

Evolutionary phenomics and the emerging enlightenment of arthropod systematics

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Abstract. Published research on the diversity and evolutionary history of Arthropoda sets a high standard for data collection and the integration of novel methods. New phylogenetic estimation algorithms, divergence time approaches, collaborative tools and publishing standards, to name a few, were brought to the broader scientific audience in the context of arthropod systematics. The treatment of morphology in these studies, however, has largely escaped innovation. Lodes rich in characters too often go unexplored, phenotype concepts are published with inadequate documentation and the way observations are textualised leaves them inaccessible to a majority of biologists. We discuss these issues, using data from recent arthropod systematics publications, and offer several that stand to restore the broad utility of morphological data. Specifically, we focus on: (1) the potential of internal soft-part characters and how to integrate their observation into arthropod systematics projects through dissection and serial sectioning; (2) the importance of capturing observations in images, especially using relatively new approaches, like laser scanning confocal microscopy and three-dimensional reconstruction; and (3) the untapped potential of established knowledge representation methods, which may help make the descriptive components of arthropod systematics research more accessible to other domains.

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Introduction

Systematists arguably benefit from the most diverse array of data sources and technological approaches in the life sciences. We use information from genomes, transcriptomes, proteomes, phenomes and whole biomes to elucidate niche restrictions, discover cryptic diversity, delimit and diagnose taxa, estimate relationships and divergence times, discover characters and to more fully understand evolution. The systematist's toolkit, therefore, is correspondingly large and continually expanding. The integration of new tools, however, appears to be uneven. Johnson (2011) recently described the latest collaborative environments, specimen data sharing resources and advances in publishing, which are percolating through the community. Molecular advances, likewise, are rapidly finding their way into systematists' workflows and will soon become mainstream (e.g. next generation sequencing; Bybee *et al.* 2011; Lemmon and Moriarty Lemmon 2012). Breakthroughs in the realm of morphology, however, are admired but rarely used outside a handful of laboratories. The promise of a 'renaissance of morphology', triggered by the proliferation of new tools (e.g. Popper and Schaffner 1959; Stuessy *et al.* 2003; Budd and Olsson 2007; Friedrich and Beutel 2008), has yet to be broadly realised in arthropod systematics.

To explore this predicament in a more structured (if crude) way, we surveyed 428 arthropod systematics (*sensu lato*) papers,

published from 2007 to early 2012 in seven journals and scored them for several variables relevant to morphology-based systematics (Table 1; complete dataset at <http://dx.doi.org/10.6084/m9.figshare.94214>). The results show, unsurprisingly perhaps, that morphology remains the primary data source for arthropod systematists. Our survey also uncovers three aspects of arthropod systematics research that we predict will benefit from the adoption of contemporary methods: (1) internal soft-part characters remain largely unexplored despite demonstrable utility for phylogenetics and diagnosis; (2) new imaging technology is vastly underutilised despite increasing accessibility; and (3) phenotypic concepts, especially anatomical entities, used in character descriptions too often remain undocumented. We discuss these deficiencies below and offer some thoughts on how to bring arthropod systematics through a renaissance and into enlightenment.

Prospecting for relevant internal phenotypes

Methodological inertia guides most arthropod systematists to digest muscles, glands, ganglia and other soft parts, so that we can cleanly prospect for characters on sclerites. The resulting soft-tissue soup is poured down the drain, so to speak, with potassium hydroxide or captured in vials after treatment with proteinase. We read >15 500 character descriptions in our literature review and found a paltry 408 that referred to internal

Table 1. Summary of our literature review of arthropod systematics papers published in *Acta Zoologica*, *Insect Systematics & Evolution*, *Invertebrate Systematics*, *Systematic Biology*, *Systematic Entomology*, *Zoological Journal of the Linnean Society* and *Zoologica Scripta*

Total papers reviewed	428
Arthropod taxa covered (order-level or higher)	47
Papers that include morphological data	219 (51%)
Total morphological characters reviewed	15 590
Internal soft part characters	408 (2.6%)
Morphology papers with line drawings	177 (80%)
With brightfield micrographs	108 (49%)
With scanning electron micrographs	81 (37%)
With μ -computed tomography	4 (1.8%)
With laser confocal micrographs	2 (0.9%)
With serial sectioning	1 (0.5%)
With glossary of anatomical concepts	5 (2.3%)
Without any references for anatomical concepts	64 (29%)

soft parts. Yet our understanding of arthropodean phenotypes, in the context of evolutionary history (a core goal of systematics), would benefit from deeper observation of these structures. The soft structures themselves serve as evidence for phylogenetics (e.g. Friedrich and Beutel 2010), but they also inform us about the hard parts, including external features.

Muscle characters, for example, are usually ignored in phylogenetic analyses but, most problematically, also in descriptions and interpretations of skeletal characters. Homology hypotheses are often built around the overall similarity of the ‘topography’ of two characters (here the shape and biospatial relationship of the structure with other anatomical entities; see discussion in Seltnann *et al.* 2012). Although a sclerite can be described based on its relationships to other sclerites, its relationship with muscles is often crucial for generating well founded homology hypotheses. The mesoscutum in Hemiptera (Insecta) looks remarkably similar to the mesoscutum in Hymenoptera; in both taxa, the median area of the mesoscutum is delimited submedially by two longitudinal sulci. These areas could easily be interpreted as homologous anatomical structures. Observations of the thoracic intima, however, reveal that the sulci are defined by different muscles and hence are most likely not homologous (Mikó *et al.* 2012). Similar examples can be found in characters that involve antennal sensilla, which are too often classified based on superficial external morphology (Romani *et al.* 2010). The functional mechanisms that underlie these cuticular modifications, common characters for species diagnosis in many insects, are impossible to understand without observing the innervations of the sensory cell, as well as their corresponding exocrine glands (Romani *et al.* 2010). The internal phenome, in these cases muscle morphology and sensillar neuroanatomy, yields critical evidence that shapes our understanding of superficially equivalent structures.

Mining the internal phenome in most arthropods can be as simple (and inexpensive) as preparing a specimen and dissecting it under a microscope. The process also requires that one be willing to divide a specimen into many smaller parts, a process to which many systematists seem have an aversion (especially if the species is rare). Dissections can be made on either wet specimens in alcohol or glycerine or on a specimen that has been put through

a regimented drying process (e.g. Quicke *et al.* 1999), with more-or-less well preserved internal soft structures. The cardinal difference between wet and dried specimens is the physical relationships between anatomical structures. In wet specimens, muscles, glands and nerves are more strongly connected to each other and to the integument, whereas in dried specimens they are easy to detach. Dried specimens, therefore, are feasible for the description of the spatial position of anatomical structures, while wet specimens can be used for checking muscle insertions and origins. Since dissections are inherently dissociative (not inevitably destructive), visualisation and imaging of the specimens are highly recommended in different phases of the mining process. Vilhelmsen *et al.* (2010) and Mikó *et al.* (2007, 2012), provide examples of how dissections and subsequent observations of internal soft-tissue features can inform character concepts.

A more involved, precise and highly dissociative way of mining the internal phenome is to serially section a specimen, either using histological or X-ray-based techniques. Histological sectioning (Fig. 1A) is common in comparative morphology studies and some physiological research but rarely used by systematists, possibly due to problems presented by heavily sclerotised cuticle, although advances in epoxy resin embedding media and microtome technology have solved most of these problems. Fixation of freshly collected specimens is required for subcellular histological observations, but semi-thin sections of alcohol-preserved specimens can provide important and useful information about the gross morphology of arthropods. Great examples of histological sectioning in the context of arthropod systematics include Wirkner and Richter (2007), Edgecombe and Koch (2008), Beutel *et al.* (2009a, 2009b, 2010), Friedrich and Beutel (2010), Camacho (2011), Szucsich *et al.* (2011) and Koch and Edgecombe (2012).

A more recent advancement in serial sectioning is to use X-rays to virtually slice the arthropod specimen in a process called microtomography (μ CT) (Fig. 1B). This method is non-dissociative but usually offers lower-resolution and much weaker tissue contrast images than histology. Stained or unstained specimens can be scanned in a pipette tip filled with water, alcohol or glycerine or embedded in Araldite or Canada balsam. Critical point drying is the best preparation technique, when staining is not an option, since it maximises the contrast between the biological structures and the surrounding medium (air). There are two primary μ CT resources used for arthropods: desktop machines (DT μ CT; e.g. machines by Phoenix, SkyScan), which are more widely accessible and relatively inexpensive, and synchrotron-radiation-based μ CT (SR μ CT), which yields much higher resolution but typically requires careful planning and access to relatively high-demand facilities. With SR μ CT, one has the opportunity to use the absorption contrasting technique (Beckmann *et al.* 2008, Friedrich and Beutel 2008), whereas only phase contrasting (Wilkins *et al.* 1996, Hörnschemeyer *et al.* 2002, Dunlop *et al.* 2012) is available with DT μ CT. Although phase contrast scans allow strong delimitation of the specimen from the surrounding media, the tissue-contrast of the resulting micrographs is very low. The tissue contrast of pure absorption contrast data are, however, very high, and therefore SR μ CT might be considered an excellent alternative to histological

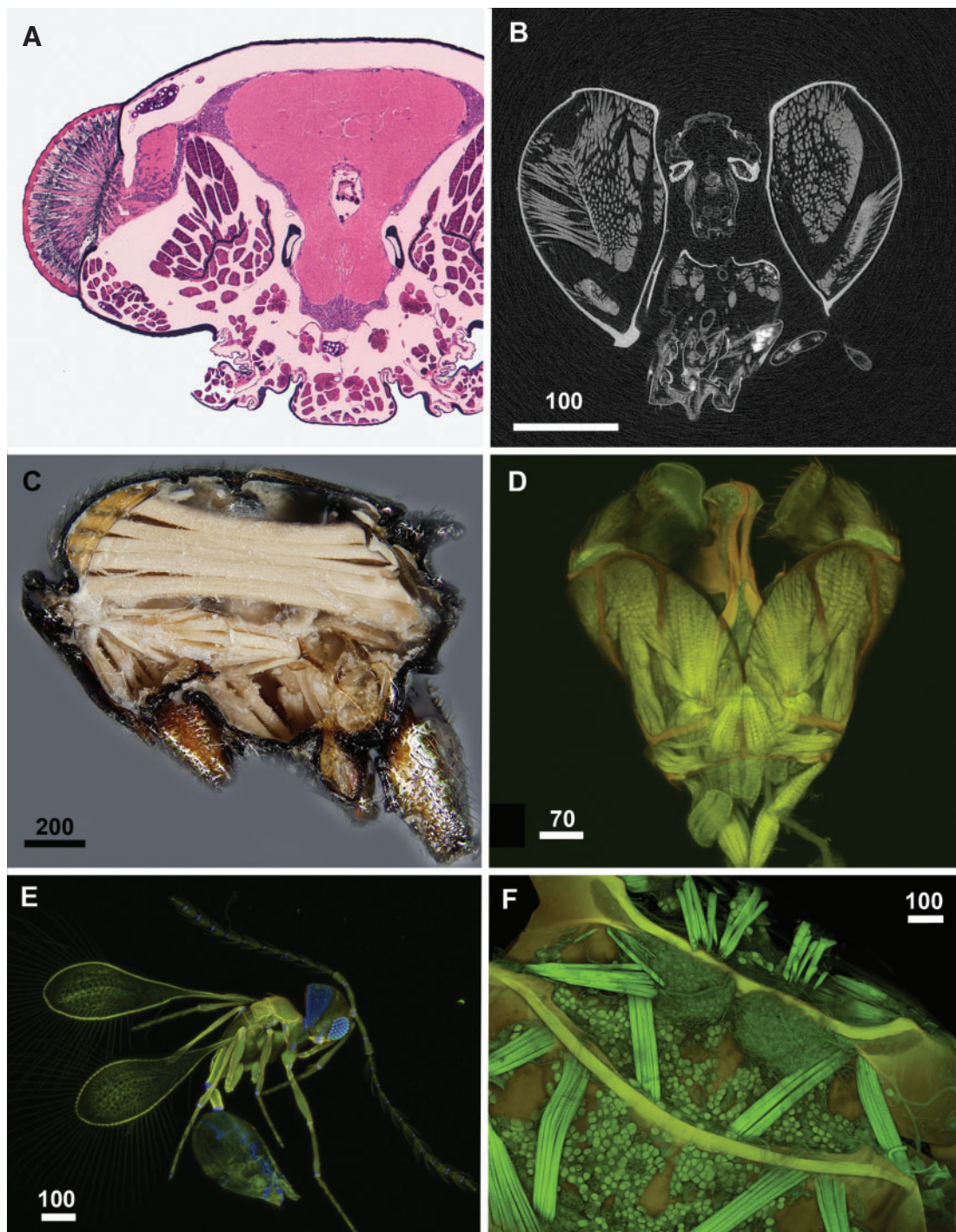


Fig. 1. Brightfield, Confocal Laser Scanning Microscopy (CLSM), histological and X-ray-based section micrographs of different Hymenoptera anatomical structures. (A) Histological section of *Xyela* sp. (Hymenoptera, Xyeloidea). (B) Virtual cross section based on SRμCT of *Tenthredo* sp. (Hymenoptera, Tenthredinoidea). (C) Brightfield image of *Ceraphron* sp. (Ceraphronoidea, Ceraphronidae), bisected mesosoma, median view. (D) CLSM micrograph of *Xyela* sp. (Xyeloidea, Xyelidae), male genitalia, ventral view. (E) CLSM micrograph of *Mymaromma* sp. (Mymarommatoidea, Mymarommataidae) showing resilin sensitivity of CLSM (resilin-rich areas in blue), habitus, lateral view. (F) CLSM micrograph of *Megalyra* sp. (Megalyroidea, Megalyridae) showing tissue contrast of CLSM (sclerotised structures yellowish-red, soft structures green), anterior metasomal sterna, dorsal (internal) view; scale bars in micrometer. Original video files are also available: doi:10.6084/m9.figshare.94275, doi:10.6084/m9.figshare.94276, doi:10.6084/m9.figshare.94277.

sections (Friedrich and Beutel 2008). Muscles, nervous system, glands and different grades of skeletal sclerotisation can easily be distinguished. However, for detailed information on thin peripheral nerves or on the cellular aspects of tissues, the data from whole-specimen scans are not yet as informative as those from histological sections. Hardware improvements (larger sensors, stable beams, etc), advances in X-ray-sensitive staining (Metscher 2009), and new developments in corrosion casting (Wirkner and Richter 2007) will help surmount these issues.

Examples of articles using SR μ CT to infer arthropod systematics include Beutel *et al.* (2009a), Friedrich and Beutel (2010), Huckstorf and Wirkner (2011), Talarico *et al.* (2011), Wipfler *et al.* (2011) and Friedemann *et al.* (2012). Researchers have also used SR μ CT to explore the characteristics of obscured fossil insects in amber (Tafforeau *et al.* 2006; Lak *et al.* 2008) and to partially reconstruct the internal phenomes of fossil insects (Pohl *et al.* 2010). Microtomography of fossil vertebrate tissues is also a possible future application of SR μ CT in arthropod systematics, for example to examine pigment composition (Wogelius *et al.* 2011).

Documenting observed phenotypes

The arthropod systematics papers we read reported >15 000 characters, and most of these publications included at least some token illustrations, almost exclusively as line drawings or light or traditional scanning electron micrographs (SEMs). While these methods are convenient, given their general accessibility, low cost, and long history, they are also limited in their efficiency. None of the studies we read (2007–2012) illustrated every character state for every taxon, despite the existence of tools that facilitate this level of documentation (Liljeblad *et al.* 2008). Yet imaging and annotating characters is crucial to the repeatability of our research and the repurposing of our observations. Refinements in brightfield methods (e.g. Buffington and Gates 2008), another common approach to illustrating observations, are easy to implement and should increase the utility of the resulting images (Fig. 1C). Two emerging classes of imaging methods, however, are poised to facilitate broader discovery and richer documentation of phenotypes for arthropod systematists: (1) Confocal Laser Scanning Microscopy (CLSM); and (2) three-dimensional reconstruction.

Confocal Laser Scanning Microscopy

Confocal Laser Scanning Microscopy, used extensively since the late 1980s by cellular and molecular biologists, has only recently been applied to arthropod systematics (Galassi 1997, Klaus *et al.* 2003, Klaus and Schawaroch 2006). CLSM has several advantages over traditional light microscopy and SEM. (1) depending on the CLSM strategy used and the size of the specimen, one can produce and annotate three-dimensional models of the object of interest, which results in a highly efficient representation strategy (see below); and (2) unlike scanning electron microscopy, or even light microscopy for some specimens, one can readily differentiate tissues – for example, skeletal structures (Michels 2007) (Fig. 1D, F), muscles, resilin-rich areas (Fig. 1E) of the integument (Neff *et al.* 2000; Andersen 2004; Burrows *et al.* 2011; Michels and

Gorb 2012), or even calcified regions (Haug *et al.* 2011) can be differentiated by lasers of different wavelengths (Fig. 1E). Specimens also do not require exotic media: Canada balsam, euparal, glycerine jelly (Michels 2007; Michels and Gorb 2012) or agarose are the usual embedding media. CLSM can also be used for visualisation of internal structures through the cuticle in transparent specimens, which rapidly yields information about the site of origin of muscles corresponding with external cuticle modifications (Fig. 1D). Specimens with strongly melanised cuticle can be made transparent with hydrogen peroxide (Stüben and Linsenmair 2008), without affecting the autofluorescence of insect anatomical structures and destroying soft structures (A. R. Deans, pers. obs.)

Three-dimensional reconstruction

Most anatomical structures are readily visualised in two-dimensions, but rendering these structures as three-dimensional interactive models is a more realistic and efficient representation. Indeed, three-dimensional reconstruction has a relatively long history in biology (Gaunt and Gaunt 1978) and even among some arthropod systematists, especially when personal computers enabled a switch from a tedious manual process to computer-assisted reconstruction (Huijsmans *et al.* 1986; Winslow *et al.* 1987; Verbeek *et al.* 1995).

Aligned image sets from CLSM, histological sectioning or μ CT can be rendered as three-dimensional models using one of several software packages (e.g. Imaris Bitplane (<http://www.bitplane.com/go/products/imaris>), Amira (<http://www.amira.com/>), Zeiss LSM Image browser (http://www.embl.de/camnet/html/body_image_browser.html)) and either the surface (SR) or volume rendering (VR) technique (Kaufman and Mueller 2006). Surface rendering provides information exclusively about the surface of selected structures of the specimen (Fig. 2D), whereas VR captures both internal and external features of the structures (Fig. 2A–C). Models rendered straight from the raw image data are immediately useful for illustrating external characters (Fig. 2A) and dissected parts (Fig. 1D). However, annotating different structures on individual image sections, an admittedly chronophagous process with current technology (Ruthensteiner 2008), facilitates the rendering of interactive models, whereby structures can be added or removed to facilitate viewing of obscured phenotypes (i.e. virtual dissection; see example in Fig. 2B, C).

The Portable Document Format (PDF) can now integrate SR three-dimensional models (supplementary file available at <http://dx.doi.org/10.6084/m9.figshare.94272>) as embedded, interactive figures (Ruthensteiner and Heß 2008), which makes distribution of these illustrations a relatively trivial exercise; only common, free software (namely Adobe Acrobat Reader) is required. Volume rendered models currently can only be published as supplementary files, but the ability to incorporate them into PDFs will likely happen in the near future (Ruthensteiner *et al.* 2010).

Explicit textualisation of concepts

Phenomic data cannot be represented by images alone, as they are difficult to datamine and require context and a history of interpretation that can only be communicated in words. Virtually

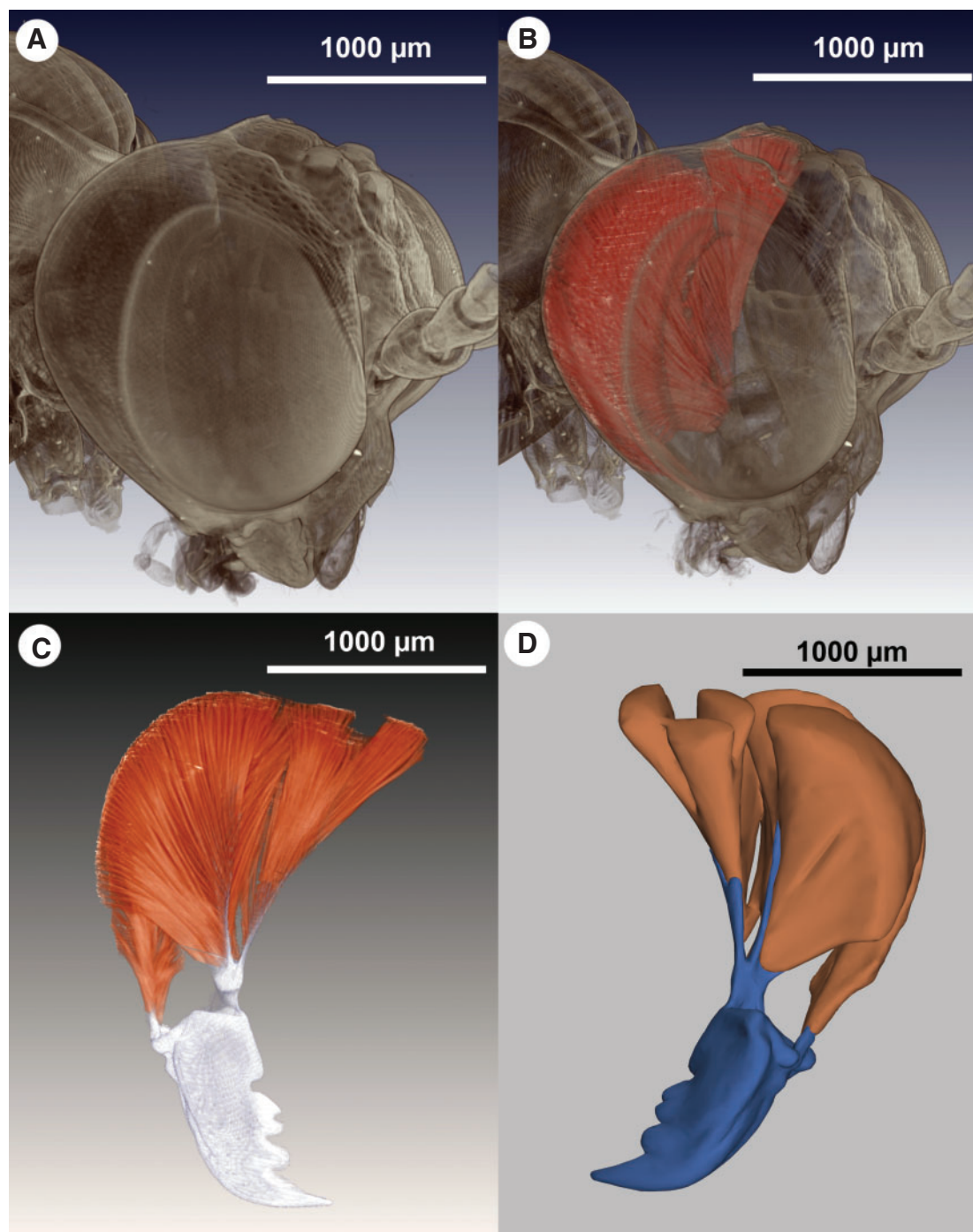


Fig. 2. Microcomputed tomography of *Tenthredo* sp. (Hymenoptera, Tenthredinoidea) habitus, anterolateral view. (A) Head, anterolateral view (volume rendering of synchrotron-radiation-based microtomography (SRμCT) data). (B) Head, anterolateral view, mandibular muscles are annotated with red (volume rendering of SRμCT data). (C) Mandible with mandibular muscles, anterior view, mandibular muscles are annotated with red (volume rendering of SRμCT data). (D) Mandible with mandibular muscles, anterior view, mandibular adductor is annotated with green, mandibular abductor with blue and mandible with brown (surface renderings of SRμCT data); original three-dimensional PDF available with doi:10.6084/m9.figshare.94272.

every named organism is associated with a textual diagnosis, and each morphology-based paper we read in our review included blocks of prosaic natural language that attempted to communicate knowledge about phenotypes. We feel strongly, as communicated by Deans *et al.* (2012), that phenomic data derived from the

systematic process, including the observations described in textual descriptions, should be availed broadly to other domains. Unfortunately, we have no standardised way of publishing phenotype data (Vogt *et al.* 2010; Deans *et al.* 2012). Nucleotide data, for example, are represented by

symbols (A, G, C, etc.) designated by the International Union of Pure and Applied Chemistry (IUPAC) and universally accepted by biologists. Strings of these symbols are submitted to an accessible, highly functional and robust database, like the National Center for Biotechnology Information's GenBank (Benson *et al.* 2012). The purpose of these standards, in part, was to 'facilitate comparisons ... as in the search for homologies' (IUPAC-IUB Commission on Biochemical Nomenclature 1970). There is no equivalent yet for arthropod morphology. Homonymy is rampant (Yoder *et al.* 2010), and phenotypes are often painstakingly qualified ('somewhat reddish') or too vague to be meaningful ('labrum narrow' versus 'labrum broad' versus 'labrum very broad').

Emerging informatics standards stand to dramatically improve the way we share phenotype data. Deans *et al.* (2012) alluded to one method that was developed initially for representing mutant model organism phenotypes, the Entity–Quality (EQ) formalism (Mabee *et al.* 2007; Mungall *et al.* 2010), but which could be adapted to systematics. In the EQ approach, anatomical and phenotype quality concepts are explicitly defined and organised as ontologies (see Washington and Lewis 2008); the concepts from the ontologies are then composed in a way that makes the phenotype data (i.e. the character states) queryable. The most successful deployment of this approach, at least in an evolutionary biology context, is found in the world of ichthyology (Phenoscape project; see Mabee *et al.* 2012). Phenoscape combines morphological data from fish phylogenetic matrices with data from mutant model organisms (primarily *Danio rerio*; <http://zfin.org>) and uses the logic inherent in ontologies to inform, in part, hypotheses about genotype–phenotype interactions. Examples of fine-grained EQs, composed in Web Ontology Language (OWL; <http://www.w3.org/TR/owl-features/>) and applied to species descriptions, are also emerging in arthropod research (Mullins *et al.* 2012).

While it is too early to apply the EQ approach broadly in arthropod systematics, we do think systematists should more rigorously document their concepts, especially for anatomy. Almost one-third of the papers we reviewed provided no references for anatomical concepts, and only 2.3% provided a glossary. In most cases, the intended readership, i.e. other specialist taxonomists, will understand which 'paramere' (=five different structures in Hymenoptera) or 'forearm' (=part of the male genitalia of some Lepidoptera) the authors mean in their character description, but homonymy could induce confusion in anyone attempting to repurpose those observations. A simple solution is to append one's publication with a glossary of terms, explicitly defined using *genus differentia* (Smith 2005). Some insect systematists, Talamas *et al.* (2011) and Mikó *et al.* (2012) for example, have started doing this in the form of a table that also includes links (Uniform Resource Identifiers or URIs) to anatomical concepts in the Hymenoptera Anatomy Ontology (Yoder *et al.* 2010) and, in some cases, to phenotypic qualities in the Phenotype Quality Ontology (PATO; Mungall *et al.* 2010) (see Seltsmann *et al.* 2012 for a description of the process).

Conclusion

Arthropod systematists are charged with researching more than half of all known life forms, a process that requires extraordinary

levels of data collection and methods innovation. The majority of data and hypotheses generated by our science – nucleotide sequences, specimen collecting events, taxonomic names, phylogenetic trees, etc. – are already availed broadly using community-developed standards, and we continue to lead the field in mechanisms of collaboration and dissemination. In our view, at least three aspects of the systematic process stand to benefit from a shift in strategy or the incorporation of emerging technology. (1) Internal anatomy remains a largely untapped resource for evidence of taxonomic association and evolutionary history. We encourage arthropod systematists to expand their character-harvesting repertoire to include more dissociative methods (dissection, sectioning) and to explore X-ray-based sectioning methods, which are increasingly accessible. (2) Phenomic observations would be more robustly documented if we utilise CLSM and three-dimensional reconstruction of serial sections, especially now that annotated and interactive three-dimensional models can be easily published. (3) We should also invest in the development of new standards (and testing of emerging standards) that facilitate sharing of textual phenotype data. Prosaic natural language is by far the most expressive way to communicate one's interpretations of phenotypes, but the resulting summary remains largely inaccessible to researchers outside of the community of domain experts (i.e. taxonomist specialists). However, biology is increasingly integrative, and arthropod systematists can no longer afford to keep these observations within their circle of experts. Of course we acknowledge that resources are limited and that wholesale adoption of our suggestions is currently impossible. We hope, however, that our viewpoint stimulates discussion about how we take the next steps to dramatically improve and extend our science.

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