneven thickness of the wing membrane. These patterns have been used to define a newly described genus from the Neotropical region (Hansson 2011), where a distinct WIP was one of the autapomorphies for the genus. Shevtsova & Hansson (2011) demonstrated the usefulness of WIPs to separate species in the genus *Achrysocharoides* Girault, and in Hernandez-Lopez et al. (in press) WIPs were used in the classification of *Pediobius saulius* (Walker), one of the major parasitoids of the highly invasive horse-chestnut leafminer (*Cameraria ohridella* Deschka & Dimic). Consequently WIPs are useful for the classification on both genus and species levels. Here WIPs are investigated for the first time in a larger revision. The above-mentioned examples are all from the Eulophidae, but as shown in Shevtsova et al. (2011) distinctive WIPs also occur in other groups of Hymenoptera.

The male genitalia in many insect groups tend to evolve divergent forms relatively rapidly compared to other structures, and display a formidable morphological diversity (Eberhard 2009). Male genitalia are therefore frequently the richest source of morphological characters in insects (e.g. Grimaldi & Engel 2005), with important characters on different taxonomic levels. Examples of this are commonly found in e.g. Diptera (Grimaldi & Nguyen 1999), Coleoptera (Hubweber & Schmitt 2009), and Lepidoptera (Sohn & Nishida 2011). However, in the Hymenoptera male genitalia usually show little variation on both species and genus level (Michener 1956). There are some exceptions to this among some of the smallest species of the Chalcidoidea, in the Aphelinidae (Viggiani & Battaglia 1984) and Trichogrammatidae (Nagarkatti & Nagaraja, 1968, 1971; Viggiani 1971, Owen et al. 2007). Male genitalia in the Eulophidae conform to the situation in remaining Chalcidoidea, i.e. with little variation and thus with little information value (e.g. Graham 1987) – with some notable exceptions. The most striking exception is genus *Perditorulus* Hansson, a recently described group confined to the Americas with its main distribution in tropical America, and currently comprising close to a hundred described species. The original description of the genus (Hansson 1996a) included 33 species, all with species-specific genitalia, and with a remarkable morphological diversity of this structure, unparalleled within the Chalcidoidea. Subsequent contributions to *Perditorulus* (Hansson 2004, Hansson & Costa in manuscript) have firmly established and expanded these initial findings. Another group with variations in male genitalia, and therefore with useful information for the classification, is *Omphale* (Hansson 1996b, 1997, 2004), which was one of the incentives for the study of the European species.

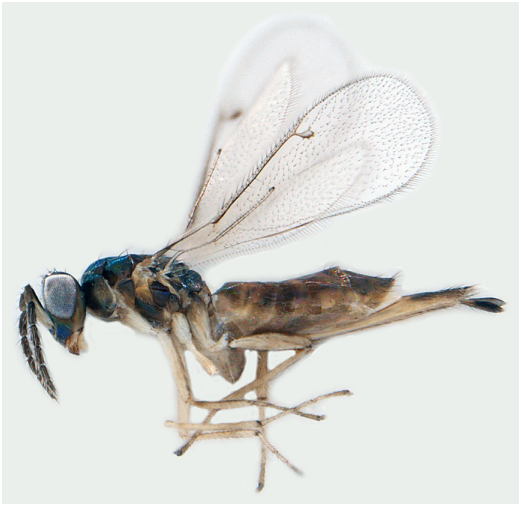


Figure 1. *Omphale theana* (Walker), female habitus, length of body 2.2 mm. This species is distributed throughout Europe and is also known from North America.

There are two hypotheses for the rapid evolution of the morphology in male genitalia. One is the "lock-and-key" hypothesis introduced by Dufour (1844), suggesting that they have evolved as species isolation devices (Shapiro & Porter 1989, Sota & Kubota, and references therein), i.e. that natural selection favours differences in genitalia to prevent cross-specific fertilizations. According to this theory only the genital key of a conspecific male could fit into the lock of the female's genitalia. Several arguments against the lock-and-key hypothesis as a general theory have been put forward by e.g. Eberhard (2009).

The other hypothesis suggests that this evolution is driven by sexual selection (Eberhard 1985, Arnqvist 1998, and references therein), more specifically by selection that is in effect after the male has achieved genital coupling (Eberhard 2009). If a female copulates with more than one male, and if one of these males is more successful than others in "promoting" his own gametes, then that male will sire more offspring and will win out over the other males. If such a female bias is associated with some particular male trait, then it can result in selection favouring that particular trait. The role of the male genitalia in this scenario is to stimulate the female to favour the sperm of the male that possess these traits, i.e. through female cryptic choice (Eberhard 1996). Reasons for the rapid evolution through sexual selection, also known as "runaway evolution", as seen in the male genitalia of many insect groups, was first put forward by Fisher (1930) and were subsequently developed and elaborated on by Lande (1981, 1982).

However, one theory does not exclude the other (Gwynne 1998). On the one hand there are species where females mate only once (monandrous species). In these the cost of cross-specific mating greatly exceeds the cost of evolving species-specific genitalia that functions as reproductive barriers, and lock-and-key devices (or some other species recognition device) will be an evolutionary advantage. On the other hand there are species with females that mate with more than one male (polyandrous species). This is a competitive situation where females have a choice which male will fertilize her eggs, and a situation where sexual selection potentially is in play.

Material and Methods

Terminology – Morphological terms follow Gibson (1997). The morphology is also illustrated in Hansson (2004) and on http://www.neotropicaleulophidae.com. The terminology for male genitalia is from Snodgrass (1941) (Fig. 2). The length of the phallobase on the slide preparations is measured from the apex of the paramere to the base of the phallobase (Fig. 2).

Specimen preparations – Fresh material collected with a sweep-net were killed and kept in 80% ethanol. The wet specimens were subsequently dried using a critical point drier and mounted on a rectangular

card as described by Noyes (1982). Preparations of male genitalia were also done according to the description in Noyes (1982). Photos of these slides were made with a Nikon phase contrast microscope, using 20x magnification, and the drawings based on these slides were made with Adobe Illustrator© and finally prepared in Adobe Photoshop©. The making of photos of wing interference patterns is described in detail by Shevtsova & Hansson (2011). The habitus pictures were made from a Nikon stereomicroscope and a Nikon camera. Photos were taken at different focus levels, and Helicon Focus Pro version 4.75 software was used to merge them into a single image. To eliminate wing colour reflections and reflections from the metallic and shiny body in the habitus pictures (Figs 1, 72-94) the light source used was a dome-light, manufactured as described on http:/www.cdfa.ca.goc/phpps/ppd/entomology/dome.html.

Phylogenetic analysis – To hypothesize the relationships and evolution of species we performed a phylogenetic analysis using the morphological characters described in Appendix 1 and coded in a matrix (Appendix 2). The analysis comprises 24 taxa, including one outgroup, and 35 characters (unweighted), two continuous and 33 alphanumerical. As outgroup the genus *Tropicharis* Hansson was chosen. In a recent phylogenetic analysis of the relationships in the Entedoninae (Burks et al. 2011) *Tropicharis* came out as the most basal taxon in the clade containing *Omphale*. The program used for the phylogenetic analysis was TNT (Tree analysis using New Technology), version 1.1, a software freely available and described by Goloboff et al. (2008). The "traditional search" was used for the phylogeny analysis, equivalent to heuristic search of other phylogeny programs.

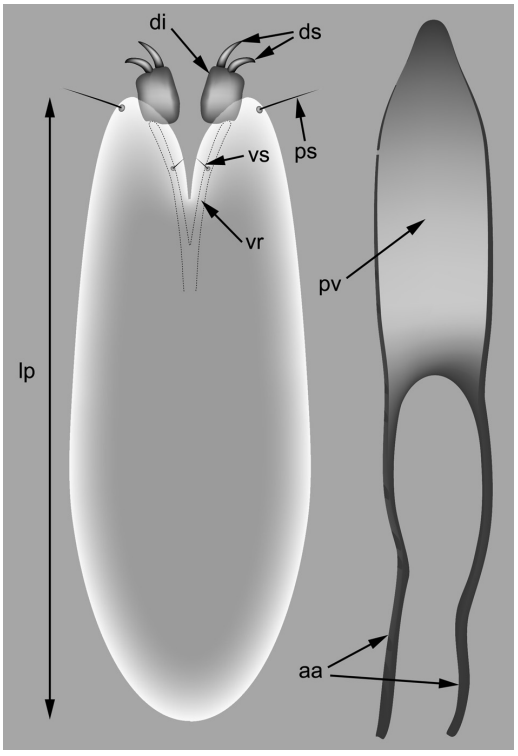
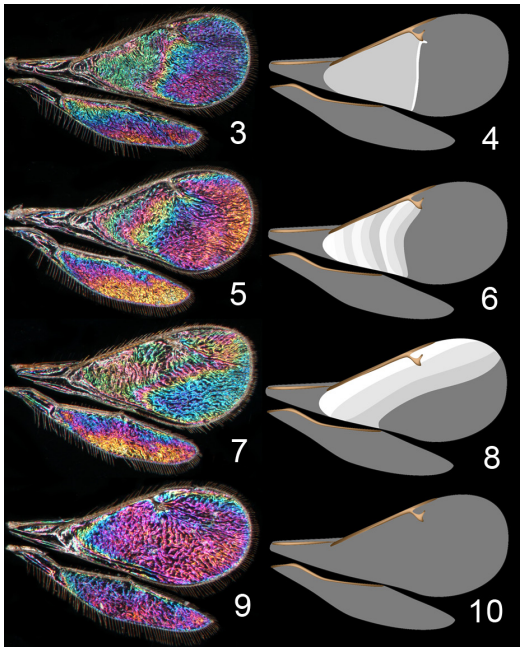


Figure 2. Male genitalia of *Entedon fufius* (Walker) (Hymenoptera: Eulophidae: Entedoninae), phallobase to the left, aedeagus to the right. Abbreviations: aa = aedeagal apodemes, di = digitus, ds = digital spines, lp = length of phallobase, ps = parameral setae, pv = penis valve, vr = volsellar ridge, vs = volsellar setae.

WIP analysis – Fore wing patterns were classified into four groups: the "**half-split**" WIP with a narrow borderline connecting the stigmal vein with the hind margin of the wing. This line divides the wing into two parts, a basal part (inside the narrow borderline) and an apical part (outside the narrow borderline) (Figs 3-4); the "**soft-gradient**" WIP with a similar division into two parts, but without a narrow borderline separating them. Instead the basal part has different colours and the apical part has only one (Figs 5-6); the "**cross-diagonal**" WIP with several differently coloured bands crossing the wing diagonally and reaching beyond the level of stigmal vein (Figs 7-8); the "**one-colour**" WIP with basically the same colour over the entire wing surface (Figs 9-10).



Figures 3–10. Categories of wing interference patterns (WIPs). **3–4**. Half-split WIP (*O. connectens*). **5–6**. Soft-gradient WIP (*O. sulciscuta*). **7–8**. Cross-diagonal WIP (*O. admirabilis*). **9–10**. One-colour WIP (*O. theana*).

Genus *Omphale*

Species of *Omphale* are very small, typically 1-2 mm long, parasitic wasps (Figs 1, 72-94). The genus is cosmopolitan and the species frequently have a wide distribution, several of the European species are found throughout Europe (Bouček & Askew 1968), and some of the species even have a Holarctic distribution (Hansson 1996b). It is one of the largest genera of the Eulophidae with 259 described species (Noyes 2011). Recent treatments of the genus are in Bouček (1988) and Hansson (1996b, 1997, 2004), the former on Australian species and the latter on species from the Americas. Currently there is no comprehensive study on the European species. This paper is a preamble to a forthcoming and comprehensive revision for this region.

Species of *Omphale* are, as far as is known, primary solitary koinobiont endoparasitoids on gall-midges (Diptera: Cecidomyiidae) (Dziurzynski 1961, Bouček & Askew 1968). There are host records for 13 of the European species (Table 1), but very little is known about reproductive strategies and nothing about mating behaviour.

Male genitalia in Omphale – Male genitalia in the Chalcidoidea are simple, consisting of two parts, the phallobase and the aedeagus (Snodgrass 1941) (Fig. 2). The phallobase is the most complicated structure, forming a semiopen tube made up from three parts, the basal ring and a pair each of parameral and volsellar plates. In Chalcidoidea the basal ring is not visible, and the parameral and volsellar plates are completely fused. The parameres are continuations of the parameral plates. The parameres have some setae at the apex, thus indicating their presence. The volsellar plates are strengthened by a ridge, the volsellar ridge, and at the apex of this ridge there is a lobe – the digitus (digitus volsellaris) – with (digital) spines at the apex. Inside the tube which is the phallobase lies the aedeagus, consisting of a larger apical part – the penis valves, and with two long "legs" – the aedeagal apodemes.

Most genera belonging to the eulophid subfamily into which *Omphale* is classified, the Entedoninae, have a phallobase and an aedeagus as in Fig. 2, i.e. a phallobase with weak volsellar setae, one parameral seta, and two digital spines, and an aedeagus with very few structures. There is little variation in this "ground-plan" within the subfamily, and species of same genus and even species of different genera are more or less indistinguishable in this structure. One rare exception from this non-variable situation is genus *Omphale* (Hansson 1996b, 1997, 2004) where both the phallobase and the aedeagus show interspecific variation (Figs 49-71). Furthermore, the phallobase in *Omphale* differs from all other genera in the subfamily in having enlarged volsellar setae, and this is the only known morphological autapomorphy for the genus.

O. aethiops	*Dasineura epilobii* (Diptera: Cecidomyiidae) on *Chamaenerion angustifolium*, collected investigating flowers of *Silene dioica* with cecidomyiid larvae; *Dasineura traili*, a gall midge associated with *Ranunculus*.
O. brevis	*Cystiphora taraxaci* (Diptera: Cecidomyiidae), *Cystiphora sonchi* on *Sonchus palustris*; *Cystiphora sanguinea* on *Hieracium sabaudum*.
O. chryseis	*Contarinia medicaginis* (Diptera: Cecidomyiidae).
O. clymene	*Dasineura pyri* (Diptera: Cecidomyiidae).
O. clypealis	*Dasineura brassicae* (Diptera: Cecidomyiidae).
O. erginnus	Associated with bracket fungi, possibly on a Cecidomyiidae (Diptera).
O. isander	From *Mycodiplosis* sp. (Diptera: Cecidomyiidae) feeding on leaf rust on *Populus*.
O. lugens	*Mikiola fagi*, *Contarinia tiliarum* & *Dasyneura alni*, *Placochela nigripes*, all hosts are Diptera: Cecidomyiidae.
O. lugubris	Associated with *Picea*, but not reared from any host.
O. obscura	*Dasineura viciae* (Diptera: Cecidomyiidae); unidentified budgall on *Galium mollugo*.
O. salicis	*Contarinia lentis* (Diptera: Cecidomyiidae) (probable record), *Contarinia loti; Contarinia vincetoxici*.
O. sp.4	*Geocrypta galii* (Diptera: Cecidomyiidae) on *Galium* spp.
O. sp.5	*Bayeria capitigena* (Diptera: Cecidomyiidae) on *Euphorbia esula*.

Table 1. Host records for European *Omphale* species.

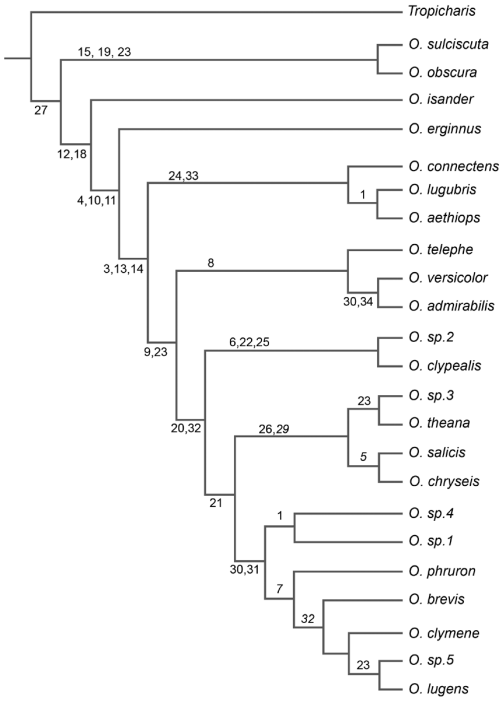
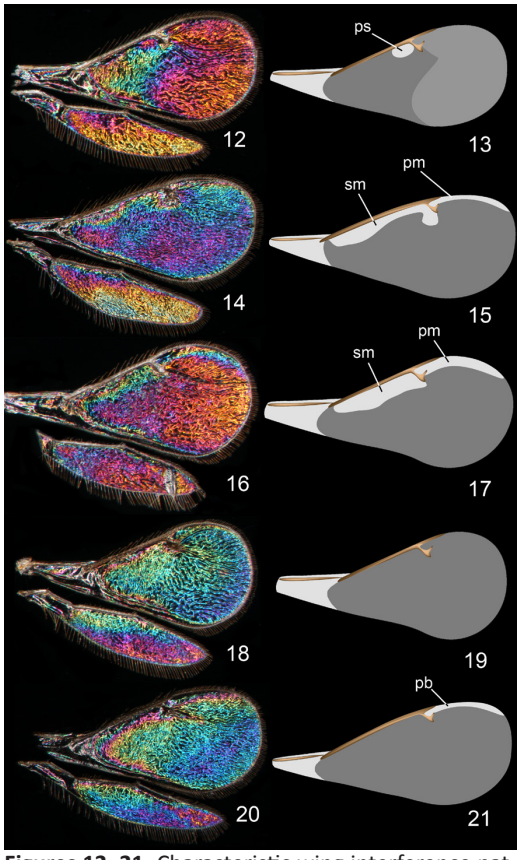


Figure 11. The single most parsimonious tree, with 104 steps, resulting from a "traditional search" using the software TNT. The derived characters, listed in Appendix 1, are shown on each branch with reversals in italics. Apomorphies for the species are not-shown.

Results

Wing interference patterns

There are no sexually dimorphic WIPs in *Omphale*, the patterns are identical in both sexes, and all WIPs illustrated here are from females (Figs 25-47, and included in the cladogram on Fig. 133). Species of *Omphale* do not display elaborate patterns as in some species of *Achrysocharoides* which may have distinct eye-catching spots in the forewing WIPs (Shevtsova & Hansson 2011). The WIPs in *Omphale* are less elaborate, but still with perceptible pattern variations. The differences between some of the four categories are sometimes small and the difference between the half-split and soft-gradient WIPs can sometimes be difficult to ascertain. The outgroup to *Omphale*, genus *Tropicharis* Hansson, has



Figures 12–21. Characteristic wing interference patterns (WIPs) in *Omphale* spp. **12–13**. *O. clymene*. **14–15**. *O. telephe*. **16–17**. *O. sp.3*. **18–19**. *O. lugens*. **20–21**. *O. sp.5*. Abbreviations: pm = poststigmal band, ps = prestigmal spot, sm = submarginal band.

a half-split WIP (Fig. 48), as have many other Entedoninae genera (see http://www.neotropicaleulophidae.com for a survey of WIPs in the subfamily Entedoninae), and we hypothesize that this is the plesiomorphic state in *Omphale*. The cross-diagonal WIP has evolved once, in the *admirabilis*-group, but the other two derived WIPs, the soft-gradient and one-colour WIPs, have evolved independently on several occasions, soft-gradient five times and one-colour WIP four times (Fig. 133). Clearly such homoplasious characters are of limited value in phylogenetic analyses. However, WIPs can also be used for species recognition within the species groups, which are defined by other morphological characters. The ***sulciscuta*-group** comprises two species, both with a soft-gradient WIP but the pattern differs between the species. In *O. obscura* the colour gradient in the basal part is fading towards the level of stigmal vein and does not reach beyond it, leaving a large apical unicoloured part (Fig. 26). In *O. sulciscuta* several wide colour bands in the basal part reach beyond the level of stigmal vein and thus leave a comparatively smaller apical unicoloured part (Fig. 25). The ***aetius*-group** includes three species, all with half-split WIPs that are very similar (Figs 29-31), and thus not useful for species identification. In the ***admirabilis*-group** WIPs are distinct in all three species. *O. admirabilis* is the only species with a cross-diagonal WIP (Fig. 34), *O. versicolor* has a soft-gradient WIP (Fig. 33). The third species, *O. telephe,* has a featured one-colour WIP with an easily recognizable differently coloured narrow area just below the marginal vein (Figs 14, 15, 32). Both species in the ***clypealis*-group** have a soft-gradient WIP, but the pattern is much more distinct in *O. clypealis* (Fig. 35) compared to the other species, *O. sp.2* (Fig. 36). In the ***salicis*-group** *O. theana* and *O. sp.3* both have one-colour WIP, but they can be separated quite easily from each other – *O. sp.3* has a submarginal band (Figs 16, 17, 38), while *O. theana* has the same colour over the entire wing surface (Fig. 37). The remaining two species in this group, *O. chryseis* and *O. salicis,* have a soft-gradient and half-split WIP, respectively (Figs 40, 39). The ***phruron*-group** is the largest with seven species of which two, *O. sp.5* and *O. lugens*, have a one-colour WIP, but can be separated through the presence (O. *sp.5*, Figs 20, 21, 46) or the absence (*O. lugens*, Figs 18, 19, 47) of a poststigmal band. They can also be separated through the shape of their forewings (Figs 46, 47). The remaining five species have a soft-gradient WIP (Figs 41-45). Of these *O. clymene* has the most featured WIP. It lacks a poststigmal band,

similar to *O. lugens*, and it is the only species with a small but distinct prestigmal spot (Figs 12, 13, 45). The remaining four species have similar WIPs and cannot be separated confidently enough through these patterns.

Male genitalia

The male genitalia in European *Omphale* are equally useful for the classification as they are for *Omphale* in the Americas, showing variation in both the phallobase and the aedeagus (Figs 49-71, and included in the cladogram on Figs 134-135). They frequently have both species specific and group specific features, as well as having the only morphological autapomorphy for genus *Omphale* – the greatly enlarged volsellar setae. Male genitalia are thus an extremely useful structure for the classification and species discrimination in this group which is so deprived in other morphological characters.

We report some new features in the male genitalia in *Omphale*. One such feature, which to our knowledge has not been described in any other Hymenoptera group, is the merged apices of the aedeagal apodemes in *O. admirabilis* and *O. versicolor* (Figs 22, 23). This feature is also present in the Nearctic species *O. oculiparva* Hansson and *O. purpurea* Hansson, that also belong to the *admirabilis* group.

In some groups singular species have very deviating genitalia compared to the other species in the same group. In *sulciscuta* group there are two species and the nominal species has a quite "ordinary" phallobase (Fig. 49) for an *Omphale* species, i.e. with strong volsellar setae attached distinctly below apex of volsellar plates and digitus with strong spines situated close to one another. The other species, *O. obscura*, differs very much from this (Fig. 50), with weak volsellar setae attached at the apex of the phallobase, and a digitus

Figures 22–23. Aedeagus of male genitalia in *Omphale* spp. **22.** *O. admirabilis*. **23.** *O. versicolor*. Arrows point at fused apices of aedeagal apodemes.

with very differently sized spines situated wide apart. The other example is *O. lugens* in the *phruron* group. In this species both the phallobase and the aedeagus (Fig. 71) are very different from all other *Omphale* species, and a classification based only on male genitalia would place *O. lugens* in a group of its own.

Discussion

One hypothesis concerning the evolution of the variations of WIPs, and the distinct patterns in e.g. some species of *Achrysocharoides*, suggested by Shevtsova et al. (2011) was that they are used for intraspecific signaling and thus serve as reproductive barriers. Such signals only apply to situations when two or more species co-exist. Hansson et al.

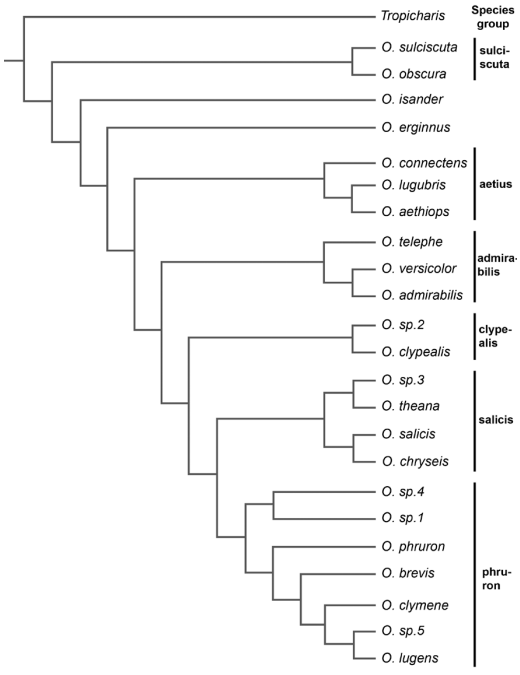


Figure 24. Phylogenetic tree with species-groups shown.

(manuscript submitted) showed that this was a probable scenario for sympatric species of *Achrysocharoides*, where co-existing species had evolved WIPs differing from other species occurring on the same host plant. Our knowledge of the biology for *Omphale* is poor, e.g. with host records for only 13 of the 23 species. However, the host records do point in a certain direction because there is no overlap of hosts between the *Omphale* species. Thus each species appears to be allopatric, and probably only encounters closely related species by accident or not at all. We do not know anything about mating behaviour in this group, e.g. where mating takes place, but if it occurs close to, or on, their respective hosts host plants this might be one explanation for the lack of elaborate WIPs in *Omphale*.

Then why are there interspecific variations in the WIPs in *Omphale*? If, as we suggest, species in this group are allopatric and wing patterns do not play an important role in species recognition, then natural selection may have optimized the wing thickness for functional purposes, e.g. for flight. The half-split WIP may thus be a result of physical constraints. The narrow vertical line that delimits the basal part from the apical part (Figs 3-4) emanates from where the veins along the fore margin end. Such a wing is distinctly divided into two parts, an inner part with a thick membrane stabilized by the marginal vein along the fore margin, and an outer thin part. Even though the outer part is thin, structural modifications such as corrugations (Shevtsova et al. 2011), act to stabilize the membrane. Having the apical part of the wing membrane thin and thus light is cost-efficient energy-wise when moving the wing. The apical part is farthest away from the "engine", i.e. the muscle package in the pterothorax, and if heavy then it is more arduous and more costly to move. The soft-gradient and cross-diagonal WIPs (Figs 5-8) both have a smooth transition between the thick basal and thin apical parts of the wing, but the discussion for the half-split WIP is applicable also to these patterns. Wings with a one-coloured WIP (Figs 9-10) have a membrane that is thin throughout, i.e. a very light wing, and might represent an alternative solution for saving energy when the wing is in motion. The small, but species specific, differences in WIPs present in *Omphale* species might be the result of genetic isolation and subsequent adaptations to different environmental parameters.

Since WIPs in *Omphale* lack the elaborate and distinct patterns present in some species of *Achrysocharoides*, where these patterns probably are used for signaling, then perhaps they are not important for species recognition in this group. Why then are male genitalia distinct for each species, and why is the interspecific variation in this structure so pronounced within this group? There are two hypotheses for rapid divergent evolution of this structure, the lock-and-key and sexual

selection hypotheses. If our suggestion that *Omphale* species are separated in space is correct, then the lock-and-key hypothesis, similar to the discussion on WIPs, is not valid because females will only encounter conspecific males. The sexual selection hypothesis is a more plausible explanation for the strong divergence of appearances of male genitalia in *Omphale*. The other, more conservative, morphological characters present in these species indicate the deeper phylogeny of the species. To be reasonably sure if the sexual selection hypothesis is applicable to *Omphale* we need to know if the species are polyandrous, where the mating takes place, etc. However, nothing is known about mating behaviour in *Omphale* so this is for now an unproven, but plausible explanation to this variation.

The high morphological diversity of this structure in *Omphale* species is in contrast with most other Entedoninae genera, including *Achrysocharoides,* that do not show any variation in male genitalia between species, or even between genera. Perhaps these groups have other mating strategies that do not involve sexual selection, or if sexual selection is in play then male genitalia are not the feature selected for. Instead other attributes, e.g. WIPs, are the target for this selection.

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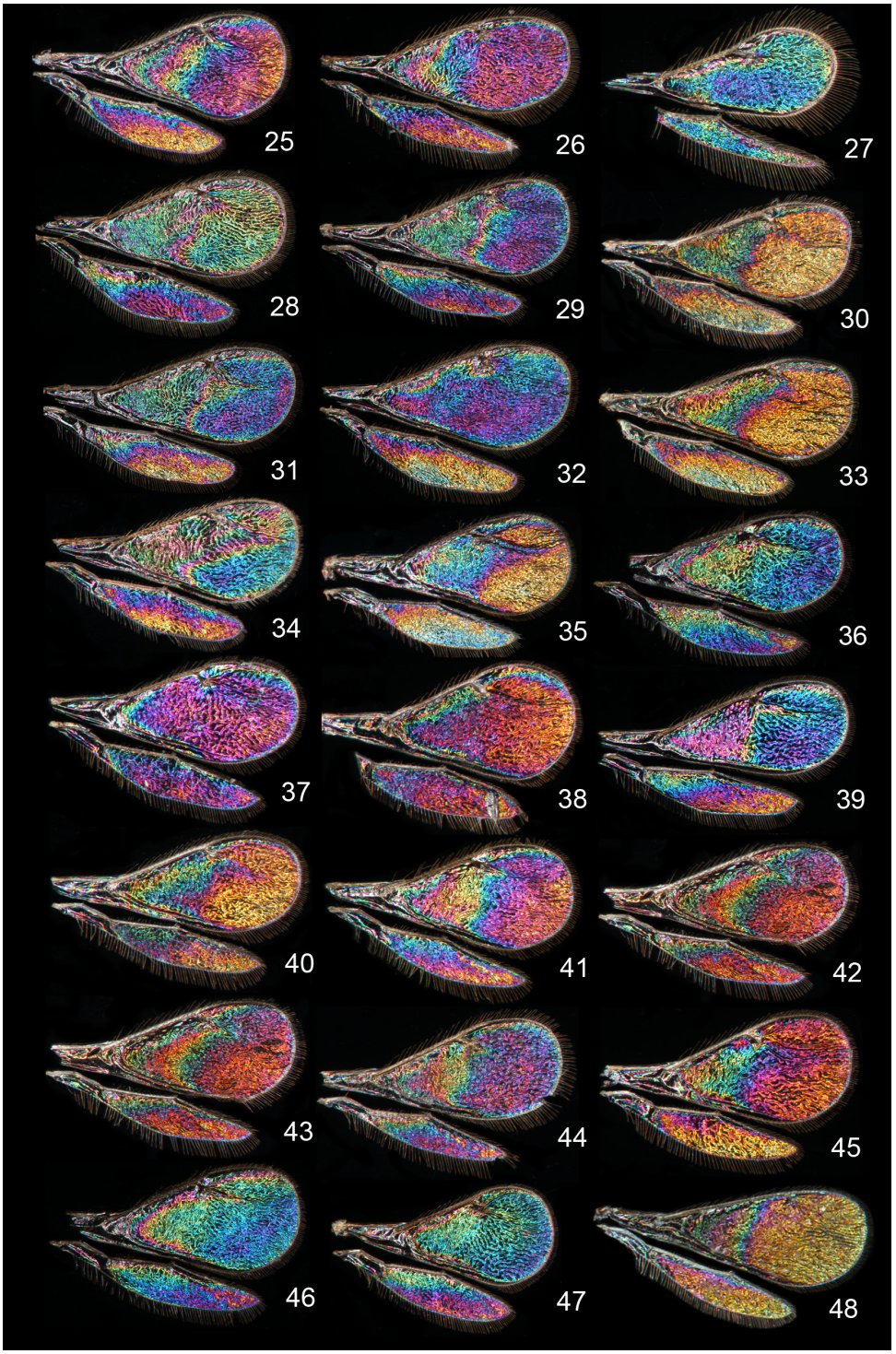
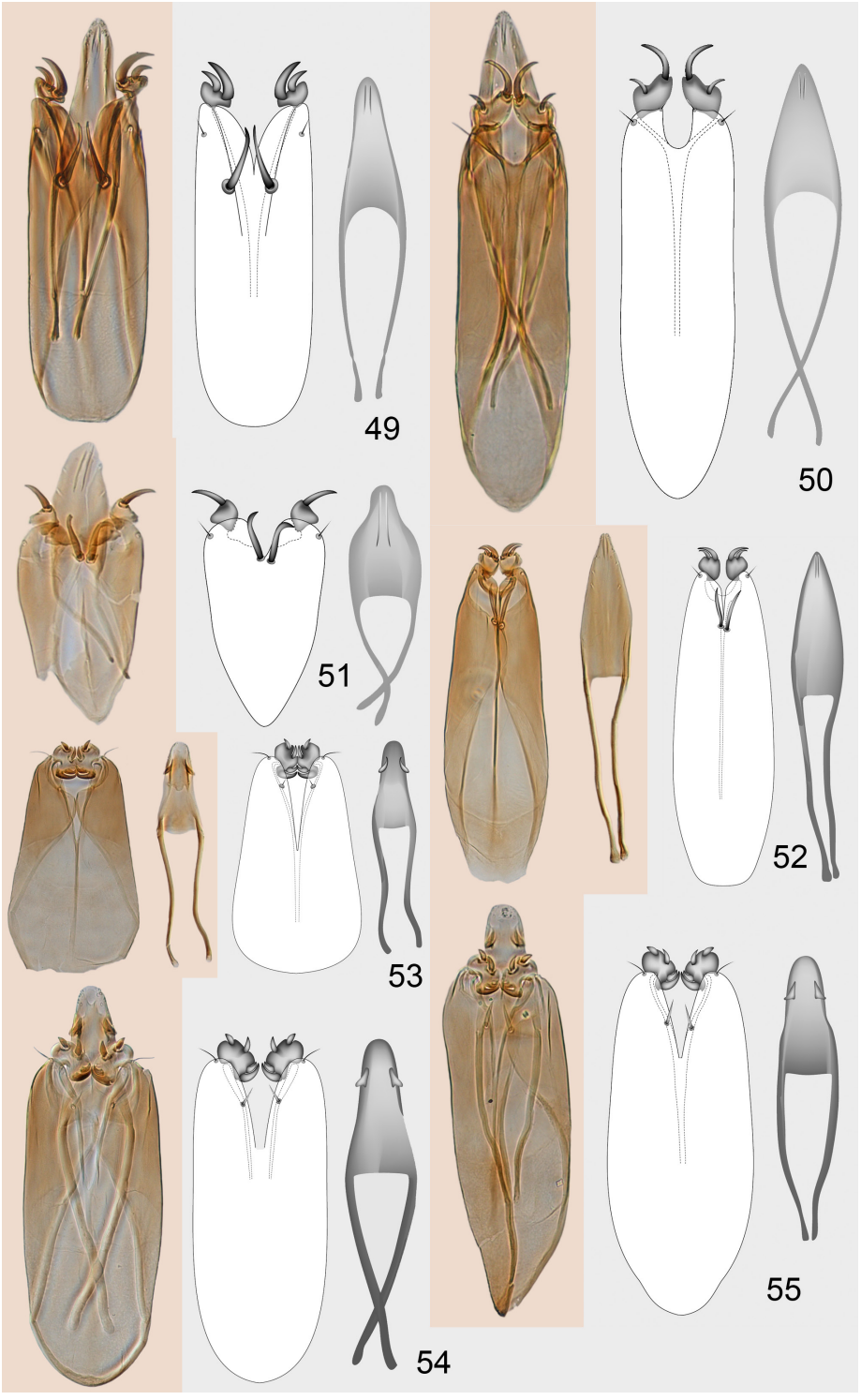
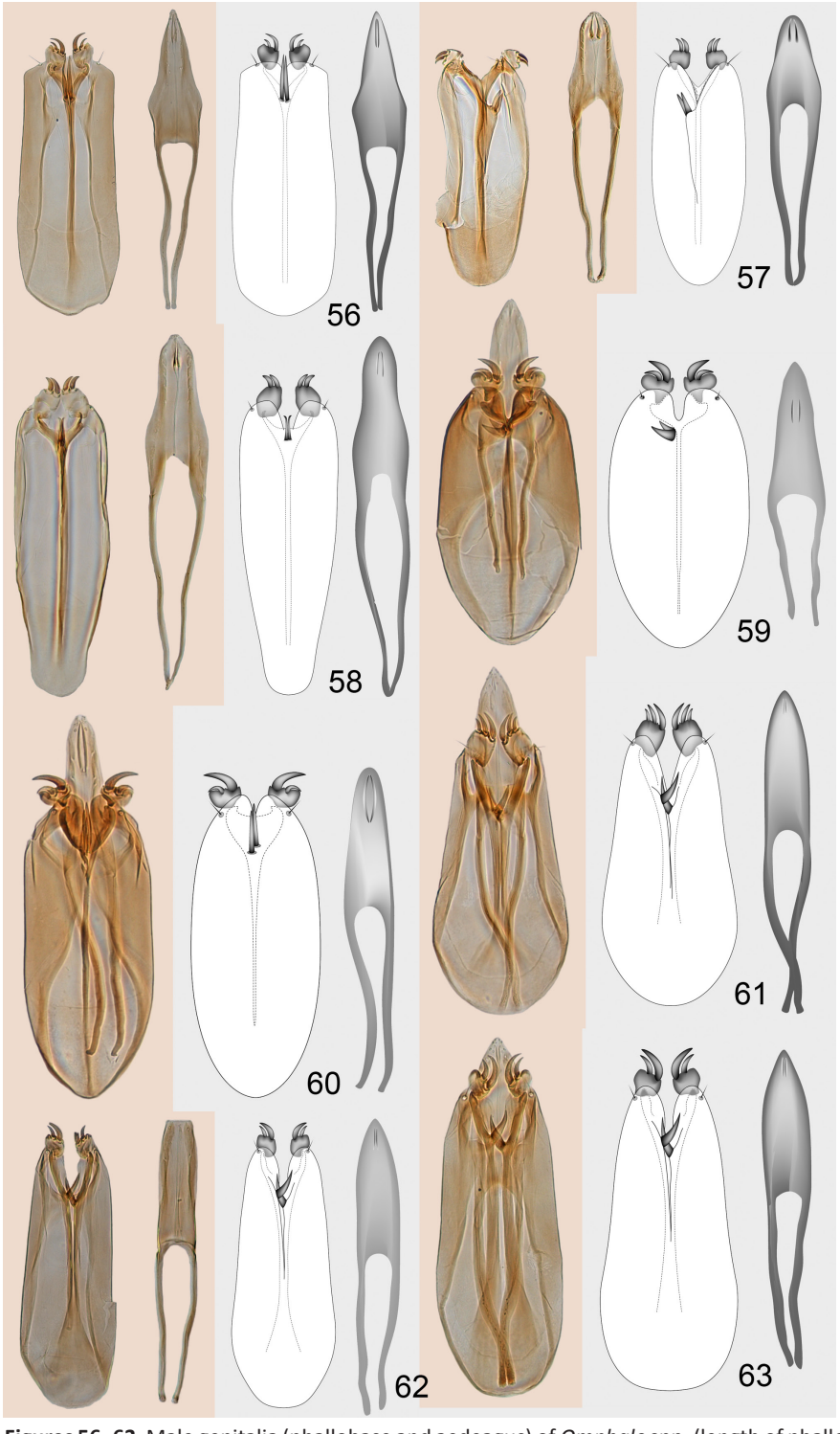


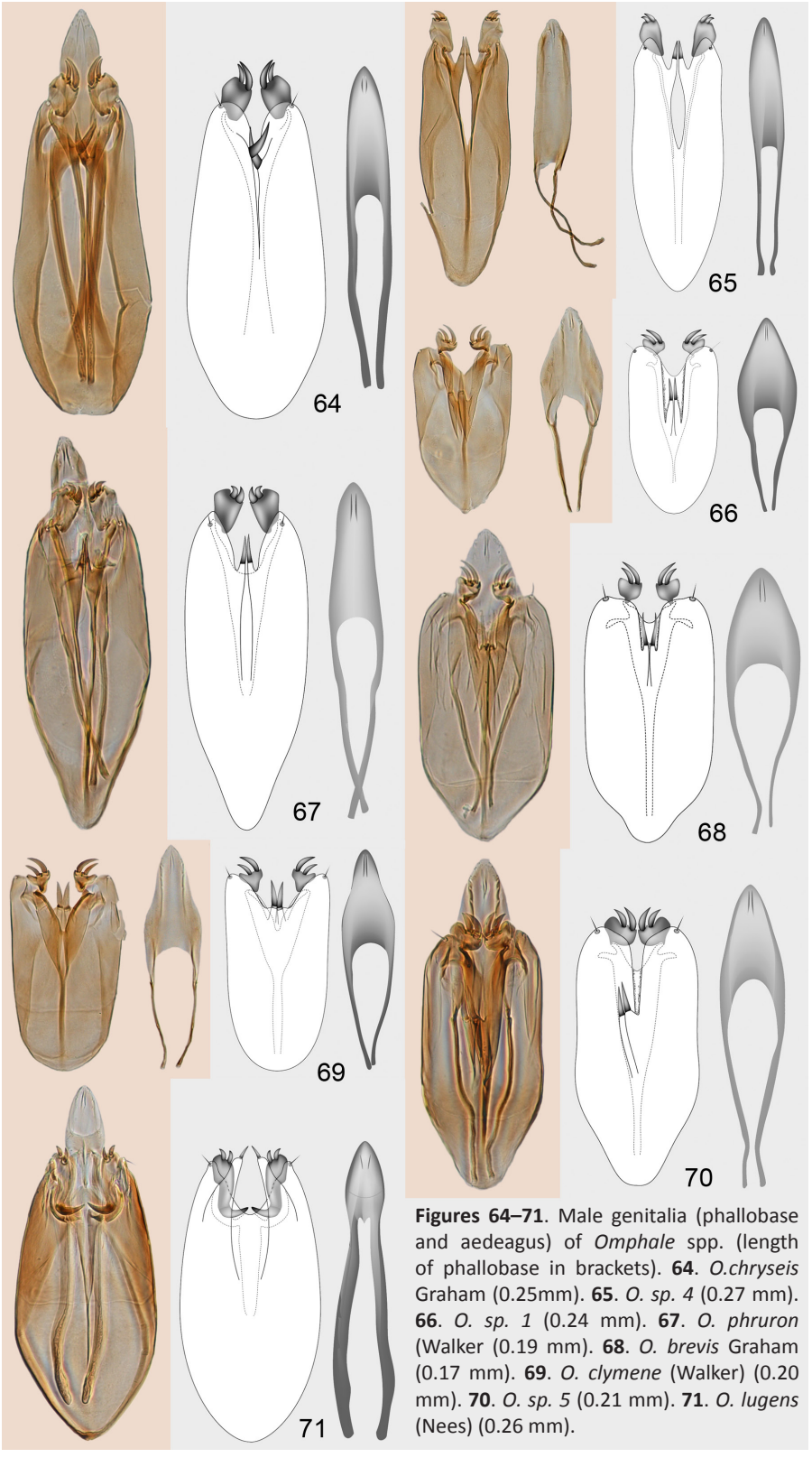
Figures 25–48. Wing interference patterns (WIPs) for *Omphale* spp., females. **25**. *O. sulciscuta* (Thomson). **26**. *O. obscura* (Förster). **27**. *O. isander* (Walker). **28**. *O. erginnus* (Walker). **29**. *O. connectens* Graham. **30**. *O. lugubris* Askew. **31**. *O. aethiops* Graham. **32**. *O. telephe* (Walker). **33**. *O. versicolor* (Nees). **34**. *O. admirabilis* (Westwood). **35**. *O. clypealis* (Thomson). **36**. *O. sp. 2*. **37**. *O. theana* (Walker). **38**. *O. sp. 3*. **39**. *O. salicis* Haliday. **40**. *O. chryseis* Graham. **41**. *O. sp. 4*. **42**. *O. sp. 1*. **43**. *O. phruron* (Walker). **44**. *O. brevis* Graham. **45**. *O. clymene* (Walker). **46**. *O. sp. 5*. **47**. *O. lugens* (Nees). **48**. *Tropicharis cecivora* Hansson (outgroup).



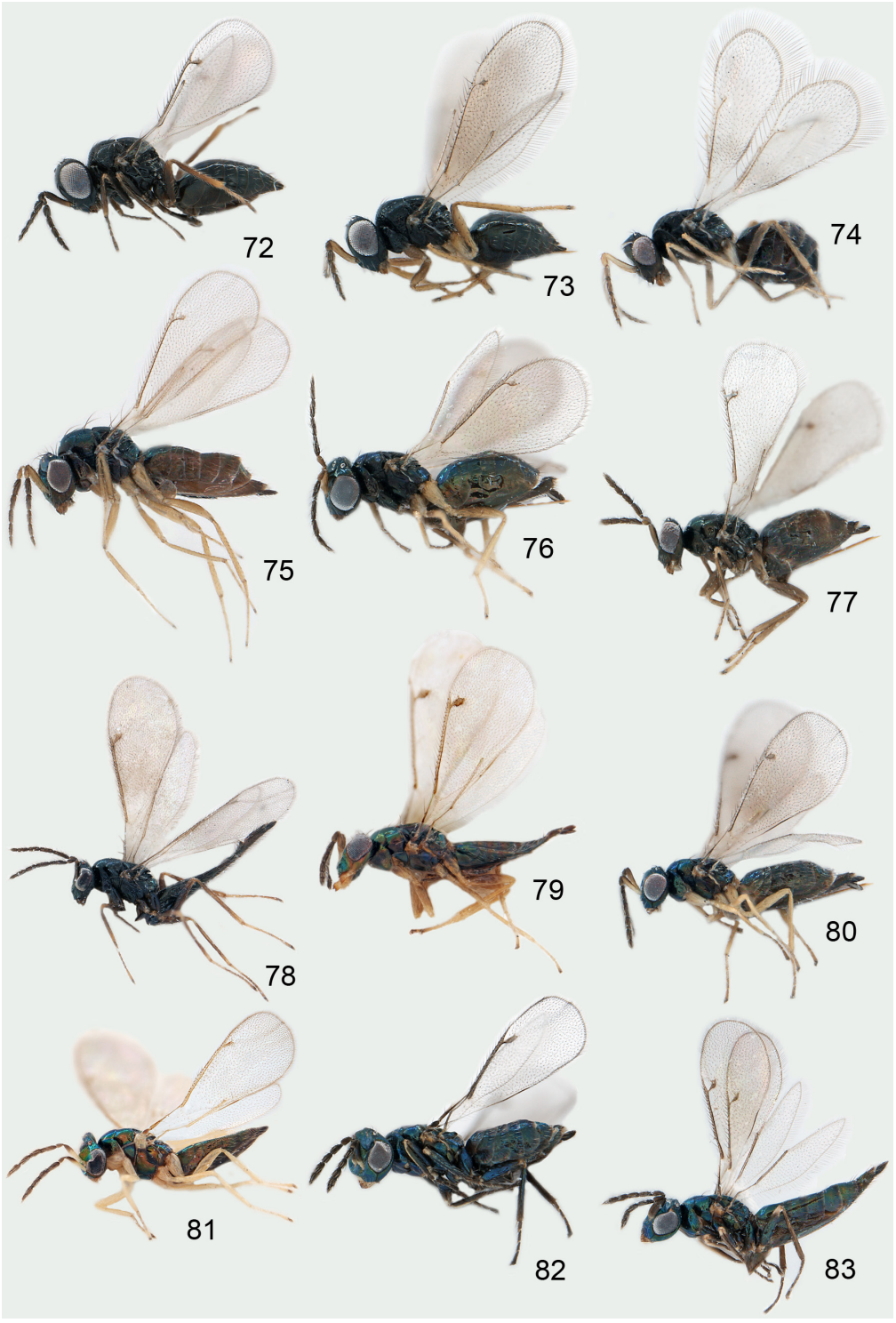
Figures 49–55. Male genitalia (phallobase and aedeagus) of *Omphale* spp. (length of phallobase in brackets). **49**. *O. sulciscuta* (Thomson) (0.24 mm). **50**. *O. obscura* (Förster) (0.22 mm). **51**. *O. isander* (Walker) (0.13 mm). **52**. *O. erginnus* (Walker) (0.22 mm). **53**. *O. connectens* Graham (0.22 mm). **54**. *O. lugubris* Askew (0.27 mm). **55**. *O. aethiops* Graham (0.30 mm).



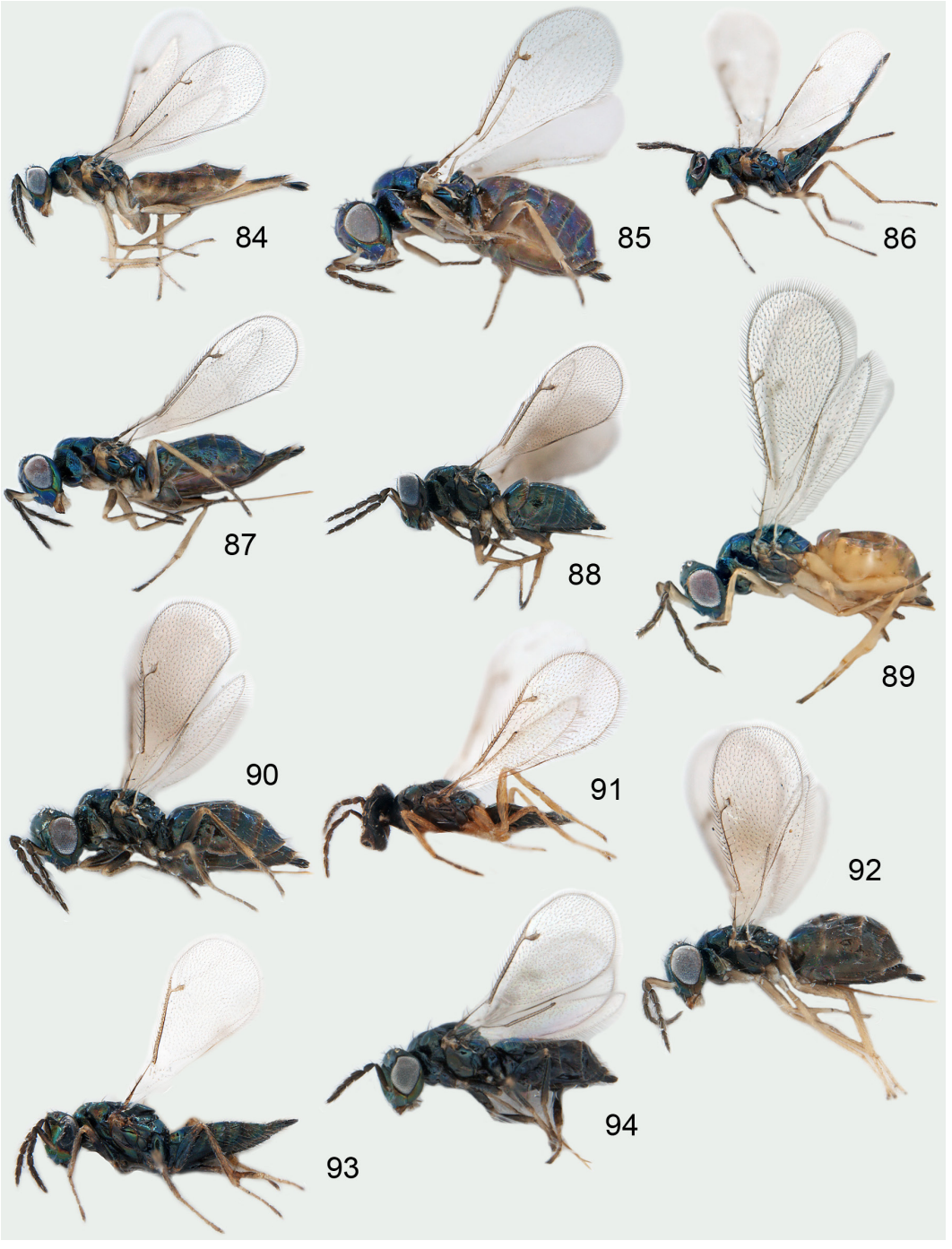
Figures 56–63. Male genitalia (phallobase and aedeagus) of *Omphale* spp. (length of phallobase in brackets). **56**. *O. telephe* (Walker) (0.32 mm). **57**. *O. versicolor* (Nees) (0.31 mm). **58**. *O. admirabilis* (Westwood) (0.30 mm). **59**. *O. clypealis* (Thomson) (0.21 mm). **60**. *O. sp. 2* (0.22 mm). **61**. *O. sp. 3* (0.24 mm). **62**. *O. theana* (Walker) (0.26 mm). **63**. *O. salicis* Haliday (0.27 mm).



Figures 64–71. Male genitalia (phallobase and aedeagus) of *Omphale* spp. (length of phallobase in brackets). **64**. *O.chryseis* Graham (0.25mm). **65**. *O. sp. 4* (0.27 mm). **66**. *O. sp. 1* (0.24 mm). **67**. *O. phruron* (Walker (0.19 mm). **68**. *O. brevis* Graham (0.17 mm). **69**. *O. clymene* (Walker) (0.20 mm). **70**. *O. sp. 5* (0.21 mm). **71**. *O. lugens* (Nees) (0.26 mm).



Figures 72–83. Habitus of *Omphale* spp., females (body length in brackets). **72**. *O. sulciscuta* (Thomson) (1.6 mm). **73**. *O. obscura* (Förster) (1.2 mm). **74**. *O. isander* (Walker) (1.0 mm). **75**. *O. erginnus* (Walker) (1.9 mm). **76**. *O. connectens* Graham (1.8 mm). **77**. *O. lugubris* Askew (1.4 mm). **78**. *O. aethiops* Graham (2.7 mm). **79**. *O. telephe* (Walker) (1.8 mm). **80**. *O. versicolor* (Nees) (1.7 mm). **81**. *O. admirabilis* (Westwood) (1.7mm). **82**. *O. clypealis* (Thomson) (1.5 mm). **83**. *O. sp. 2* (2.0 mm).



Figures 84–93. Habitus of *Omphale* spp., females (body length in brackets). **84**. *O. theana* (Walker) (2.2 mm). **85**. *O. sp. 3* (1.5 mm). **86**. *O. salicis* Haliday (2.3 mm). **87**. *O. chryseis* Graham (1.7 mm). **88**. *O. sp. 4* (1.6 mm). **89**. *O. sp. 1* (1.5 mm). **90**. *O. phruron* (Walker) (1.5 mm). **91**. *O. brevis* Graham (1.4 mm). **92**. *O. clymene* (Walker) (1.5 mm). **93**. *O. sp. 5* (2.0 mm). **94**. *O. lugens* (Nees) (1.4 mm).

Appendix 1. List of morphological characters used in the phylogenetic analysis

Continuous characters

1) Length of penis valves: continuous value, length/width.
2) Length of digitus: continuous value, length/width.

Antenna

3) Shape of sensilla ampullacea on flagellomeres: elongate asymmetric (Fig. 95) (0); short asymmetric (Fig. 96) (1).
4) Attachment area vs free apical part of multiporous plate sensilla (MPS) on female flagellomeres: with short attachment area and with major part free, i.e. setae-like (Fig. 97) (0); with long attachment area and short free apical part (Fig. 98) (1).
5) Setation on ventral part of female flagellomeres 2-4: scattered setae (Fig. 101) (0); two sets of setae, one set attached basally and one attached medially to apically (Fig. 99) (1); one set attached basally and reaching beyond apex of flagellomere attached to (Fig. 100) (2).
6) Number of flagellomeres in antennal clava in female: 1-2 (Fig. 102) (0) (frequently difficult to tell if the apical two flagellomeres are separated or not); 3 (Fig. 103) (1).
7) Setation on male flagellomeres 1-4: with scattered setae (Fig. 104) (0); with a basal whorl and with setae apical to whorl (1); with a single basal whorl (Fig. 105) (2).
8) Shape of male scape: narrow throughout (Fig. 112) (0); widest medially, but wide also at base (Fig.113) (1); widest apically, narrow at base (Fig. 114) (2).

Head

9) Sculpture on face (i.e. part between lower margin of eye and mouth margin): smooth (Fig. 115) (0); reticulate-strigose (Fig. 116) (1).
10) Shape of clypeus: triangular (Fig. 117) (0); rounded (Fig. 118) (1); quadrangular (Fig. 120) (2).
11) Sculpture on frons: smooth (Fig. 121) (0); at least partly reticulate (Fig. 116) (1).
12) Antennal scrobes join: below frontal suture (Fig. 121) (0); frontal suture separately (Fig. 116) (1).
13) Frontal cross carina: absent (Fig. 121) (0); present (Fig. 122) (1).
14) Occipital margin: carinate (Fig. 123) (0); rounded (Fig. 124) (1).

Mesosoma

15) Midlobe of mesoscutum: without median groove (Fig. 125) (0); with median groove (Fig. 127) (1).
16) Scutellum: without median groove (Fig. 126) (0); with median groove (Fig. 129) (1).
17) Posterior ½ of notauli: indistinct (Fig. 125) (0); as distinct grooves (Fig. 128) (1).
18) Propodeum: with median carina (Fig. 130) (0); without median carina (Fig. 132) (1).
19) Propodeum: without lateral carinae (Fig. 132) (0); with lateral carinae (Fig. 131) (1).

Forewing

20) Radial cell: hairy (Fig. 106) (0); bare (Fig. 107) (1).
21) Attachment of admarginal setae: predominantly or entirely on membrane (Fig. 111) (0); predominantly or entirely on marginal vein (Fig. 110) (1).
22) Forewing speculum: closed (Fig. 108) (0); open (Fig. 109) (1).
23) Wing interference patterns: half-split (Figs 3-4) (0); soft-gradient (Figs 5-6) (1); cross-diagonal (Figs 7-8) (2); one-colour (Figs 9-10) (3).

Phallobase

24) Digitus: without lateral spines (e.g. Fig. 49) (0); with lateral spines (e.g. Fig. 54) (1).
25) Size of (two) digital spines (apical spines): outer spine more than ½ as long as inner spine (e.g. Fig. 56) (0); outer spine less than ½ as long as inner spine (e.g. Fig. 57) (1).
26) Shape of volsellar setae: round (e.g. Fig. 49) (0); flattened (e.g. Fig. 61) (1).
27) Volsellar setae, strength 1: like ordinary setae (0); enlarged (1).
28) Volsellar setae, strength 2: weak, just a little stronger than ordinary setae (e.g. Fig. 53) (0); very strong (e.g. Fig. 52) (1).
29) Orientation of volsellar setae: converging and not crossed (e.g. Fig. 55) (0); converging and crossed (e.g. Fig. 61) (1); parallel to slightly divergent (e.g. Fig. 68) (2).
30) Volsellar setae, attachment on longitudinal level: different levels (e.g. Fig. 52) (0); same level (e.g. Fig. 56) (1).
31) Attachment of volsellar setae: directly from volsellar ridge (e.g. Fig. 52) (0); on a short extension from volsellar ridge (e.g. Fig. 65) (1); on a very long extension from volsellar ridge, extension about 5X as long as length of volsellar seta (Fig. 71) (2).

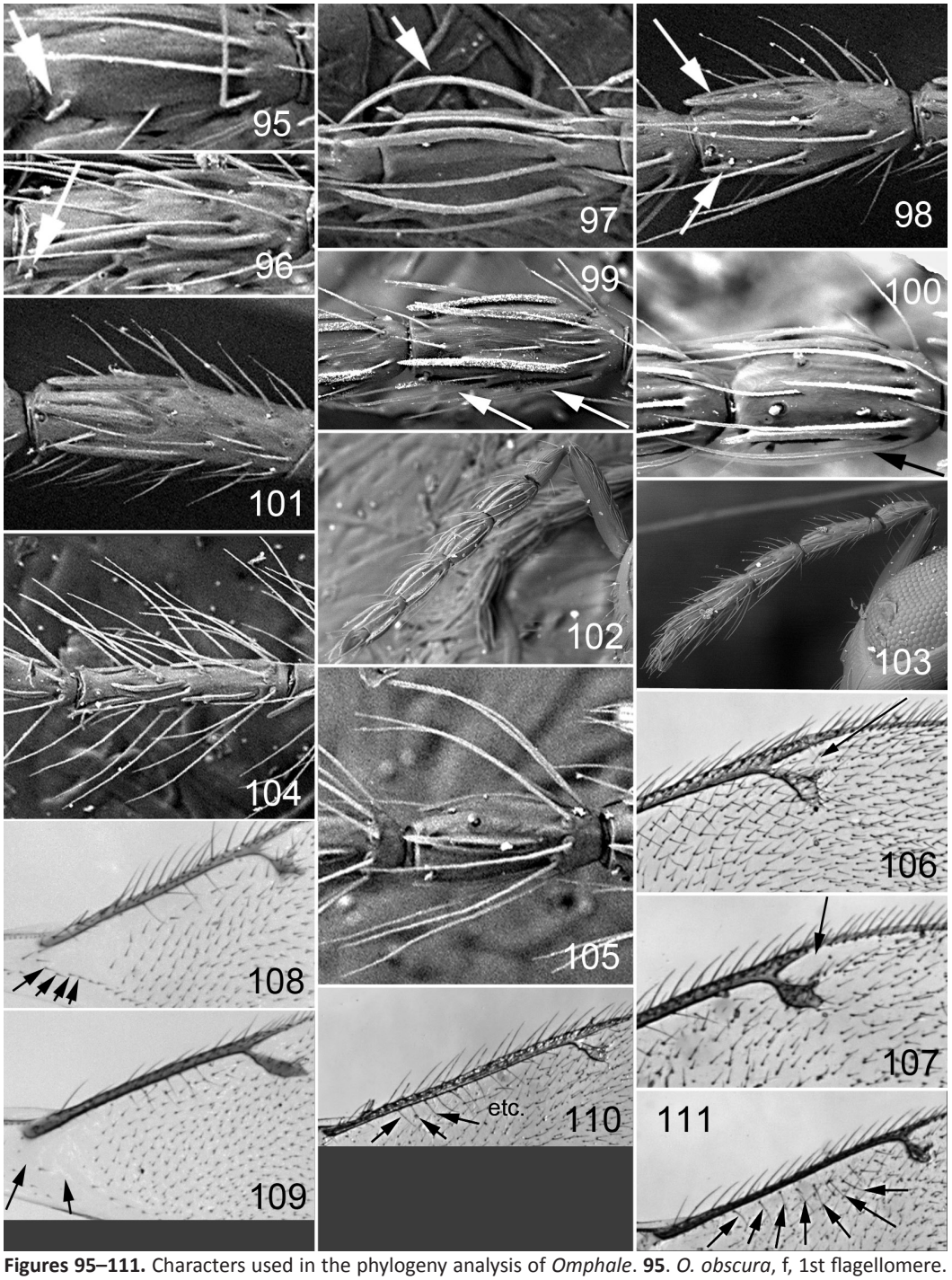
32) Attachment point of parameral seta: at or close to apex (e.g. Fig. 53) (0); distinctly below apex (e.g. Fig. 49) (1).

Aedeagus

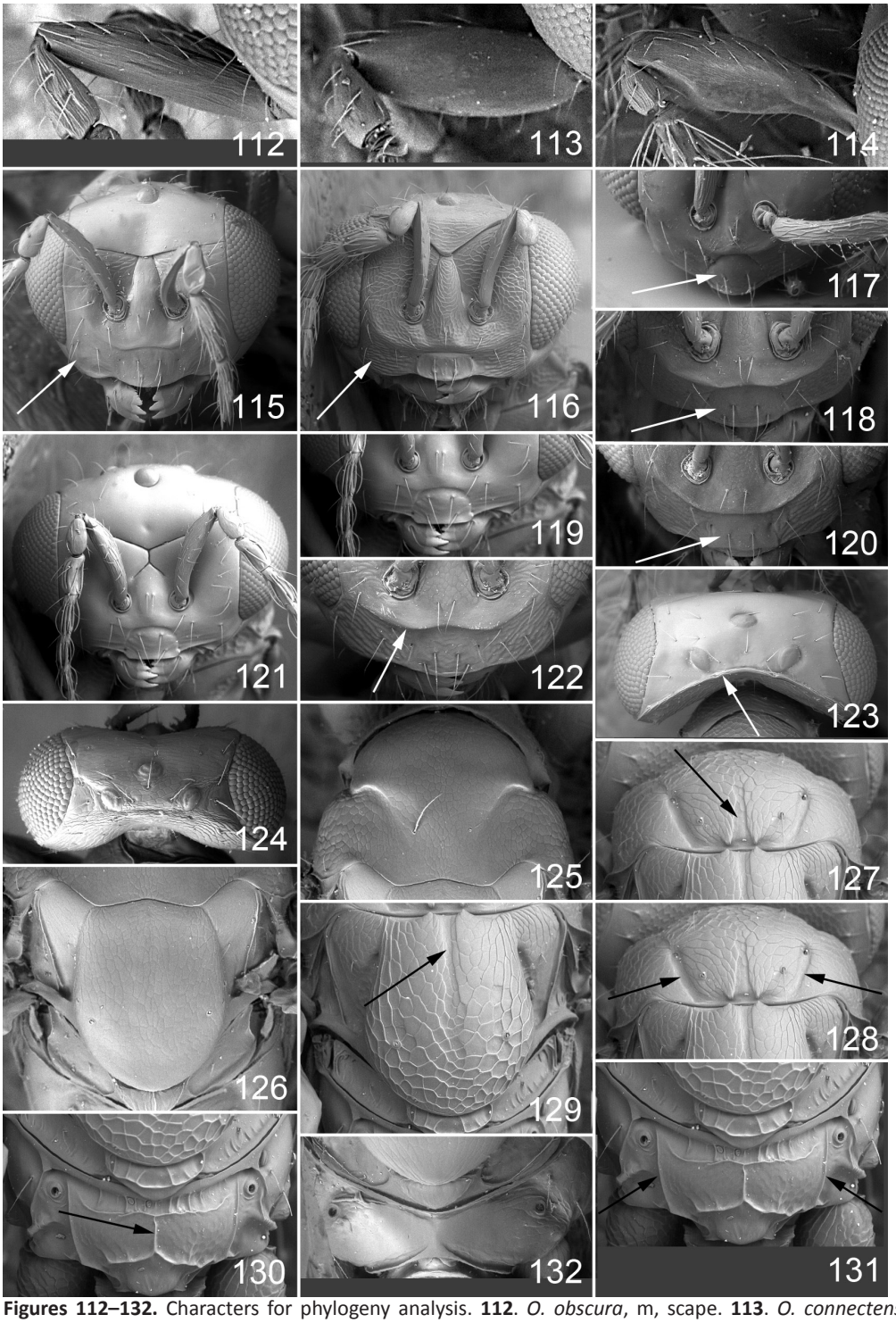
33) Apically with small lateral plates extending from surface: no (e.g. Fig. 50) (0); yes (e.g. Fig. 53) (1).

34) Apex of aedeagal apodemes: free (e.g. Fig. 60) (0); fused (Figs 57, 58) (1).

35) Membrane between upper part of aedeagal apodemes: curved, rounded (e.g. Fig. 49) (0); straight (e.g. Fig. 54) (1); drawn out to a tip (Fig. 71) (2).



Figures 95–111. Characters used in the phylogeny analysis of *Omphale*. **95**. *O. obscura*, f, 1st flagellomere. **96**. *O. admirabilis*, f, 1st flagellomere. **97**. *O. sulciscuta*, f, 2nd flagellomere. **98**. *O. sp.2*, f, 2nd flagellomere. **99**. *O. chryseis*, f, 2nd flagellomere. **100**. *O. lugens*, f, 2nd flagellomere. **101**. *O. sp.2*, 1st flagellomere. **102**. *O. clymene*, f, antenna. **103**. *O. sp.2*, f, antenna. **104**. *O. admirabilis*, m, 2nd flagellomere. **105**. *O. phruron*, m, 2nd flagellomere. **106**. *O. aethiops*, f, forewing part. **107**. *O. salicis*, f, forewing part. **108**. *O. phruron*, f, forewing part. **109**. *O. clypealis*, f, forewing part. **110**. *O. connectens*, f, forewing part. **111**. *O. sulciscuta*, f, forewing part. (f=female, m=male)



Figures 112–132. Characters for phylogeny analysis. **112**. *O. obscura*, m, scape. **113**. *O. connectens*, m, scape. **114**. *O. admirabilis*, m, scape. **115**. *O. connectens*, f, head. **116**. *O. lugens*, f, head. **117**. *O. obscura*, f, clypeus. **118**. *O. salicis*, f, clypeus. **119**. *O. sulciscuta*, f, clypeus. **120**. *O. versicolor*, f, clypeus. **121**. *O. sulciscuta*, f, head. **122**. *O. acuminata*, f, lower part of head. **123**. *O. sulciscuta*, vertex. **124**. *O. acuminata*, f, vertex. **125**. *O. chryseis*, m, mesoscutum. **126**. *O. chryseis*, m, scutellum. - **127-131**. *O. sulciscuta*, f. **127-128**. Mesoscutum. **129**. Scutellum. **130-131**. Propodeum. **132**. *O. acuminata*, f, propodeum. (f=female, m=male)

Appendix 2. Character matrix

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Tropicharis		2	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	?	?	?	?	?	?	0	0	0	0
O.admirabilis	1.1	1.9	1	1	1	0	1	2	1	2	1	1	1	1	0	0	0	1	0	[01]	[01]	0	2	0	0	0	1	1	2	1	0	1	0	1	0
O.aethiops	0.7	1.7	1	1	0	0	1	1	0	2	1	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1
O.brevis	0.9	1.3	1	1	2	0	2	1	1	1	1	1	1	1	0	0	0	1	0	1	[01]	0	1	0	0	0	1	1	2	1	1	0	0	0	0
O.chryseis	1.1	2.8	1	1	1	0	1	1	1	[12]	1	1	1	1	0	0	0	1	0	1	1	0	1	0	0	1	1	1	1	0	0	0	0	0	0
O.clymene	0.9	1.8	1	1	2	0	2	1	1	[12]	1	1	1	1	0	0	0	1	0	1	[01]	1	1	0	0	1	1	1	2	0	1	0	0	0	0
O.clypealis	0.8	2.4	1	1	2	1	1	1	1	2	1	1	1	1	0	0	0	1	0	1	0	1	1	0	1	1	1	1	2	1	0	1	0	0	0
O.connectens	0.8	2.1	1	1	1	0	1	1	0	[12]	1	1	1	1	0	0	0	1	0	1	0	0	0	1	0	0	1	0	0	1	0	0	1	0	1
O.erginnus	0.8	2.7	0	1	1	0	1	1	0	2	1	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	1	2	0	0	0	0	0	1
O.isander	1	2.1	0	0	2	0	2	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	3	0	?	0	1	1	2	0	0	1	0	0	0
O.lugens	2.4	2.1	1	1	2	0	2	1	1	2	1	1	1	1	0	0	0	1	0	1	1	0	3	1	0	0	1	0	0	1	2	0	0	0	2
O.lugubris	0.6	2.1	1	1	2	0	1	1	1	2	1	1	1	1	0	0	0	1	0	0	0	0	0	1	0	0	1	0	0	0	0	0	1	0	1
O.obscura	0.7	2.3	0	1	3	0	2	1	[01]	0	0	[01]	1	0	1	[01]	0	0	1	0	0	0	1	0	1	0	1	0	0	1	0	1	0	0	0
O.phruron	0.7	1.5	1	1	2	0	2	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	2	1	1	1	0	0	0
O.salicis	0.5	2.8	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0	0	0	0	1	1	1	1	0	0	1	0	0	0
O.sulciscuta	0.7	2.5	0	0	3	0	2	1	0	2	0	0	0	0	1	1	1	0	1	0	0	0	1	0	0	0	1	1	0	1	0	1	0	0	0
O.telephe	0.8	2.5	1	1	1	0	1	2	1	1	1	1	1	1	0	0	0	1	0	0	0	0	3	0	1	0	1	1	2	0	0	0	0	0	0
O.theana	0.8	3	1	1	2	0	2	1	1	[12]	1	1	1	1	0	0	0	1	0	1	1	0	3	0	0	0	1	1	1	0	0	1	0	0	0
O.versicolor	0.9	1.6	1	1	1	0	1	2	1	2	1	1	1	1	0	0	0	1	0	0	0	1	1	0	0	0	1	1	2	1	0	0	0	1	0
O.sp1	1	2.5	1	1	1	0	1	1	1	2	[01]	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	2	1	1	1	0	0	0
O.sp2	0.6	2.9	1	1	2	1	2	1	1	[12]	1	1	1	1	0	0	0	1	0	1	0	1	1	0	1	0	1	1	2	0	0	1	0	0	0
O.sp3	1.2	2.4	1	1	2	0	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0	3	0	?	1	1	1	1	0	0	1	0	0	0
O.sp4	1.5	3.8	1	1	2	0	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0	1	0	0	0	1	1	2	1	1	1	0	0	0
O.sp5	0.6	2.1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	0	1	0	1	1	0	3	0	0	0	1	1	2	1	1	0	0	0	0

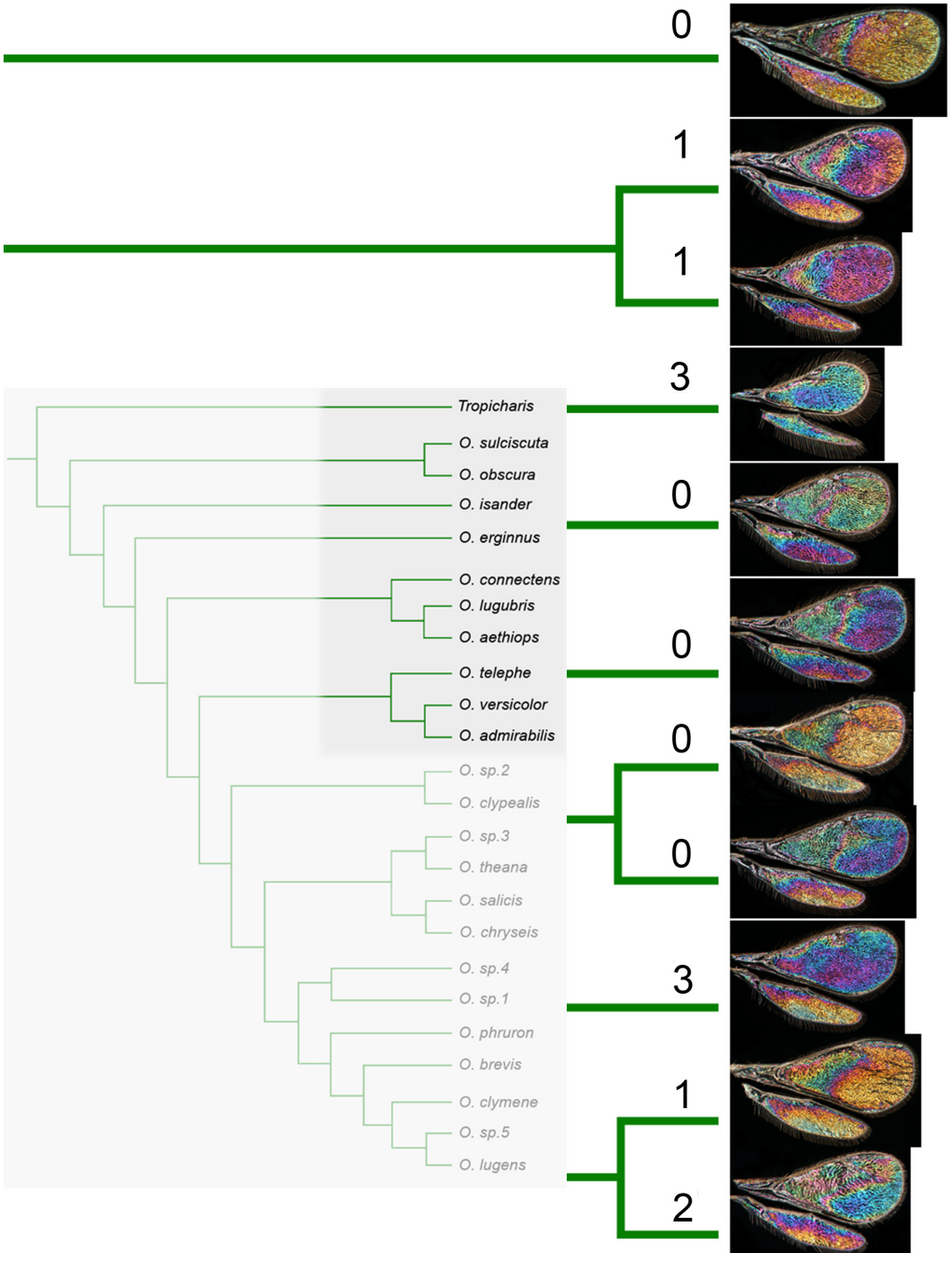
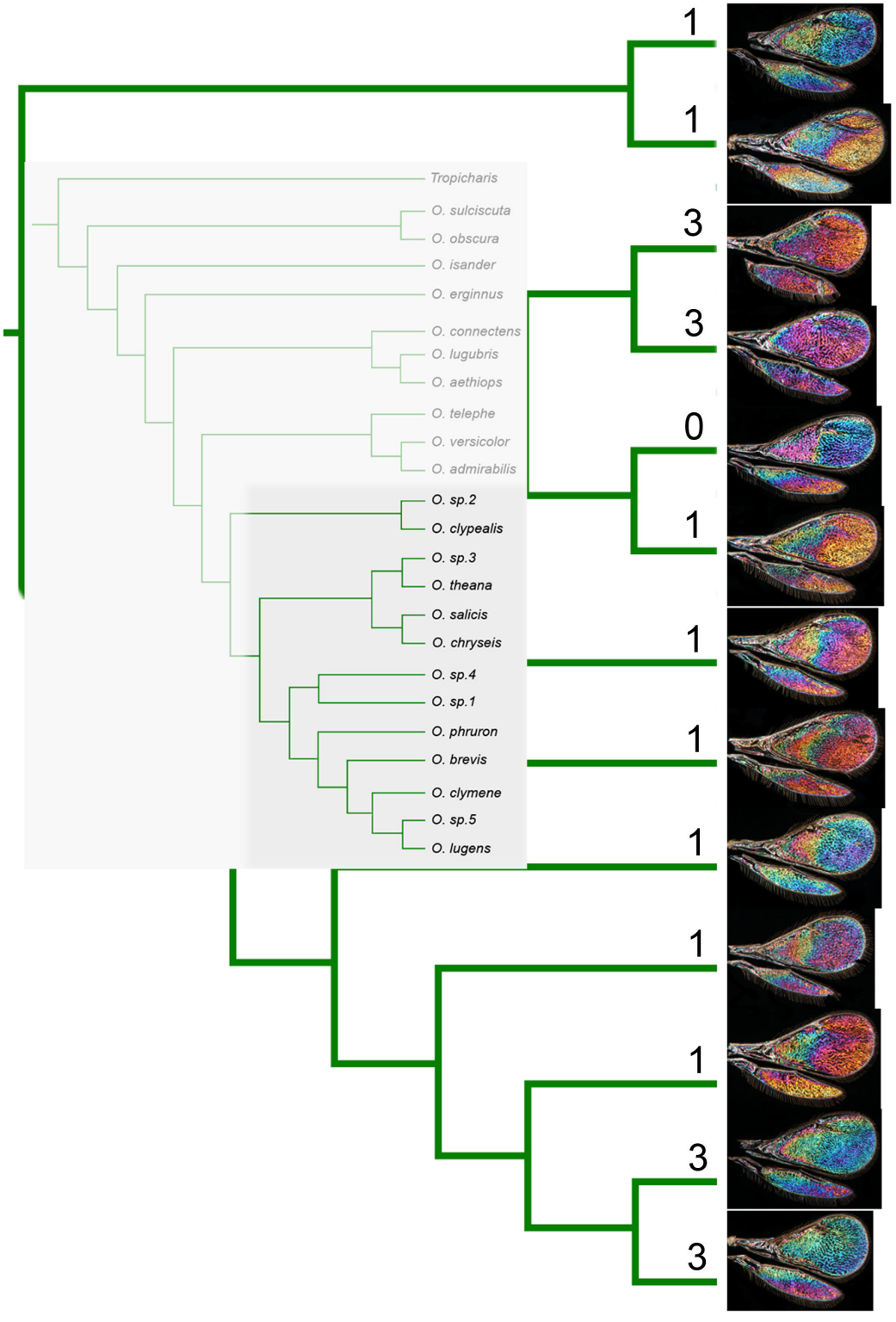


Figure 133. Cladogram including wings displaying their interference patterns (WIPs). The character state (0-3) of the WIP is shown for each species. The cladogram continues on next page.


Tropicharis
O. sulciscuta
O. obscura
O. isander
O. erginnus
O. connectens
O. lugubris
O. aethiops
O. telephe
O. versicolor
O. admirabilis
O. sp.2
O. clypealis
O. sp.3
O. theana
O. salicis
O. chryseis
O. sp.4
O. sp.1
O. phruron
O. brevis
O. clymene
O. sp.5
O. lugens
1
1
3
3
0
1
1
1
1
1
1
3
3

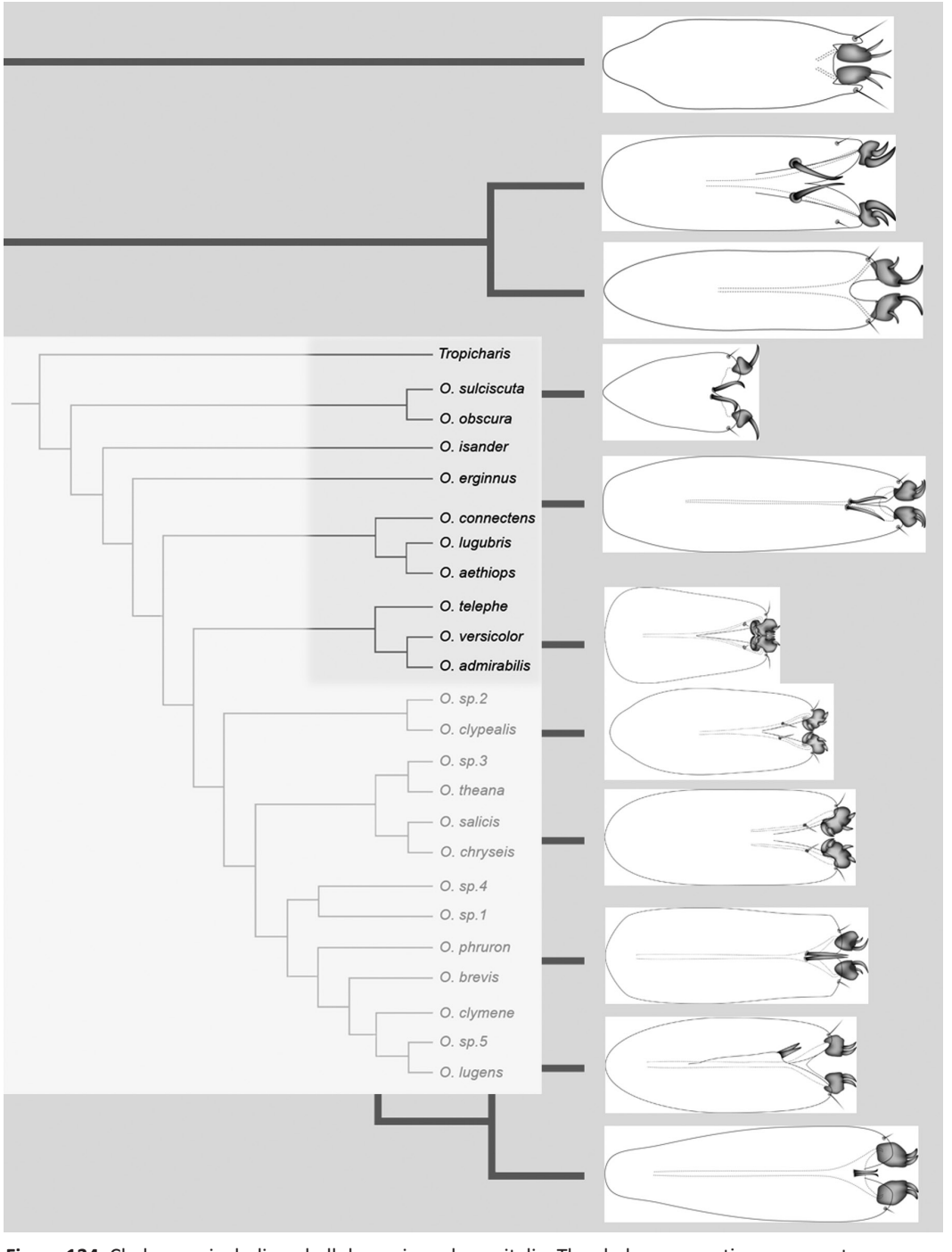
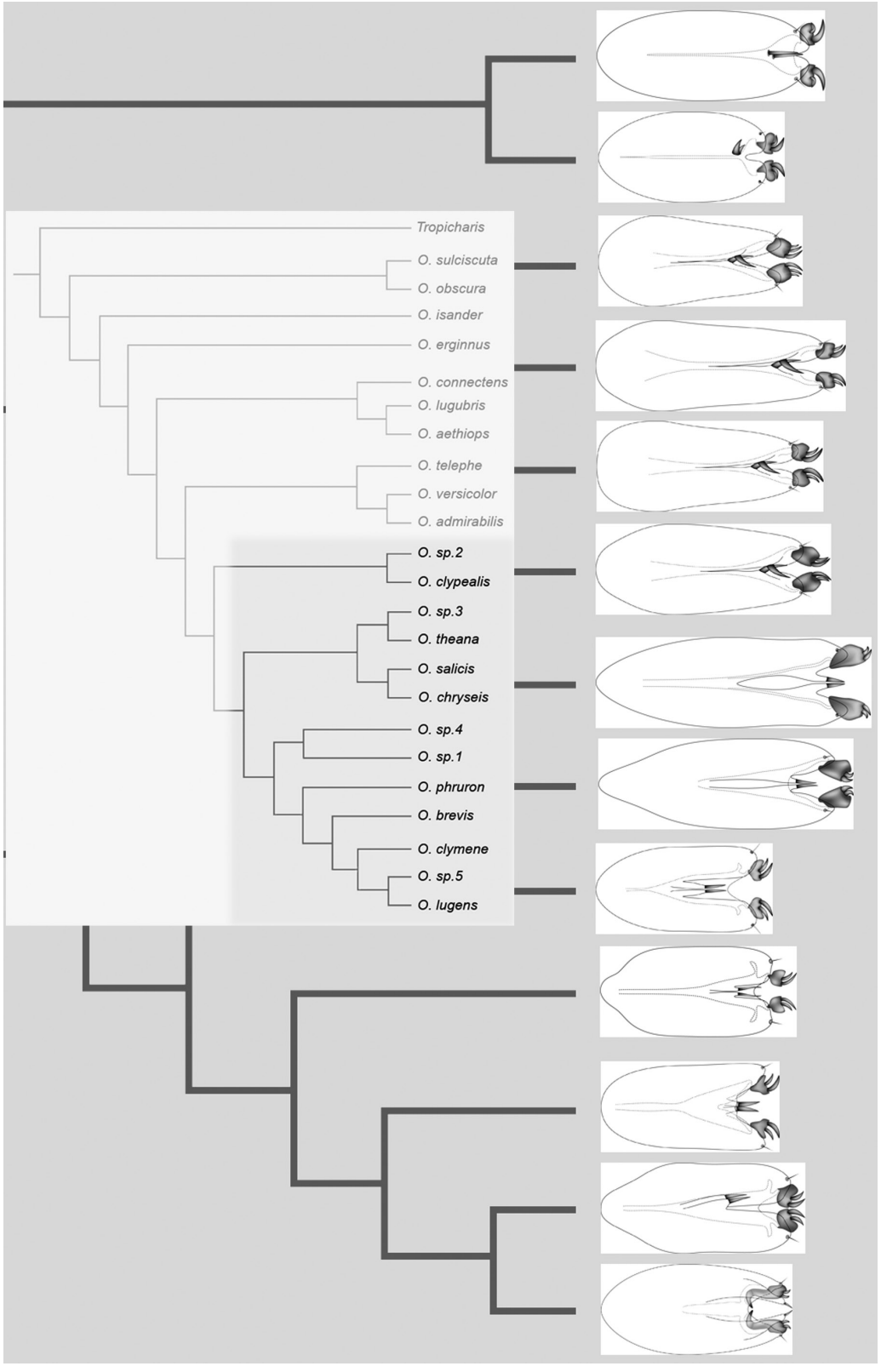


Figure 134. Cladogram including phallobases in male genitalia. The cladogram continues on next page.


Tropicharis
O. sulciscuta
O. obscura
O. isander
O. erginnus
O. connectens
O. lugubris
O. aethiops
O. telephe
O. versicolor
O. admirabilis
O. sp.2
O. clypealis
O. sp.3
O. theana
O. salicis
O. chryseis
O. sp.4
O. sp.1
O. phruron
O. brevis
O. clymene
O. sp.5
O. lugens

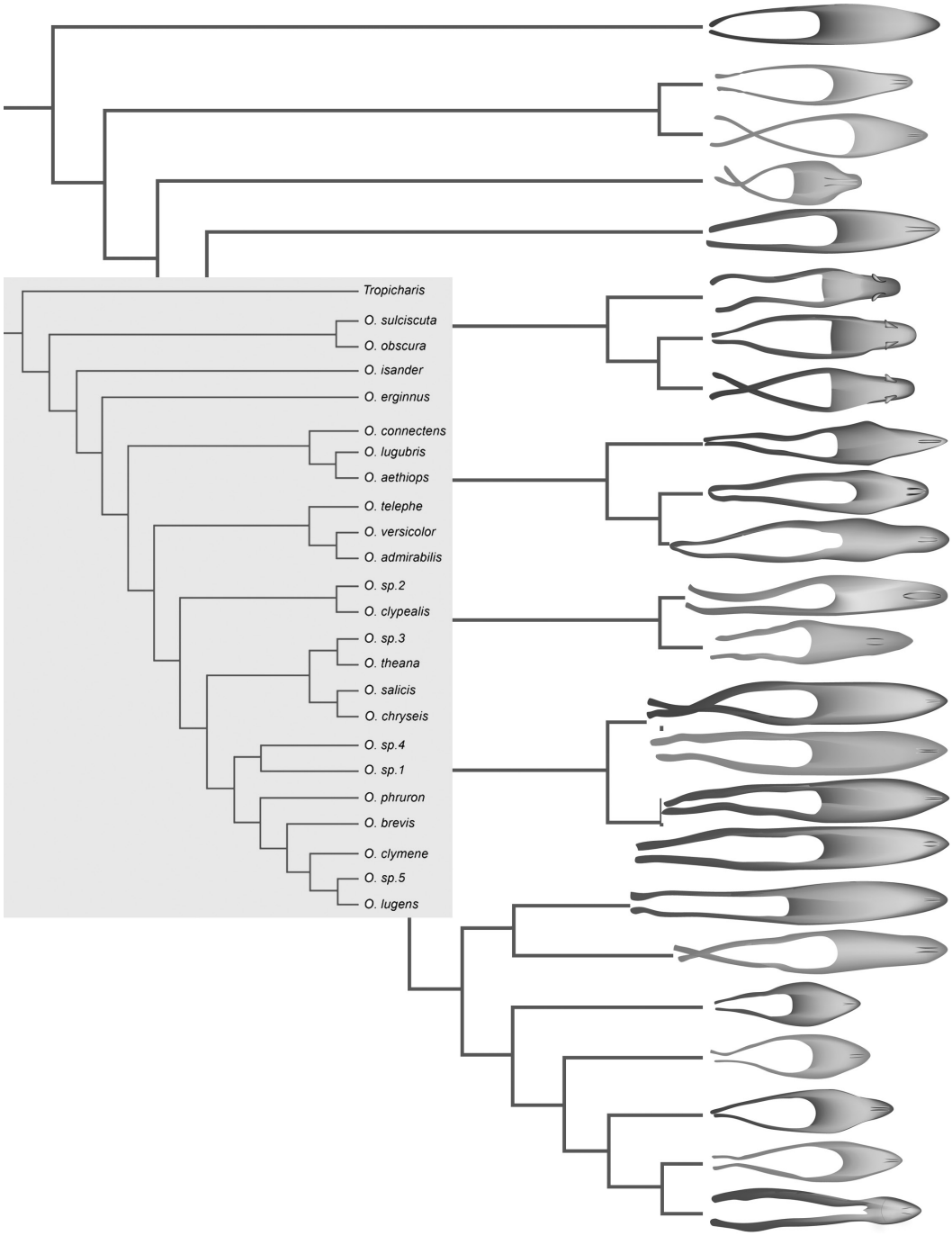


Figure 135. Cladogram including aedeagi in male genitalia.

Appendix 3. List of species in their respective group

Species-group *admirabilis*
O. admirabilis (Westwood)
O. telephe (Walker)
O. versicolor (Nees)

Species-group *aetius*
O. aethiops Graham
O. connectens Graham
O. lugubris Askew

Species-group *clypealis*
O. clypealis (Thomson)
O. sp.2

Species-group *phruron*
O. brevis Graham
O. clymene (Walker)
O. lugens (Nees)
O. phruron (Walker)
O. sp. 1
O. sp. 4
O. sp. 5

Species-group *salicis*
O. chryseis Graham
O. salicis Haliday
O. theana (Walker)
O. sp. 3

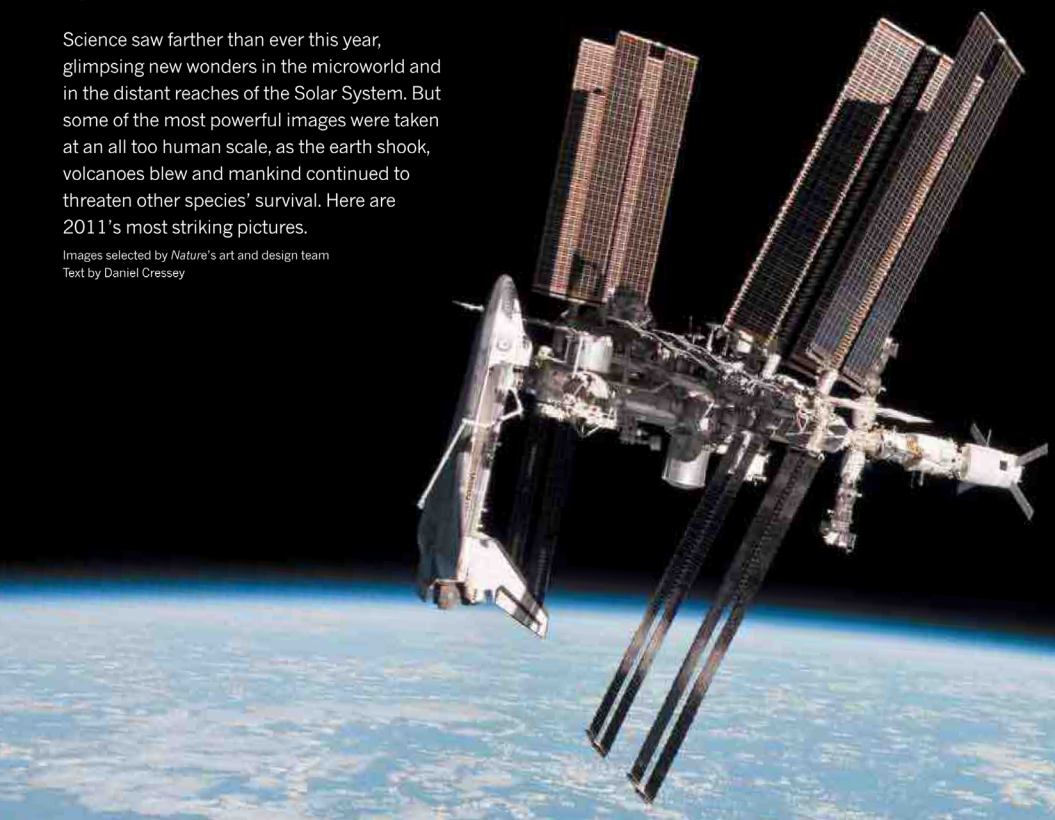
Species-group *sulciscuta*
O. obscura (Förster)
O. sulciscuta (Thomson)

Singular species
O. erginnus (Walker)
O. isander (Walker)

IMAGES OF THE YEAR

Science saw farther than ever this year, glimpsing new wonders in the microworld and in the distant reaches of the Solar System. But some of the most powerful images were taken at an all too human scale, as the earth shook, volcanoes blew and mankind continued to threaten other species' survival. Here are 2011's most striking pictures.

Images selected by *Nature*'s art and design team
Text by Daniel Cressey



JAPAN'S PAIN

The shattered village of Ōtsuchi testifies to the sav
subsequent tsunami that hit Japan's Tohoku regio
hundreds of thousands homeless and triggered a

CAGED FURY

Rats don't deserve their bad name, but this ball
Russian scientists bred this aggressive rat strain
study on domestication that has teased out seve

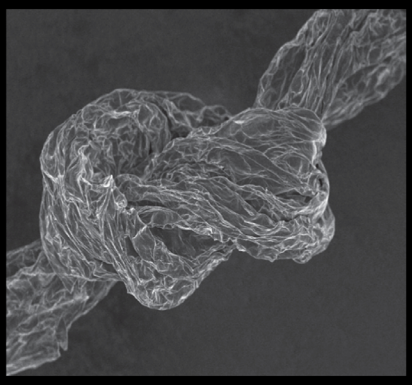
365 DAYS: *the year in science*

earthquake and
ns of thousands, left
na nuclear plant.

ny murophobes.
docile creatures in a
l to tame traits.

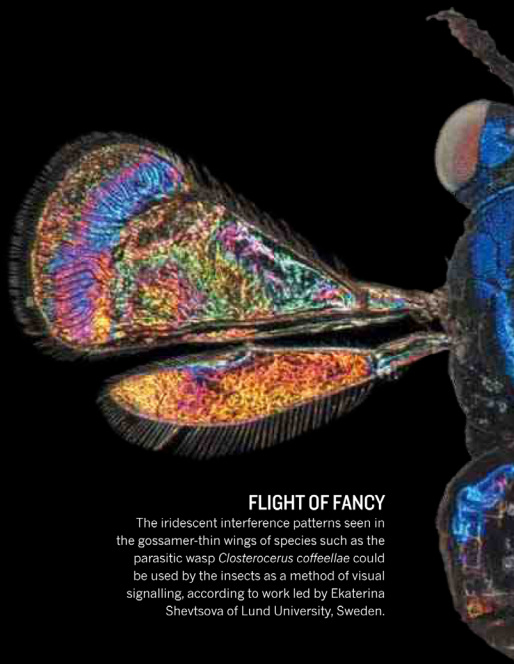
CELLULAR CHRISTMAS

Donna Stolz created a festive wreath by assembling images of mammalian cells from more than 25 experiments. The picture adorned her Christmas card last year and won recognition for the University of Pittsburgh biologist in the 2011 Nikon Small World photography contest.



UNFORGETTABLE

This 400-μm-long knot in graphene, tied by Zhen Xu and Chao Gao at Zhejiang University in China, shows exquisite control at the nanoscale. Xu and Gao spun flat liquid crystals of graphene oxide into flexible fibres metres long, and then converted them into graphene threads.



FLIGHT OF FANCY

The iridescent interference patterns seen in the gossamer-thin wings of species such as the parasitic wasp *Closterocerus coffeellae* could be used by the insects as a method of visual signalling, according to work led by Ekaterina Shevtsova of Lund University, Sweden.