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Cryptic species in ants (Hymenoptera: Formicidae) revisited: we need a change in the alpha-taxonomic approach

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Abstract

Cryptic species are a major challenge for alpha-taxonomy in ants. Their reliable identification requires the application of elaborate methods such as Numeric Morphology-Based Alpha-Taxonomy or analysis of DNA and cuticular hydrocarbons. Complications caused by intraspecific polymorphism and interspecific hybridisation necessitate integrating these methods in multi-source approaches. The frequency of cryptic species was estimated in three ant genera subject to a thorough analysis as ± 46% of about 94 Palaearctic *Lasius* species, ± 43% of about 67 Palaearctic *Formica* species and ± 52% of about 77 *Cardiocondyla* species worldwide. Similarly high ratios were predicted for other ant genera, although testable data are missing. Cryptic biodiversity is not evenly distributed within the evaluated ant genera. The indicative value of the following investigation methods was assessed in ants: Morphology-Based Alpha-Taxonomy (MOBAT), Numeric MOBAT (NUMOBAT), analysis of nuclear and mitochondrial DNA, cuticular hydrocarbons, pheromones, allozymes, karyotypes, ethology, and ecology. NUMOBAT is arguably the "backbone" of a testable integrative taxonomy, the deciding link to Zoological Nomenclature, the only useable method for DNA-degraded specimens and the only method to examine vouchers in which no damage is allowed. The unacceptably high ratios of paraphyly in mtDNA barcoding forbid its application as primary decision finder. In conclusion, no single method but only an integrative, multi-source alpha-taxonomy offers the most convincing approach towards recognition of real biodiversity.

Key words: Review, integrative taxonomy, alpha-taxonomy, multi-disciplinary biodiversity research, species concepts, sibling species, sister species, nuclear DNA, mtDNA barcoding, cuticular hydrocarbons, allozymes, pheromones, karyotype, behavioural test.

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Content

Introduction

"The deciding advances in science were achieved by measuring"

JAMES CLERK MAXWELL (1831 - 1879)

We are living in the initial phase of a catastrophic man-made extinction of biodiversity (BROOK & al. 2006) and it is predictable that a large percentage of species will vanish from the globe within the next 100 years often before their existence is recognised by taxonomists. "Cryptic species" form a large fraction of biodiversity and the number of publications mentioning them in the title, key words or ab-

stract exponentially rose during the last two decades – caused to a great deal by the advent of the PCR technique (BICKFORD & al. 2007). The negative consequences of misidentification of medically and economically important cryptic species in fields as for example pest control, pharmaceutics, fisheries, and nature conservation are increasingly recognised (e.g., RHOADES 1979, PATERSON 1991, WÜSTER & MCCARTHY 1996, WÜSTER & al. 1997, WANG & al. 1998, BESANSKY 1999, DAVIDSON & HAYGOOD 1999, BIDOCHKA & al. 2001, KOUFOPANOU & al. 2001, BESANSKY & al. 2003a, PRINGLE & al. 2005, GARROS & al. 2006, SANDERS & al. 2006).

Expectably, there is no taxonomic group within the animal kingdom without cryptic species but it is disputed if their distribution is random or if they are particularly abundant in certain animal groups or ecosystems (BICKFORD & al. 2007). Since most information processed by the human brain is visual, morphological characters feature more prominently than chemical and auditory characters in our recognition of the natural world. MAYR (1963) wrote in this context: "Sibling species are apparently particularly common in those kinds of species in which chemical senses (olfactory and so on) are more highly developed than the sense of vision. Although indistinguishable to the eye of man, these sibling species are evidently dissimilar to each other. Sibling species are apparently rarest in organisms such as birds that are most dependent on vision in the role of epigamic characters." This most plausible hypothesis might then predict cryptic biodiversity to be especially frequent in ants. Weakly developed visual systems of intra- and interspecific recognition and absence of bioacoustic, air-borne signalling make many ant species hardly separable in terms of our innate human senses.

In this paper, I wish to evaluate the problem of cryptic species of ants in particular and to address the consequences for research strategies. After reading some 40 publications on the discrimination or discovery of cryptic or sibling species, in which the authors reported species to be inseparable or not safely separable by morphology, I got the impression that serious attempts to make use of the information offered by external morphology were only performed exceptionally. Either the personal or material conditions did not allow this approach or the authors, usually coming from other disciplines of biology, believed that Morphology-Based Alpha-Taxonomy (MOBAT) was a blunt weapon compared to the methods they favoured. This problem is largely a consequence of research policy of the last four decades which, with the rise of molecular biology, degraded MOBAT to an inferior rank within the biosciences – cutting funding, jobs and university-based courses. This inferior ranking is also obvious within the discipline of taxonomy and systematics taken alone. WARD (2007) wrote in this context: "A further disincentive is the great scarcity of jobs for those engaged primarily in descriptive taxonomy. Museum and university positions in systematics that would have been filled by such individuals fifty years ago are increasingly going to those whose primary focus is molecular phylogenetics ... the imbalance needs to be redressed if we wish to have the capability of using morphology to confidently identify terminal taxa on the tree of life."

MOBATists, however, are not only innocent victims of a misguided development. They also take responsibility because the majority of them continue to publish hypotheses without presenting reproducible and testable data sets – hypotheses not susceptible to any verification or falsification. Is it then surprising that scientists coming from physics, chemistry, biochemistry or engineering, where each statement must be supported by sufficient measuring data and hundreds of tests, consider a whole discipline in the friendliest way as some sort of fine art? The introductory quote by J.C. Maxwell – our modern society would be unthinkable without his pioneering research – stands by itself and needs no further comment.

It is also true that MOBATists have the huge problem of facing an overwhelmingly large biodiversity while at the same time having so sadly depleted working capacities and this is perhaps the main reason to refrain from applying the time-consuming numeric description and analysis of multiple characters. Even so, a real possibility exists to develop Numeric Morphology-Based Alpha-Taxonomy (NUMOBAT) into one of the basic and most widely used tools of integrative taxonomy that is, once fast and reliable technologies and working procedures for geometric morphometrics are available, at least in a semi-automatic form. NUMOBAT is already a powerful tool for detecting cryptic species in its conventional, slow form of direct recording and analysis of multiple characters – even when operating in isolation (e.g., SEIFERT 1995, 1996, 1997, 1999, 2000b, CSŐSZ & SEIFERT 2003, SEIFERT 2003a, 2003b, 2003c, 2003d, 2004a, 2004b, 2005, 2006a, 2006b, CSŐSZ & al. 2007, MODER & al. 2007, SEIFERT 2008a). On the other hand, as I will show, this method also works most effectively when communicating with both genetical and biochemical systems.

Terminology and the problem with estimates of crypsis

Alpha-taxonomy is delimiting and naming biodiversity at species level or below – irrespective of the tools a scientist applies. Molecular biologists working in this field are in the same sense alpha-taxonomists as classical MOBATists. The underlying species concept for this review is the so-called Unified Species Concept (USC) of DE QUEIROZ (2007). It considers a separately evolving metapopulation lineage as the only necessary conceptual property of species and recognises the species criteria of other species concepts (e.g., reproductive isolation, niche separation, phenotypic and genetic cohesion and clustering) only as operational criteria. The USC has the advantage of being open for any further operational criterion and of being applicable to all organisms, both reproducing sexually and asexually as well as species of hybrid origin. Local or occasional genetic exchange between species can be acommodated if it does not affect their integrity over most of their range. As with any other species concept, the USC cannot offer a logical solution for the "eternal" allopatry problem of taxonomists.

The USC allows to hypothesize heterospecificity if only a single operational criterion supports this while all others may contradict. This does not deny that coincident multiple lines of evidence are the best indication of separate species. The oversplitting bias of the USC in case of single-source decisions requires that each discipline must find their own remedy against this illness. In case of NUMOBAT data and the operational criterion "forming separate phenetic clusters", I applied a confidence threshold of $p > 98\%$. Nevertheless, checking for possible polymorphism by other operational criteria, whenever available, was an

essential part of my working routine since intraspecific morphs may be separable with > 98%.

The taxonomic literature is full with terms such as "sister species", "sibling species", "cryptic species", or "hidden species" and often these terms are used synonymously or arbitrarily in most different meanings. With many elements adopted from BICKFORD & al. (2007), the following terminology should hopefully reduce this confusion:

(a) **Sister species** are two taxa that are derived from the same immediate common ancestor. The term only refers to genealogical relationships but does not imply any statement on phenotypic similarity – though a close phenotypic resemblance between sister species is usually apparent.

(b) **Cryptic species** are two or more species which are not separable by primary visual or acoustic perception of an expert. This reflects the immediate sense of the word and restricts such species to truly cryptic cases – i.e., to species not safely separable by training of innate pathways of the human cognitive system. Rather, their reliable identification requires the application of elaborate methods such as numeric recording and analysis of phenotypic characters, DNA analysis, biochemistry or analysis of sound spectograms. As a reasonable threshold to declare a species as cryptic, I propose a subjective expert determination error of > 10%. Cryptic species must not necessarily be the closest of relatives, though a high relatedness is usually the case.

(c) **Sibling species** in effect represent the interception of sister and cryptic species – i.e., they are derived from the same immediate common ancestor and are not separable without application of special identification methods. This conception of cryptic species deviates from that of BICKFORD & al. (2007) who wrote "We consider two or more species to be "cryptic" if they are, or have been, classified as a single nominal species because they are at least superficially morphologically indistinguishable." This rather broad concept also includes species inseparable on first glance but nevertheless easily distinguished by the eye after having trained specific recognition skills. The expression "have been classified" implies the majority of currently recognised species to be cryptic.

A detailed evaluation of the historic revision of the ant genus *Lasius* (WILSON 1955) illustrates this. Omitting taxa of which Wilson could not see specimens but rather based his decisions on interpreting original descriptions, his material account indicates access to at least 42 Palaearctic *Lasius* species. However, within this material he recognised only 16 species. Upon following BICKFORD & al.'s (2007) conception, as much as 62% of these 42 species are cryptic but many of these are, based on recent knowledge, easily separable, even for amateur entomologists. When following the restricted conception preferred here, the number of cryptic species in WILSON's (1955) material reduces to about 29%.

It is clear that delimitations between cryptic and non-cryptic in my sense must remain diffuse to some extent because training of "innate pathways of visual or acoustic perception" would not lead to identical results in different experts. This is a weakness, but in contrast to BICKFORD & al.'s (2007) idea, the present conception offers the chance for more stable estimates and better comparability of figures between different species groups when growth of taxonomic knowledge has approached the asymptotic phase.

The economy of our expectations – when alleged crypsis is a matter of fixed recognition schedules

Evolution has apparently installed or "hardwired" in our brain principles of neuronal economy which aim to avoid superfluous confusion and help us to find the quick decisions which are necessary for our survival (RIEDL 1979). One of these is the principle to extrapolate from the similar to further similarities – that is to say, all things which look, with some variation, like an apple are expected to be apples and nothing else. Translated to taxonomy this means that recognition schedules of phenotypic patterns, apparently having worked in our experience hundreds of times, are likely to become fixed in our brain. We are then blind to things freely exposed to our vision and, worse, we build up incredible resistance against learning new lessons.

The history of *Lasius* taxonomy shows exemplary cases for such a fixation. The revision of WILSON (1955) resulted in a weakly conflicting system of species delimitation which was most attractive for ant students because of the simplicity of the keys. "Scape and tibia with or without standing setae" almost always worked well in separating *Lasius* "*alienus*" and *L.* "*niger*" everywhere within the Holarctic and the revision as a whole seemed to be translucent. The glass dome of "Mount Wilson" formed a towering monument – at least in America. In Europe, some ant taxonomists soon began to correct WILSON's (1955) lumping (COLLINGWOOD 1963, PISARSKI 1975, KUTTER 1977, SEIFERT 1983) or described new species (FABER 1967, SEIFERT 1982, 1988a) in the subgenera *Chthonolasius* and *Austrolasius* – but nobody including me challenged WILSONS's (1955) concept regarding the subgenus *Lasius* s.str.

Take alone the classical example *L.* "*niger*" sensu WILSON (1955). Being most abundant in the Palaearctic and easily to collect, this ant has been the object of many investigations on ant biology, ecology, faunistics and taxonomy – I guess that more than 500 publications since Linnaeus have concerned it. During extensive field studies in a diversity of habitats in the years 1979 - 1988, I collected some 4,000 nest samples of this ant and inspected around 30,000 specimens under the microscope. The experience of thousands of determinations apparently without conflict fixed WILSON's (1955) recognition schedule in a way that even the alerting finding that gynes from woodland and bogs had much flatter mesosomas could not prompt me to have a closer look. At the same time, I also noted strange things within *L.* "*alienus*" sensu WILSON: there were brownish specimens with a weak yellowish colour component in open sandy grasslands and *Calluna* heath of Brandenburg, Sachsen and Sachsen-Anhalt (today recognised as *L. psammophilus*), more medium-brown workers in xerothermous oak forests of Sachsen (today the true *L. alienus*), and blackish-brown ants with swarming as late as September / October on semi-dry grasslands of Pontic hillsides in east Sachsen (today *L. paralienus*). Again, no investigation was started – the economy of expectations was assisted by the comfortable idea of explaining all these differences by environmental modification or intraspecific polymorphism.

The picture of a tiny pebble being gently thrown against a glass dome that is under tension and which causes it to collapse in a sudden burst is a good metaphor for what happened next. The tiny pebble was a *Lasius* sample that luckily passed the Iron Curtain in October 1989. It was sent by

Jacobus Boomsma who had collected it near Budapest. In his accompanying letter, he called these ants "Blackish alienus" and noted that they showed a strikingly different electromorph pattern for both Malic enzyme and Esterase A (see also BOOMSMA & al. 1990). A microscopic side-by-side comparison of this sample with *L.* "*alienus*" from a German *Calluna* heath showed shocking differences and mobilised all my energies for the next couple of weeks. I scrutinised my whole *Lasius* s.str. collection and a numeric analysis convinced me that the five German variants of *L. alienus* and *L. niger* each were separate morphospecies with clear-cut differences in pubescence density, pilosity distribution and length, body proportions, and ecology. WILSON's (1955) species recognition schedule had completely collapsed. Within a few weeks I learned a new view of reality which also influenced the approach to other ant genera. The woodland and bog population of "*L. niger*" was quickly recognised as being the new species *L. platythorax* and "*L. alienus*" was divided into three German species (SEIFERT 1991b, 1992b). This view has been subsequently confirmed by all European ant taxonomists.

Another example of a well-studied ant yet with late recognition of a non-cryptic two-species identity is the fungus-growing ant *Cyphomyrmex longiscapus* s.l. Found abundantly along stream embankments in the wet forests of Panama, it has become a model organism for the study of behaviour, ecology, mating frequency, cultivar specificity, pathogenesis, and social parasitism. SCHULTZ & al. (2002) found *C. longiscapus* s.l. to consistently cultivate two distantly related fungal symbionts and distinct allozyme patterns of the ants completely to correlate with DNA data of the fungi. Since both ant forms occurred sympatrically, they interpreted them to represent reproductively isolated cryptic species which are adapted to different symbionts. A subsequent morphological study demonstrated, however, very clear-cut morphological differences visible by simple eye-inspection, and SCHULTZ & al. (2002) described one form as the new species *Cyphomyrmex muelleri*.

The list of ants which are not cryptic in terms of phenotypic recognition but fell victim to the innate economy of our expectations is very long but I only mention the Neotropical ponerine *Pachycondyla villosa* which was the subject of 24 publications in different fields of biology during the years 1984 - 2002 before it was recognised to consist of three different species (LUCAS & al. 2002).

Cryptic species, intraspecific polymorphism and interspecific hybridisation – the diffuse border

Taxonomists consider species as separable units and inevitably they must decide YES or NO when giving a name, even when nature herself has not made a decision. Gradual transition is one aspect of evolution but discrete character states may also cause taxonomic problems. Setae characters, for instance, are most powerful species discriminators in wood ants of the Palaearctic *Formica rufa* group but in two members of this group, *F. pratensis* and in Fennoscandian *F. lugubris* there are intraspecific pilosity morphs with differences so strong as typically observed between "good" species (SEIFERT 1992a, 2003c). The intraspecific character of this polymorphism was concluded from the high frequency of the morphs occurring within the same nest and additionally, that the hairy morph is no socially parasitic species occurring in the nests of a less hairy host. Allozyme and DNA analyses also did not provide evidence to split up *F. pratensis* and Fennoscandian *F. lugubris* (PAMILO & al. 1992, GOROPASHNAYA & al. 2004b). In Fennoscandian *F. lugubris*, which are monogynous, the two pilosity morphs can be produced by the same queen.

The interpretational history of the size-dimorphism in *Myrmica rubra* gynes shows – in an exemplary way – how a large body of apparently harmonic observations made by different scientists may lead, via mutual reinforcement, to coincident conclusions which are accepted as a sort of established truth. The very discrete size-dimorphism is associated with a number of biological traits typical for the relation between a permanent socially parasitic species (the microgyne) and its host (the macrogyne). ELMES (1976, 1991), PEARSON & CHILD (1980), PEARSON (1981) and ELMES & BRIAN (1991) reported the following traits: (a) microgynes do not appear to occur independently of the macrogynes; (b) microgynes produce microgynes but no macrogynes; (c) microgynes may produce workers in laboratory cultures but do so only occasionally in the field; (d) macrogynes produce macrogynes but no microgynes and plenty of workers; (e) there is inhibition of macrogyne production but plenty of microgynes produced in nests parasitised by microgynes; and (f) microgynes mate intranidally whilst macrogynes fly off to external mating places. ELMES & BRIAN (1991) concluded that the microgyne population represents a different socially parasitic species. SEIFERT (1993) took up this argumentation and described the microgyne morph as the new species *Myrmica microrubra*. SAVOLAINEN & VEPSÄLÄINEN (2003) supported this hypothesis by proposing that the social parasite *M. microrubra* is an example for sympatric speciation. The forgotten suggestion of BUSCHINGER (1997) of macrogynes possibly producing microgynes then prompted a reinvestigation by STEINER & al. (2006a). They found ncDNA data not to support separate gene pools and an exceptionally large ratio of mtDNA haplotype sharing between micro- and macrogynes all over the European range of *Myrmica rubra*. Even when considering the high frequency of mtDNA paraphyly found in several ant genera (see below), the situation in *M. rubra* seems extreme and is better explained by occasional hybridisation and introgression than assuming a complicated scenario of incomplete lineage sorting and parallel evolution. The observation of BUSCHINGER (1997), who found overwintered, alate and mated (!) macrogynes in a spring nest of *M. rubra*, indicates occasional intranidal mating of macrogynes. This ethological deviation is probably the leak in the isolation of reproductive cycles of both morphs which presumably occurred several times in different areas of Europe. As a consequence, STEINER & al. (2006a) synonymised *M. microrubra* with *M. rubra*, but VEPSÄLÄINEN & al. (2006) and SEIFERT (2007) also suggested a beginning speciation process.

Discrete colour polymorphism, often seen in the form of a light vs. a dark morph, represents another problem for taxonomists. In ants, it is found, for instance, in *Myrmica* (ELMES & ABOTT 1981, SEIFERT 1988b, 2003d), *Cardiocondyla* (SEIFERT 2003a), *Formica* (SEIFERT 1997, 2003b) and *Lasius* (SEIFERT 1992b) but in none of these cases morphological and biological data suggested that colour morphs could represent separate species. Colour morphs are frequently controlled by a single gene locus (e.g., LUS 1932, BARRION & SAXENA 1987, MAJERUS 1998, ANDRES & COR-

DERO 1999, MAJUMDAR & al. 2008) and a single point mutation may be sufficient for shifts to another colour morph which illustrates the risk of using such simple characters in determination keys.

The high percentage of cryptic species in some ant genera is shown in the next section. It presents a most uncomfortable situation and a big challenge for morphology-based, genetical and other disciplines of alpha-taxonomy. This scenario is further complicated by interspecific hybridization obliterating the species borders. SEIFERT (1999) estimated as much as 11% of ant species within the well-investigated Central European fauna to hybridise occasionally or frequently with other species. Investigations since then have supported this view and added further examples (GOROPASHNAYA & al. 2004b, SEIFERT & GOROPASHAYA 2004, SEIFERT 2006a, SAAPUNKI & al. 2008), giving an up-to-date estimate of at least 14% of the 175 Central European species. Hot spots of hybridisation are certain species groups of *Temnothorax*, wood ants of the *Formica rufa* group and soil ants of the subgenus *Chthonolasius* in which hybridization may be part of an evolutionary strategy. Different cases of hybridisations have also been reported from North America – I only mention *Solenopsis* (ROSS & al. 1987, HELMS CAHAN & VINSON 2003) or *Acanthomyops* species, here with social cleptogamy as in Eurasian *Chthonolasius* (UMPHREY & DANZMANN 1998), or the fascinating scenario of symmetric social hybridogenesis in *Pogonomyrmex* harvester ants (HELMS CAHAN & KELLER 2003, ANDERSON & al. 2006, SCHWANDER & al. 2007). Traditional ant taxonomists should not only begin to recognise an unexpected number of cryptic species but also consider hybridisation as an accessory factor of evolution (MALLET 2007).

Estimates for crypsis in ants

The precondition for reasonable estimates of true crypsis are (a) availability of methods having the potential to visualise hidden biodiversity; (b) having applied these broadly in the ant group under question; and (c) background information from other fields of biology to distinguish cryptic species and intraspecific polymorphism. This qualification restrict my estimates to very few adequately studied ant groups.

The first group is the Palaearctic members of the genus *Lasius* in which WILSON (1955) distinguished only 18 species. According to the most recent state of knowledge obtained by unpublished NUMOBAT investigations in workers, about 94 Palaearctic species can be separated, 16 of these are new species and perhaps 43 species or 46% of the total are cryptic species according to my restricted conception. When reading these figures, consider the poor NUMOBAT survey and lack of broad molecular genetic studies in the Asian fauna. This suggests a larger number of cryptic species and a Palaearctic total of > 100 species. Crypsis within the best studied and largest subgenera of *Lasius* seems to have comparable ratios: 52% of the currently recognised 52 *Lasius* s.str. species and 44% of the 25 *Chthonolasius* species. The opposite extreme is absence of cryptic species within the subgenus *Dendrolasius* – if we can trust published descriptions, all eight species currently recognised (RADCHENKO 2005) should be separable without the application of elaborate methods. Let us hope that *Dendrolasius* remains taxonomically comfortable after application of high-resolution techniques.

A large proportion of the Palaearctic *Formica* species has been studied by NUMOBAT techniques (DOUWES 1979, 1981, SEIFERT 1991a, 1992a, 1996, 1997, 2000a, 2003b, 2003c, 2004b, SEIFERT & SCHULTZ 2008) and there are some DNA and allozyme analyses of alpha-taxonomic significance (PAMILO & al. 1992, CHAPUISAT 1996, GOROPASHNAYA & al. 2004a, 2004b, SEIFERT & GOROPASHNAYA 2004, GOROPASHNAYA & al. 2007). Including eleven undescribed species, NUMOBAT techniques resolve 67 Palaearctic species, 43% of which are truly cryptic. Crypsis seems to be distributed rather equally over the species groups of *Formica* but there are no suggestions so far for cryptic species within the monospecific *F. uralensis* and *F. subrufa* clades and the two Palaearctic species of the subgenus *Raptiformica*.

The world fauna of the genus *Cardiocondyla* is currently subject to a NUMOBAT investigation using more powerful methods than presented in SEIFERT (2003a). The number of recognised species has almost doubled since BOLTON (1995): as of October 2008, 77 species are recognised, 16 of which are to be described as new, whilst the curve of recognised species vs. the number of investigated samples has not yet reached an asymptote. Over the entire genus *Cardiocondyla*, the percentage of cryptic species is estimated to be 52%. Crypsis is most frequent (up to 75%) in the *C. batesii*, *C. elegans*, *C. bulgarica*, *C. nuda*, and *C. minutior* groups. The often reduced capacity of mated gynes for long-range dispersal in combination with isolation in less arid regions of desert and semidesert zones results in a high ratio of endemism and crypsis. On the other hand, some clades with exclusively Indo-Malayan primary forest distribution seem to have rather few cryptic species though extension of available sample size could possibly change this optimistic view.

The Central European fauna of the genus *Tetramorium* currently comprises approximately eleven species, as integrative approaches including NUMOBAT studies indicate (SCHLICK-STEINER & al. 2006). Five of these species have as yet no zoological name and only three species can be recognised in the worker caste by simple eye inspection with an acceptable error rate (SEIFERT 2007). This means a ratio of about 72% of cryptic species. If this value, valid for a small geographical region, proves true for the whole Palaearctic, we have an enormous problem.

EASTWOOD & al. (2006) conducted a mtDNA study of Australian *Iridomyrmex* ants attending larvae of the butterfly, *Jalmenus evagoras*, believed, so far to live with only two *Iridomyrmex* species. The analysis showed the existence of seven clades, aligned with "independent morphological determinations" but the corresponding morphological data and identification methods were not given. Among the mtDNA clades, there was a mean sequence divergence of 8.50% and within-clade divergence was 0.65%. These seven mtDNA clades were interpreted as being different closely related, ecologically dominant species having similar gross morphology and behavioural characteristics and none of these included the previously identified ant associates, *I. anceps* and *I. rufoniger* (A. Andersen, pers. comm.). Even if one assumes a certain percentage of mtDNA paraphyly and difficulties with the morphological determination of species in this large and complex group (ANDERSEN 1995), it seems that there are some 70% cryptic species sensu BICKFORD & al. (2007).

Assessing alpha-taxonomic tools for identification of cryptic species

Any biological information on an organism may have relevance in detecting a cryptic species. In the following sections, I try to assess the alpha-taxonomic value of methods originating from very different fields of biology. This cannot be a complete story – some further sources of information such as host species, symbionts or zoogeography are not treated. The sequence of treatment gives some sort of ranking according to my personal view, although integrative, multi-source taxonomy is always the best approach.

MOBAT and NUMOBAT – the backbone of integrative taxonomy and the only link to Zoological Nomenclature

Morphology-Based Alpha-Taxonomy (MOBAT) is the only discipline providing the link of biosystematic research to Zoological Nomenclature from 1756 up to the present (SCHLICK-STEINER & al. 2007) and Numeric Morphology-Based Alpha-Taxonomy (NUMOBAT) is the only way to do this in a testable, verifiable form, fitting the standards of modern natural sciences. NUMOBAT is any form of investigation that describes morphological traits numerically. This may be, for instance, simple statistical analysis of single characters, complex multi-character discriminant analysis or geometric morphometrics – independent if the data originate from direct evaluation of specimens or their pictures.

The provocative sentence that DNA analysis is incapable of establishing the link to Zoological Nomenclature needs explanation. Any species identification during taxonomic revisions is done by referring either to the type specimen or the original description of a taxon. These descriptions, accumulated during 250 years after Linnaeus, almost always consider external morphology – genetic information has been included only very recently. One and a half million animal taxa have been described in this way and this language is only understood by MOBAT. Identifying and describing cryptic species inevitably requires the direct investigation of type specimens and this investigation must be non-destructive. Though extraction of DNA from freshly collected arthropod material seems possible with rather little damage to external morphology (ROWLEY & al. 2007), the problem of non-destructive and yielding extraction from dried, older arthropod vouchers (i.e., from the vast majority of existing animal type specimens !) remains unsolved. To sacrifice, for instance, a small spot of foot epithelium of a large-bodied bird type represents no problem but no responsible curator of a museum collection would allow to destroy types of small-bodied arthropods, more especially knowing that sequencing will often fail because of DNA degradation. The clear conclusion is: a non-destructive investigation of type specimens and verifiable identification of cryptic species is only possible by NUMOBAT techniques. If we want to maintain the actual system of Linnean Nomenclature and a common language in communication between different disciplines of bioscience, DNA taxonomy cannot replace MOBAT. Moreover, the proper long-term preservation of DNA from freshly collected vouchers, which would be mandatory if DNA taxonomy took over, remains largely unexplored (cf. QUICKE & al. 1999, KING & PORTER 2004).

The irreplaceable nomenclatural function of morphology is one aspect – the other is the high performance of NUMOBAT in the detection of cryptic biodiversity. The flow of information between working groups detecting cryptic biodiversity by methods of molecular biology and me personally (as a MOBATist) has now lasted for 18 years and within this period there was only one multi-character NUMOBAT analysis which failed to demonstrate a cryptic species as suggested by molecular and chorological data: *Tetramorium* sp. B (SCHLICK-STEINER & al. 2006) which is a putative sibling species of *Tetramorium caespitum*. NUMOBAT is predicted to be less powerful than ncDNA data in identification of F_1 hybrids between structurally very similar species and should fail in detecting backcrosses of such F_1 hybrids with a parent. We have so far no convincing tests of this prediction because cross-validations of NUMOBAT against reliable ncDNA systems are almost missing in ants. In contrast, hybrids between less similar species are clearly demonstrated by morphology (e.g., SEIFERT 1984, SEIFERT 1999, SEIFERT 2006a).

One test of NUMOBAT versus a powerful ncDNA system does however exists: a 16-character NUMOBAT system offers a 100% separation of *F. polyctena* and *F. aquilonia* in a critical Leave-One-Out-Cross-Validation Discriminant Analysis (LOOCV-DA) across their whole Palaearctic range and placed a Finnish hybrid population *F. polyctena* × *aquilonia*, proposed following ncDNA and mtDNA data, between the clusters of the parent species (SAAPUNKI & al. 2008).

Apart from weakness in difficult cases of hybrid identification, the dialogue of NUMOBAT systems with molecular systems of other authors has been a success story. Cryptic species of *Lasius alienus* first identified by allozymes (BOOMSMA & al. 1990) were duly confirmed (SEIFERT 1992b), two sibling species of *Formica lugubris* first indicated by allozyme data and pupae carrying tests (PAMILO & al. 1992, ROSENGREN & al. 1994) were established (SEIFERT 1996), whilst two siblings of *Temnothorax nylanderi* first identified by allozyme data (P. Douwes, pers. comm. 1989) were also demonstrated (SEIFERT 1995) and later confirmed by genetic systems (PUSCH & al. 2006).

The initial information on cryptic species can also flow from the morphologist to the molecular biologist: my own NUMOBAT investigation of 1990 showing that British *Temnothorax* "*tuberum*" is not true *T. tuberum* but the same as continental *T. albipennis*, prompted allozyme and karyotype studies that fully upheld this view (FRANKS & al. 1992, ORLEDGE 1998). In addition, the sibling species *Formica foreli* and *F. pressilabris*, first demonstrated by NUMOBAT (SEIFERT 2000a), were later confirmed by DNA analysis (GOROPASHNAYA 2003), whilst the NUMOBAT separation of the cryptic species *Cardiocondyla mauritanica* / *C. kagutsuchi* / *C. atalanta* and *C. obscurior* / *C. wroughtoni* (SEIFERT 2003a) was confirmed by an analysis of several mtDNA genes (HEINZE & al. 2005 and J. Heinze, pers. comm.). A successful synergy of NUMOBAT with genetics was also given in Palaearctic ants of the genus *Lasius* (STEINER & al. 2004), of the *Formica rufa* group (GOROPASHNAYA & al. 2004a, 2004b, SEIFERT & GOROPASHNAYA 2004) and in European and Asian *Tetramorium* species (STEINER & al. 2006b, SCHLICK-STEINER & al. 2006).

Isolated NUMOBAT approaches without cross-reference to genetic or biochemical systems dominate in taxonomy but, anyway, the sum of both integrated and isolated NUMOBAT studies makes up only a tiny fraction of the taxonomic ant literature. ELMES (1978) was probably the

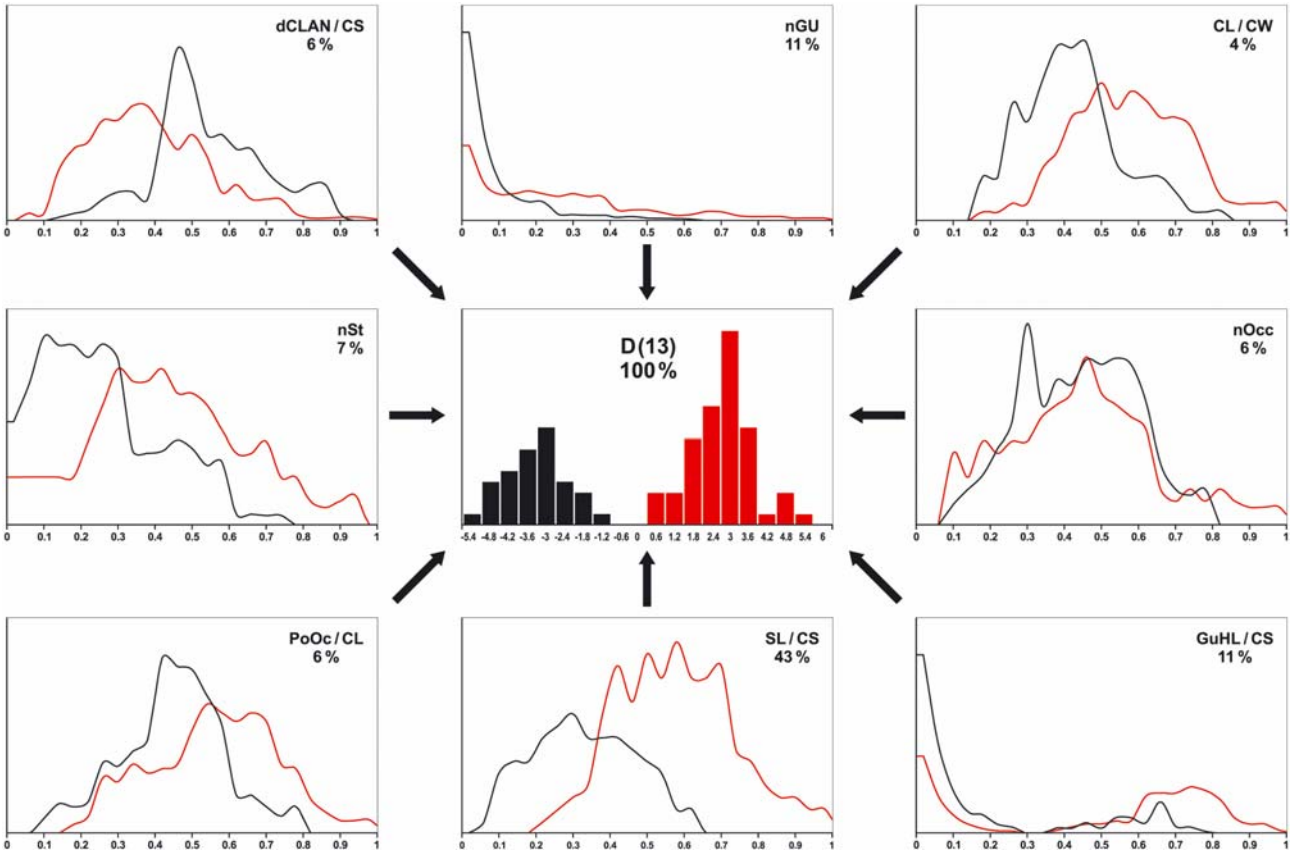

Fig. 1: Results of a 13-character discriminant analysis for 91 nest sample means (central histogram) and frequency distribution of the eight most discriminative of these characters for 247 worker individuals (peripheral diagrams) of *Lasius lasioides* (red lines and bars) and *L. barbarus* (black lines and bars); percent values indicate the number of cases outside the overlap range.

first to apply canonical discriminant analysis in ant taxonomy in separating three *Myrmica* species, whilst DOUWES (1981) applied such a system to distinguish Swedish species of the *Formica rufa* group, although in both cases the species considered were not cryptic. The following published examples for successful application of multi-character NUMOBAT for demonstrating cryptic or very similar species can be added to those already reported in the former section – eleven species in *Myrmica* (SEIFERT 1988a, 2000b, RADCHENKO & al. 2002, SEIFERT 2005), nine species of the subgenus *Serviformica* (SEIFERT 1997, 2004a, 2004b, SEIFERT & SCHULTZ 2008), four species in the subgenus *Coptoformica* (SEIFERT 2000a), three species of *Pachycondyla* (LUCAS & al. 2002), two species of *Ponera* (CSŐSZ & SEIFERT 2003), two species of *Hypoponera* (SEIFERT 2004a), four species of *Temnothorax* (SEIFERT 2006b), two species of *Cardiocondyla* (SEIFERT 2008a) and four species of *Tetramorium* (CSŐSZ & al. 2007). NUMOBAT has also been used to show intraspecific polymorphism in *Formica* ants (SEIFERT 1992a, 2003c) or in *Tetramorium* (SCHLICK-STEINER & al. 2005). The list of cases in which NUMOBAT rejected the existence of alleged interspecific differences is long – one classical example is the synonymisation of *Formica tamarae*, *F. naefi* and *F. goesswaldi* with *F. foreli* and of *F. rufomaculata* with *F. pressilabris* (SEIFERT 2000a).

The examples presented above are suggestive but do not really give a measure of the discriminatory power of NUMOBAT. This can be communicated if we consider the best studied ant fauna worldwide – that of Central and North Europe. For this region, the author's data files on numeric characters currently include the genera *Camponotus*, *Crematogaster*, *Formica*, *Hypoponera*, *Lasius*, *Leptothorax*, *Myrmica*, *Myrmoxenus*, *Plagiolepis*, *Ponera*, *Tapinoma*, *Temnothorax*, and *Tetramorium*. Differing from genus to genus, the number of characters recorded ranges from 6 to 32. Excluding workerless species, the species of these genera total 129 species. When only worker determination is considered, 34 - 43% of the species are cryptic or easily confused. The numeric data sets allow 1097 pairwise, intrageneric species separations. As much as 99.7% of these discriminations were performed by a canonical DA with a confidence > 98%. Only two analyses clearly failed: a 31% error rate in the two above mentioned *Tetramorium* siblings and one of 7% in *Lasius balcanicus* vs. *L. distinguendus* which, however, are easily separable by the gynes.

SEIFERT (2002) described several procedures to reduce errors during numeric stereomicroscopic data recording and gave recommendations on the equipment to be used. SEIFERT (2008b) assessed which forms of data processing might be advantageous to achieve the highest resolution in discriminant analyses and where they have no importance. Most remarkably, calculation of nest sample means in combination with discriminant analysis frequently offers surprisingly clear-cut separations when primary data sets of worker individuals appear as a hopeless case (Fig. 1). With-

in 247 worker individuals of the sibling species *Lasius lasioides* and *L. barbarus*, the most discriminative character, scape length ratio, separates only 43% and each of the remaining twelve characters less than 12% of cases. In contrast to this, a DA based upon 91 nest sample means of these 13 characters separated 100% of the cases with an error rate of 0% (primary DA) or of 1.1% (pessimistic LOOCV-DA). Dozens of similar examples from other ant genera could be added.

However, the splendid performance of such NUMOBAT techniques is clouded by a disadvantage: they are currently performed only by direct manual microscopy. This is enormously time-consuming and physically exhausting. Recording a 18-character data set in a *Temnothorax* worker by an experienced scientist requires some 40 minutes of working time. This limits the wider application of this system in the course of large taxonomic revisions. Assuming only 33% of the 600 Palaearctic *Temnothorax* species to be truly cryptic and a minimum need of 30 three-worker nest samples per species, we end at the incredible working time of 1050 ten-hour days. The way to overcome this dilemma is developing a microscopic system of automated character recognition and analysis (ACRA) based on geometric morphometry.

A feasibility study in preparation of an intended research project (B. Seifert, unpubl.) estimates that a landmark-based ACRA, using two-dimensional z-stack images, would allow measuring a 65-fold number of characters within ± 63% of the working time compared to manual non-geometric morphometry. Explicitly, ACRA of dorsal head and dorsal and lateral mesosoma of a *Temnothorax* ant could fix, with the pre-processed pattern vectors, some 85 geographic landmarks within a total working time of 25 minutes per specimen. This estimate refers to a semiautomatic system and the working speed of the z-stack technology currently available. Some 65% of the characters can be recognised in a fully automatic way but extension of this system to the complete character set requires enormous programming expense, long-term payment of high-calibre image processing experts and long testing series. As for 3-dimensional recording of landmarks, there is presently no technology available that allows processing of small arthropods within an acceptable working time. Apart from these current confinements, it is a realistic hope that a semi-automatic two-dimensional system will become available in the near future. Once such methods have been established for routine work, scientists can be unburdened by delegating these tasks to technicians. I see no alternative to ACRA-systems if NUMOBAT is to keep pace with the expected development of modern DNA analysis.

Analysis of ncDNA – offering a big future when adequate systems are available

Nuclear DNA directs the biological identity of a species and accordingly appropriate ncDNA systems are in better agreement to species trees or morphological species delimitations than mtDNA barcoding (e.g., SOTA & VOGLER 2001, SHAW 2003, MENDELSON & SHAW 2005, KNADEN & al. 2005, WELLS & al. 2007, WILD 2009). Analysis of ncDNA is apparently in a process of orientation and self-discovery. There are presently very few nuclear genes known with equal or higher level of variability than shown in mtDNA genes. However, ncDNA most probably will develop in future to become the most informative alpha-taxonomic molecular genetic indicator and will outperform mtDNA when laboratories are prepared to evaluate large numbers of nuclear genes. The latter is not only required to increase resolution but also to reduce the noise of data sets mainly caused by incomplete lineage sorting and null alleles. Null alleles can be detected in ants if there are also males available but this is rarely given.

NcDNA is currently mostly applied for higher classification of ants – among the genes used are 18S rDNA, 28S rDNA, abdominal-A, long wavelength rhodopsin, wingless, EF1-alpha F1 and EF1-alpha F2 (WARD & al. 2005). The majority of these markers obviously have a coarse resolution making them adequate to describe meta-phylogenies and there is so far only one published example indicating their alpha-taxonomic value in ants (WILD 2009). The introns of protein-coding genes are typically more variable than exons and they show some promise of being useful in alpha-taxonomy.

The situation with regard to the high-resolution ncDNA markers, such as microsatellites, is different. These have been shown to be most powerful tools within a regional context but application over a wide geographic area as it is mandatory for alpha-taxonomy often remains problematic in closely related species even after enlarging the number of markers.

Published examples of applying ncDNA in alpha-taxonomy of ants are to date few. These mainly involve microsatellites, occasionally ITS1 or ITS2 (STEINER & al. 2006a, WILD 2009) but regrettably not the promising AFLP method so far. The distinctness of the sibling species *Formica lugubris* and *paralugubris* using microsatellite markers within a Swiss population (CHAPUISAT 1996) was shown after allozymic, ethological and morphological evidence had demonstrated their heterospecificity (PAMILO & al. 1992, ROSENGREN & al. 1994, SEIFERT 1996). MACARANAS & al. (2001) used five microsatellite loci to demonstrate two morphologically similar species of the Australian *Camponotus ephippium* complex. This is the first documented case in which ncDNA data primed the recognition of unknown species but it remains unclear if these species were cryptic. GOODISMAN & HAHN (2005) separated three morphologically and biologically separable forms of *Camponotus festinatus* in the region of Tucson, Arizona using five polymorphic DNA microsatellite markers. Microsatellites have also been used to differentiate the sibling species *Lasius neglectus* and *L. turcicus* and the highland and lowland forms of the latter species in Anatolia (CREMER & al. 2008). These socially, ecologically and morphologically different forms are apparently in the process of divergence and can be considered either as subspecies or species in statu nascendi. WILD (2009) used combined evidence from the nuclear loci wingless, long wave rhodopsin and ITS2 to separate 16 Neotropical *Linepithema* species.

Analysis of ncDNA is probably the only tool to disentangle the most complicated scenarios of interspecific relationship. SCHWANDER & al. (2007) revealed a system of reproductively isolated genetic lineages in harvester ants of the *Pogonomyrmex* cf. *barbatus* and *P.* cf. *rugosus* species complex by a study of eight microsatellite markers. Eight of these lineages are of hybrid origin, can reproduce by symmetric social hybridogenesis only with a single complementary line, but probably are reproductively isolated from

the six other lines and from the lines of their putative parent species. This scenario is a Gordian Knot for each existing species conception and there is possibly no chance to realistically depict the situation by NUMOBAT.

Cuticular hydrocarbons and pheromones – good indicators with application problems

Weakness or absence of visual cues and lack of bioacoustic, air-borne signalling in intra- and interspecific recognition in ants led to an increase of information transported via biochemical systems – in ants these are cuticular hydrocarbons (CHCs) and other pheromones. CHCs have a number of functions in insects: water-proofing, protection from predators, chemical communication, thermal insulation, reproductive isolation and kin and species recognition. They are stable end-products of biosynthetic pathways endogenous to insects (BLOMQUIST & DILLWITH 1985), are expected to be species-specific (LOCKEY 1991, HOWARD 1993) and have been shown to be largely genetically determined (FERVEUR 1991, KAIB & al. 1991, PAGE & al. 1991, COYNE & al. 1994, FERVEUR & JALLON 1996, COYNE & al. 1999). Growing experimental evidence suggests that ant CHC compositions are not only species- but also colony- and caste-specific (SINGER 1998). This complicates their use – the more as chemo-taxonomic studies on insects often result in similar qualitative profiles with only quantitative differences (LOCKEY 1991, El MESSOUSSI & al. 1994). Some of these problems may be attributed to non-specific factors such as age, diet, habitat and other environmental factors (TOOLSON 1982, VANDER MEER & al. 1989, DAHBI & al. 1996, BUCZKOWSKI & al. 2005) and require a careful experimental design.

In spite of these general objections, CHCs have a most promising alpha-taxonomic value in ants. Like any phenotypic character that is a direct expression of nuclear DNA, CHCs are true indicators of species identities – and they have the resolution to demonstrate cryptic species or hybrids. This has been shown first in the imported North American *Solenopsis* species (VANDER MEER & al 1985, VANDER MEER 1986). A measure of the reliability of CHCs is their high correlation with morphology, as shown for cryptic species of the Neotropical *Pachycondyla villosa* complex (LUCAS & al. 2002), of European *Tetramorium* siblings (SCHLICK-STEINER & al. 2006), and in the discrimination of sibling species *Lasius neglectus* and *L. turcicus* and the two forms of the latter species (CREMER & al. 2008).

The chemical composition of pheromone glands has been rarely used as a tool for alpha-taxonomy in ants. Nevertheless, the few published attempts to separate related species seem encouraging. BILLEN & al. (1983) and BERGSTRÖM & LÖFQVIST (1968) found striking qualitative differences in the Dufour gland content between *F. cunicularia* and *F. rufibarbis*. CAMMAERTS & al. (1985) and MORGAN & OLLET (1987) showed significant qualitative differences in the trail pheromones of *Tetramorium caespitum* and *T. impurum*, whilst JACKSON & al. (1991) used Dufour and mandibular gland pheromones to detect an undescribed species of *Myrmica*. For eight American and 15 African *Monomorium* species, JONES & al. (1982) and JONES & al. (2003) found interspecific differences in the alkaloids of the venom gland to be expressed both by variation of relative amounts and presence / absence of certain substances. JONES & al. (1988) concluded from venom gland alkaloid composition that *Monomorium* "*antarcticum*" from New Zealand should be split into a minimum of four cryptic species but – as with all these investigations – there was no background information on morphology.

For *Solenopsis* species, DESLIPPE & GUO (2000) reported venom alkaloid composition to differ in the worker with age but not with size and JONES & al. (1996) also showed differences between workers and queens of this genus. Apart from these data, comprehensive investigations are still lacking on how the chemical composition of exocrine glands varies with age and physiological condition. Such a noise of data sets seems likely (in analogy to the situation in CHC) and calls for uttermost care when using them as taxonomic discriminators. The complex methodology of biochemical analysis and the need for living material will prevent a wider application in the alpha-taxonomic context.

Allozyme analysis – the reliable "old friends"

Electromorph patterns even of multiple allozyme studies provide a lower information content than DNA analysis. Nevertheless, allozyme studies have played (and continue to play) a productive role in biodiversity research. In contrast to the omnipresent mismatches of mtDNA barcoding in relation to species identities (see next section), few if any published allozyme analyses produce such confusion. Allozymes are encoded by the recombinant nuclear genome which explains their strong correlation with morphology, CHCs or ncDNA, their close affinity to fine-scale evolution and their indicative value in case of hybridisations (PEARSON 1983, DOUWES & STILLE 1991, PUSCH & al. 2006).

Within the European ant fauna, there are several cases in which allozyme analyses initiated the later identification of cryptic species by NUMOBAT approaches. Thus BOOMSMA & al. (1990) initiated SEIFERT (1992b) in cryptic species of *Lasius*, PAMILO & al. (1992) primed SEIFERT (1996) in cryptic species of *Formica* and P. Douwes (pers. comm. 1989) SEIFERT (1995) in sibling species of *Temnothorax*. An example of a fine correlation of allozyme pattern, morphology and cuticular hydrocarbons is given by LUCAS & al. (2002) for three cryptic species of the Neotropical P*achycondyla villosa* complex. In the following I discuss several allozyme studies from America or Australia, probably indicating cryptic species but problematic in interpretation.

Allozymic and nest-form differences have been demonstrated between colour "forms" of the Australian meat ant *Iridomyrmex purpureus* (HALLIDAY 1975, GREENSLADE 1974, HALLIDAY 1981). These authors found eight colour forms which were correlated with certain social types and ecologies but were "not separable by clear-cut morphological differences". Absence of common alleles in sympatry in several of these colour forms indicated reproductive isolation. These forms were later taxonomically described in the rank of species by SHATTUCK (1993) who used largely verbal descriptions of worker integument colour and iridescence for separating the species. He expressed the view that morphometry was of restricted alpha-taxonomic value in this species group but did not apply multi-character NUMOBAT methods.

CROZIER (1977) investigated allozymes and karyotypes in different colour forms of the Nearctic *Aphaenogaster rudis* complex. He distinguished a lighter coastal phenotype, fixed for a null allele of esterase and having a haploid chromosome number of n = 20, a darker mountain pheno-

type with different highly active esterase alleles and n = 22, and another mountain form with n = 18 that differed from the sympatric n = 22 karyotype also by a marked microhabitat segregation and by allele frequencies in all four loci examined. Mean allozyme genetic differences within the populations of these three entities were 0.03, whereas differences between the entities were 0.19 – a value typical between already recognised *Aphaenogaster* species. CROZIER (1977) wrote: "The most likely explanation of the observed associations between alleles and colour forms seemed to be that the colour forms are in fact sibling species." Unfortunately, we have so far no confirmation by a NUMOBAT or ncDNA study – a later NUMOBAT study of UMPHREY (1996) was not designed to give such proof. He studied ten forms of the North American *Aphaenogaster fulva-rudis-texana* complex by a 12-character discriminant analysis but the *a priori* hypotheses in his DA were largely based on chromosome numbers and not on allozyme indication. He found correlations between chromosome numbers and morphometry but 19% of the determinations differed from the *a priori* hypotheses. It remained unclear if intraspecific karyotype polymorphism, inadequate morphometrics or both these factors were responsible for this mismatch.

WARD (1980) studied 22 allozyme loci in the Australian *Rhytidiponera impressa* group and found clearly higher F_{ST} estimates within *R. confusa* (0.294) and *R. chalybaea* (0.380) than those reported for most outbreeding animals and the genetic cluster analysis showed a deep intraspecific branching. These findings suggested the existence of cryptic species but the clues were not followed further at this time – in particular there were no attempts to find a morphological correlate. The linking of genetics with morphology was then done by CROZIER & al. (1986) who used allozyme evidence to separate about 17 putative Australian *Rhytidiponera* species and found a high correlation with numeric morphological data. An extended NUMOBAT analysis and nomenclatoric treatment of these proposed entities is still missing.

ROSS & TRAGER (1990) studied allozymes in six mostly allopatric Neotropic *Solenopsis* species and found each morphologically determined nominal species separable from the others by unique suites of alleles at one or more loci. The conception of WILSON (1952) that most of the observed fire ant diversity in South America represents geographical variation within a single widespread polytypic species was clearly rejected. Unfortunately, the MOBAT methods of ROSS & TRAGER (1990) were inadequate to detect cryptic species – using a dissecting microscope with a magnification of only 25×, they performed a subjective assessment of colour pattern, of shape of head and of proportions, shape and surface sculpture of postpetiole.

ROSS & SHOEMAKER (2005) reinvestigated the electromorph pattern of seven allozymes and a CO1 gene fragment of mtDNA in three of these species (*S. invicta*, *S. richteri*, and *S. quinquecuspis*). Morphological determinations correlated completely with electromorph composition of GPI whilst the NJ tree of mtDNA (given in their fig. 3) was chaotically paraphyletic. Regrettably, the methods of morphological species identification were only vaguely given in the paper (these identifications were done by J. Pitts who was not included as a coauthor). Apparently there was no NUMOBAT approach: indirectly concluded from PITTS & al. (2005), a cladistic system of YES / NO characters, considering also sexuals and larvae was applied. However, I personally do not know a single pair of cryptic species in ants to be separable with simple cladistic YES / NO characters – if so, the species are unlikely to be cryptic.

mtDNA Barcoding – the one-eyed cyclops

In its current meaning, DNA barcoding is a large-scale screening of a mtDNA reference gene in order to assign unknown individuals to species and to discover new species. The belief in the power of this method was at one time in the recent past so strong that some authors demanded that "DNA taxonomy" should completely replace morphological methods (TAUTZ & al. 2002, 2003, MARKMANN & TAUTZ 2005). mtDNA barcoding has received an enormous public response ranging from popular media (e.g., The Times: HENDERSEN 2005, Der Spiegel: EVERS 2007) to top-ranking science journals (e.g., Nature: BLAXTER 2003). Apparent reasons for this echo are its propagandised and indeed impressive simplicity and the familiarity with the real product-barcoding in today's supermarket world (cf. SPERLING 2003, MORITZ & CICERO 2004). There is no doubt that the idea of barcoding has deeply infiltrated science funding and policy all over the world and obviously the Consortium for the Barcoding of Life presently acts as a big money aspirator. The advantage of mtDNA barcoding compared to currently applied ncDNA markers is procedural simplicity and a speed of molecular change just adequate for most alpha-taxonomic purposes. Such mtDNA trees allow a quick formation of hypotheses on possibly existing cryptic species and the system allows comparison of nucleotide sequences over geographic distances of some 10,000 km.

However, is mtDNA barcoding really such a good tool to discover cryptic species and what is its general alpha-taxonomic significance? Opposition against barcoding is not new and it is prominent. There are a series of general problems afflicting work with mtDNA on several levels. The most important source of error is paraphyly, mainly induced by incomplete lineage sorting or hybridisation (NICHOLS 2001, BESANSKY & al. 2003b, FUNK & OMLAND 2003, BALLARD & WHITLOCK 2004, KOCHER 2004, SEIFERT & GOROPASHNAYA 2004, HURST & JIGGINS 2005, LORENZ & al. 2005, MEIER & al. 2007) while nuclear-mitochondrial pseudogenes (NUMTs, BENSASSON & al. 2001) seem to be less frequent. NUMTs have been found in almost all groups of animals and easily remain undetected if a study is not controlled or guided by a skilled MOBATist or a discriminative ncDNA system. NUMTs can be detected by ambiguous base calls at variable codon positions when carefully scrutinising sequence diagrams but the only conclusive method to demonstrate a NUMT are laborious transcription experiments – these procedures are surely an obstacle to using mtDNA barcoding as a routine method for the mass screening of samples.

Paraphyly of mtDNA is most abundant in animals. Analysing 584 studies of 526 Eumetazoan genera with 2319 species, FUNK & OMLAND (2003) found paraphyletic mtDNA haplotypes in 23% of species. In birds, the group for which probably the best taxonomic knowledge exists worldwide, 17% of species deviated from mtDNA monophyly. In a letter to the author (29 January 2008), Kevin Omland suggested this ratio to be a gross underestimation. He wrote: "... Published studies of Australian bird phylogeography

and speciation include 18 species that were tested for mitochondrial paraphyly (studies that did not include any outgroups do not test for paraphyly and were excluded). Eight of these eighteen species revealed species-level paraphyly in their mtDNA gene trees, or 44% of the bird species evaluated. This number is much higher than the 17% reported in the study of Funk and Omland (2003) that included 331 bird species worldwide (Fisher's exact test $p < 0.01$). One possible cause for the difference is that Funk and Omland also included phylogenetic studies of genera and families with only two to four individuals per species meaning a very low chance of revealing paraphyly ..."

Low sample size per species will also reduce the chances of detecting mtDNA paraphyly in ants. Studies with extensive sampling are exceptions. SEIFERT & GOROPASHNAYA (2004) analysed mtDNA from as many as 128 samples of the ants *Formica pratensis* and *F. lugubris* from their whole Palaearctic range and found 14% of paraphyletic samples. This mismatch was detected by a parallel NUMOBAT analysis having an error rate of only 0.4%. It should be emphasised that this paraphyly occurred in distantly related ants: they had already diverged by the mid Pleistocene (GOROPASHNAYA & al. 2004a) with *F. lugubris* and its related species *F. paralugubris* and *F. aquilonia* belonging to the boreo-montane zone whilst *F. pratensis* is a thermophilic species of the woodland-steppe with clearly deviating morphology and biology.

Estimation of average genus-specific ratios of mtDNA paraphyly is problematic because this requires a testable and discriminative supervising system as given in the *F. lugubris* vs. *pratensis* example. A bad, untestable MOBAT could lead to an overestimation of mtDNA paraphyly (FUNK & OMLAND 2003, VOGLER & MONAGHAN 2007) but, in the other extreme, underestimations could also result because such a MOBAT could opportunistically accept species delimitations proposed by barcoding. Which data do we have so far in ants?

EASTWOOD & al. (2006) investigated *Iridomyrmex* ants attending larvae of the butterfly *Jalmenus evagoras* by sequencing a 585 bp segment of mtDNA. I cite one key sentence of this paper, namely "Genetic data grouped *Iridomyrmex* ants into seven clades which aligned with independent morphological determinations." This sentence and the colouration in fig. 2 claim a perfect matching of mtDNA and morphology but simultaneously, the authors write of "difficulties of identifying morphologically similar *Iridomyrmex* ants at the species level in this large and complex group". In fact, this big paper does not provide any information on the methods of morphological identification used and nowhere does it give data on how good the correlation between mtDNA and morphology really was. To believe in this zero paraphyly we must have testable data.

MCARTHUR & LEYS (2006) studied the Australian *Camponotus maculatus* group by a parallel investigation of the CO1 mtDNA gene and DA of five morphometric characters. The six Australian mtDNA clades P, Q, R, S, T, and U had been collected from only a single and very small geographical area (maximum within-spot distance only 15 km in P; in the other four clades much smaller) and there were only 9, 3, 3, 2, 7, and 4 specimens genetically investigated per clade, respectively. Such small sample sizes beg the question if a credible genetic analysis was possible. The DA of morphometric characters could not confirm the six mtDNA clades – only the most distinctive species (clade U) was resolved but presented pictures of the ant workers and verbal statements suggest a minimum of three morphologically clearly separable species. It remains unclear if this strong mismatch between mtDNA and morphology is explained by: (1) selection of inadequate morphometric characters; (2) strong error of morphometry; (3) mtDNA paraphyly; or (4) a misguided genetic analysis.

The interpretation of the following examples seems to be clearer. GOROPASHNAYA & al. (2004a) found mtDNA paraphyly in 37% of eight investigated species of West Palaearctic *Formica rufa* group ants. This ratio is probably close to reality since all suspicious samples were checked by NUMOBAT systems with proved performance and is remarkably high considering that only 25% of the species were represented by five or more sequenced specimens. SHOEMAKER & al. (2006) found mtDNA paraphyly in 31% of 14 species of the *Solenopsis saevissima* group if referenced to a subjective MOBAT system. A possible overestimation of paraphyly by a weakly discriminative MOBAT should be compensated by the fact that only 43% of the species were represented by five or more sequenced individuals. FISHER & SMITH (2008) found mtDNA paraphyly in 37% of eight species of Malagasy *Anochoetus* and *Odontomachus* when referenced to a subjective MOBAT system. Sample size was sufficient in all but one species and there remains the question if MOBAT or mtDNA would better reflect the species identity. Paraphyly of mtDNA, however, is clearly documented by *A. goodmanni* and *A. boltoni*, two morphologically very different species, which showed a chaotic haplotype pattern. KNADEN & al. (2005) found agreement of morphology and ncDNA data but a mtDNA paraphyly of 33% in three sufficiently sampled Tunisian *Cataglyphis* species. In 16 Neotropical *Linepithema* species, total evidence from three nuclear genes correlated well with MOBAT data but mtDNA paraphyly was 19% – a very high ratio against the background of insufficient sampling in 81% of species (WILD 2009). A low ratio of 16% of mtDNA-paraphyletic species is suggested by a NUMOBAT supervision for 19 *Cardiocondyla* species (HEINZE & al. 2005) but in this particular study only 26% of the species were represented by ≥ 5 sequenced individuals.

Even if the lower of these estimates should apply, it is clear that the linking of mtDNA with species identities is too loose to use it as a leading alpha-taxonomic tool. Phenotypic traits are almost entirely encoded by nuclear rather than mitochondrial genes. Furthermore, because of the probabilistic nature of inheritance, the distribution of variation at any single gene may not reflect patterns of ancestry of the majority of nuclear genes (and thus may not reflect species boundaries); this is especially true in recently diverged lineages. These basic considerations may explain why parallel investigations found high correlations between morphology and ncDNA but incongruence of mtDNA to both these systems (SOTA & VOGLER 2001, SHAW 2003, KNADEN & al. 2005, MENDELSON & SHAW 2005, WELLS & al. 2007, WILD 2009).

The above mentioned ratios of mtDNA paraphyly are particularly high in genera with much interspecific hybridisation – certain species groups of *Solenopsis* and the *Formica rufa* group are exemplary. It was quoted above that at least 14% of the 175 Central European ant species hybridise and there is no logical argument to believe that ant fau-

nas of other Holarctic regions should behave in a different way. mtDNA barcoding, using a single-strand, non-recombinant DNA sequence with matrilinear inheritance, cannot indicate hybridizations.

After having presented so many arguments against mtDNA barcoding, the question remains: Making further use of this method? The answer is that it should be replaced, once ncDNA systems adequate for large-scale alpha-taxonomic purposes will be available in the working routine. Nevertheless, information provided by mtDNA is actually still of considerable use in the context of multi-source integrative taxonomy. mtDNA can help to discover the history of hybridisation or to estimate divergence times but there must be control systems that filter out confusing information.

Karyotypes – suggestive but ambiguous

Karyotype polymorphisms are considered as factors promoting speciation (KAINA & RIEGER 1979) which suggests them to possibly have significance for species delimitation (IMAI & al. 1977). The real situation, however, is ambiguous and thus they probably should not be used for alpha-taxonomic decisions – yet they may give a signal to start further investigations with other taxonomic tools. CROZIER & al. (1986) found in 17 putative *Rhytidoponera* species differences in chromosome number not to correlate well with allozymic and morphological ones, indicating that the speed of karyotype change within a genus can be highly variable. There are genera or species complexes in which karyotypes gave the first indications for heterospecificity – for example in *Myrmecia* (CROSLAND & al. 1988) or in the Nearctic *Leptothorax muscorum* species complex (LOISELLE & al. 1990). On the other hand, there are numerous reports for intraspecific karyotype polymorphism (CROZIER 1975, IMAI & al. 1977, ROSENGREN & al. 1980, HAUSCHTECK-JUNGEN & JUNGEN 1983, CROZIER & al. 1986, FISCHER 1987, LOISELLE & al. 1990, BUSCHINGER & FISCHER 1991). Some of these karyotype polymorphisms may possibly refer to cryptic species but, due to missing knowledge of the particular species involved, I am unable to make a qualified assessment. However, at least in the European karyotype-polymorphic species *Manica rubida*, *Myrmica sulcinodis*, *Aphaenogaster subterranea*, *Leptothorax muscorum, L. kutteri, L. pacis*, and *Formica truncorum*, I see no suggestion from various sources of information for further splitting these taxa into cryptic species. The question if karyotypes have ever helped to detect truly cryptic species cannot be answered clearly. In the *Myrmecia pilosula* complex, five species were distinguished on the basis of karyotypes ranging between 2n = 2 to 32 (CROSLAND & CROZIER 1986, CROSLAND & al. 1988, summarised in IMAI & al. 1994). These separations were basically supported by a later mtDNA study (CROZIER & al. 1995). The five species plus one hybrid were said to be "cryptic species differing minimally in morphology" but there were no attempts made to separate these species with powerful NUMOBAT methods and there was no objective information how "cryptic" the involved species really were. The verbal description of one of these species (TAYLOR 1991) suggests very clear morphological differences to related species.

Ethology – the first signal

Behaviour is largely genetically determined but it is also the most plastic "organ" of an animal when environmental conditions change – with the exception of signal and mating behaviour which cannot vary too much. Ethology may give the first suggestions to cryptic ant species. ELMES & al. (1994) distinguished a type A with "rather timid workers living in shallow nests among vegetation roots" and a type B that was "more aggressive when disturbed and lived in deeper nests" within the *Myrmica scabrinodis* ants of the Netherlands, Spain and France. According to these authors, the morphology of these ants was identical. The idea of two cryptic species in *M. scabrinodis* is also supported by an analysis of microsatellites: J.R. Ebsen (pers. comm.) found two syntopically occurring, genetically separated populations of this ant in meadows but S. Csősz (pers. comm.), when investigating these samples by a discriminant analysis of external morphology, was not able to corroborate this finding. The case needs an extensive approach of integrative taxonomy and search for new morphological characters.

The pupae acceptance test of ROSENGREN & al. (1994) and MAEDER & al. (2005) to discriminate the cryptic species *Formica lugubris* and *F. paralugubris* provides a clear ethological support for heterospecificity but is not a really practicable tool for ant determination. It involves transport and maintenance of living ants over long distances and times, whilst the testing itself, with its many replicates and difficult design, is very time-consuming and fails to offer a determination in as many as 15% of cases investigated. No preference of conspecific against heterospecific sexual pupae was found by ROSENGREN & al. in 20% of 61 tests and no preference of conspecific worker pupae by MAEDER & al. in 11% of 91 tests. Both test systems, however, never resulted in wrong determinations. It should be noted in this context that the recent NUMOBAT system, a LOOCV-DA of seven morphometric characters, distinguished 125 samples of *F. paralugubris* and *F. lugubris* with an error rate of only 0.8% and a working time of 80 minutes per sample.

Ecology – the post-hoc confirmation

It is difficult to use ecology to separate cryptic species because ecological differences usually are not recognised b e f o r e the species have been distinguished by other methods. To give a typical example – *Lasius* "*niger*" sensu WILSON (1955) has been considered as extremely euryoecious species found in very different habitats from semi-desert to the wettest bogs and woodland. Today we know that the "jack-of-all-trades theory" is wrong and that there are some 16 ecologically different species in the Palaearctic. On the other hand, enormous ecological plasticity does not necessarily indicate unrecognised biodiversity. The Palaearctic facultative slave-holder *Formica sanguinea* is found in any open habitat from south Italy to the North Cape, Norway, in bogs as well as in xerothermous grasslands, and it can apparently use any Palaearctic *Serviformica* species as a host – yet there are no indications so far from multiple sources of information to split it into different species.

In other words, ecology can hardly detect cryptic biodiversity but it can give a *post-hoc* support to a hypothesis on heterospecificity. Such support may even follow a clear mathematic model. SEIFERT (1987) found big differences between measured overlaps of fundamental and realised niche spaces in sibling species and a reduction of these differences with decreasing relatedness. Strong overlap of fundamental niche spaces in sibling species is explained by

their high morphological and physiological similarity resulting in similar space and resource utilisation and strong interspecific competition. Consequently, sibling species are rarely found syntopically even if being largely sympatric which reduces the overlap of their realised niche space to a minimum. As typical figures for sibling species, 2 - 5% overlap was reported for realised niches but up to 65 - 75% overlap for fundamental niches, as found in the species pairs *Lasius flavus* vs. *myops* and *Tapinoma erraticum* vs. *ambiguuum* (SEIFERT 1987).

Integrative taxonomy – the most conclusive approach towards recognition of real biodiversity

It is clear from what has been said above that integrative taxonomy, which makes use of different disciplines of biology, is the most powerful, most conclusive alpha-taxonomic approach. DAYRAT (2005) laconically noted in this context: "Whether or not biologists with diverse training, competencies and perspectives will collaborate is a question that is probably more sociological than scientific." This is one aspect but it is also obvious that this ideal form of alpha-taxonomy is costly, in terms of time, manpower and money: only problematic cases can be considered and we need criteria for selection of those to be subject to full study. All disciplines can be useful in a particular context. However, if forced to reduce the number of presently available diagnostic tools at hand, I would select the following: (1) NUMOBAT as name finder and investigator of material of any age and preservation status; (2) ncDNA as best genetic indicator of species identities, hybridization and evolutionary scenarios and; (3) CHCs as indicator of species-specific recognition cues.

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Zusammenfassung

Kryptische Arten sind eine große Herausforderung für die Alpha-Taxonomie von Ameisen. Ihre verlässliche Identifizierung erfordert die Anwendung von hochentwickelten Methoden wie numerische Analyse phänotypischer Merkmale, von DNA oder kutikulären Kohlenwasserstoffen. Studien kryptischer Biodiversität werden stark durch intraspezifischen Polymorphismus und interspezifische Hybridisierung erschwert, was einen integrativen Einsatz dieser Methoden erforderlich macht. Die Häufigkeit kryptischer Arten wurde für drei gründlich untersuchte Gattungen geschätzt: ± 46 % der etwa 94 paläarktischen *Lasius*-Arten, ± 43 % der etwa 67 paläarktischen *Formica*-Arten und ± 52 % der etwa 77 *Cardiocondyla*-Arten weltweit. Kryptische Biodiversität ist offenbar nicht gleichmäßig über die Artengruppen dieser Gattungen verbreitet. Es wird vorausgesagt, dass mehrere andere Ameisengattungen ähnlich viele kryptische Arten besitzen. Es werden Aussagen über den taxonomischen Wert von aus verschiedenen Gebieten der Biologie stammenden Informationsquellen gemacht: Morphologie-Basierte Alpha-Taxonomie (MOBAT), Numerische MOBAT (NUMOBAT), nukleare und mitochondriale DNA, kutikuläre Kohlenwasserstoffe, Pheromone, Allozyme, Karyotypen, Ethologie und Ökologie. NUMOBAT wird herausgestellt als das Rückgrat einer testbaren integrativen Taxonomie, da sie die entscheidende Verbindung zur Zoologischen Nomenklatur und einziger Untersucher von DNA-degradiertem Material und Sammlungsgut ist, bei dem Beschädigung nicht zulässig ist. Die unakzeptabel hohe Häufigkeit von Paraphylien verbietet die Anwendung des mtDNA Barcoding als primären Entscheidungsfinder. Es wird betont, dass nur eine integrative, multiple Informationsquellen nutzende Taxonomie die schlüssigste Annäherung an die reale Biodiversität gestattet.

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