Polyphasic approach applying artificial neural networks, molecular analysis and postabdomen morphology to West Palaearctic *Tachina* spp. (Diptera, Tachinidae)

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Abstract

Artificial neural networks (ANN) methodology, molecular analyses and comparative morphology of the male postabdomen were used successfully in parallel for species identification and resolution of some taxonomic problems concerning West Palaearctic species of the genus *Tachina* Meigen, 1803. Supervised feed-forward ANN with back-propagation of errors was applied on morphometric and qualitative characters to solve known taxonomic discrepancies. Background molecular analyses based on mitochondrial markers CO I, Cyt b, 12S and 16S rDNA and study of male postabdominal structures were published separately. All three approaches resolved taxonomic doubts with identical results in the following five cases: case 1, the four presently recognized subgenera of the genus *Tachina* were confirmed and the description of a new subgenus was recommended; case 2, the validity of a new boreo-alpine species (sp.n.) was confirmed; case 3, the previously supposed presence of *T. casta* (Rondani, 1859) in central Europe was not supported; case 4, West Palaearctic *T. nupta* (Rondani, 1859) was contrasted with East Palaearctic specimens from Japan, which seem to represent a valid species not conspecific with central European specimens; *T. nupta* needs detailed further study; case 5, *T. nigrohirta* (Stein, 1924) resurrected recently from synonymy with *T. ursina* Meigen, 1824 was confirmed as a valid species. This parallel application of three alternative methods has enabled the principle of 'polyphasic taxonomy' to be tested and verified using these separate results. For the first time, the value of using the ANN approach in taxonomy was justified by two non-mathematical methods (molecular and morphological).

Introduction

To transform the taxonomic process, it is necessary to increase the productivity of identification of biodiversity, including the description of new species, by using new tools (e.g. by Miller, 2007; La Salle *et al.*, 2009). These approaches speed up identification and make it more precise by using

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parallel alternative methods, resulting perhaps in a semi- or fully automated process of identification. Among such methods, there are artificial neural networks (ANN) methodologies based on the artificial intelligence principle (Weeks & Gaston, 1997, Vaňhara *et al.*, 2007, 2010; MacLeod, 2008; Fedor *et al.*, 2008, 2009). Molecular analysis provides valuable results, as it is commonly understood, and has been well used in dipterology (our review Tóthová *et al.*, 2006). Classical comparative morphology remains widely used, for example in identification keys (both classical dichotomous and multi-entry computer based, e.g. Moritz *et al.*, 2001 and others). Knowledge of taxonomy and species relationships within the genus *Tachina* has been insufficiently deeply studied; the existence of 45 synonyms for the 12 species currently recognised as valid in the West Palaearctic fauna is a result of a persistent state of taxonomic uncertainty in the genus (Herting, 1984; Herting & Draskovits, 1993; Tschorsnig *et al.*, 2004).

Polyphasic taxonomy in entomology

The principle of integrating different sources of data used for identification was initiated by Colwell (1970), who introduced the term 'polyphasic taxonomy' to microbiology.

Such a polyphasic approach takes into account all known phenotypic and genotypic information and integrates them not only for the purpose of taxonomy and identification, but also for the full reciprocal validation of methods used and for the results obtained. We compared the three basic processes; identification is based on (i) ANN, (ii) molecular analyses deduced from up to four mitochondrial markers and (iii) the male postabdominal structure at different taxonomic levels (see Novotná *et al.*, 2009). In entomology, independent identification based on several methodological sources is not common. ANN used here, as a part of a polyphasic taxonomic approach, enabled us to incorporate a further method of identification, which is now possible thanks to a wide background of computational strategies. This idea is not speculative, as this ANN technology is able to evaluate a vast amount of data, which might form a stable basis for a good and reliable system for biodiversity identification (Vandamme *et al.*, 1996). A wide polyphasic taxonomic approach for other insect groups is now required.

An analogous approach to achieving an 'integrated taxonomy' is the integration of web resources into taxonomic effort, the work of Deans & Kawada (2008) being an example.

ANN in insect taxonomy

In recent years, the use of ANN has spread to many branches of science; but, in entomology and arachnology, as far as we know, ANN applications are still rare.

Usually, ANN constitute bases of automatic species recognition systems (Weeks & Gaston, 1997; MacLeod *et al.*, 2007; MacLeod, 2008). Its advantage as a computational method is that ANN can evaluate different kinds of input data, e.g. qualititative and quantitative (e.g. morphometric) morphological characters, transformed digital images, optical or acoustic spectra, etc. In the framework of particular insect/spider groups, for example, Chesmore (2001, 2004) used ANN for acoustic recognition of several Orthoptera species, Moore & Miller (2002) for optic recognition of wing flaps of five aphid species (Hemiptera), Aldrich *et al.* (2007) for near-infrared reflectance spectroscopy used for termite species and Fedor

et al. (2008) identified 18 species of four genera of Thysanoptera on the basis of 20 mostly morphometric characters. The system ABIS was developed for identification of bees (Francoy *et al.*, 2008) and the system DAISY (O'Neill, 2007) for several insect groups (e.g. moths, bumblebees, ceratopogonids, lycosiids, butterflies, caterpillars, etc.). Do *et al.* (1999), Platnick *et al.* (2005) and Russell *et al.* (2008) successfully used ANN for automatic identification, respectively of lycosid and trochanteriid spiders, based on transformed digital images of male pedipalp and female epigynium and even developed an on-line automated identification system SPIDA for 121 spp. and 15 genera of Australian Trochanteriidae. In Diptera, ANN were applied in Culicidae where Moore (1991) evaluated frequency of wing-beat in both sexes of two species. Marcondes & Borges (2000) distinguished the morphologically identical males of two species of Psychodidae; as inputs (classification variables), they used a ratio of measurements of different parts of the male body. Vaňhara *et al.* (2007) tested the methodology of ANN identification in the family Tachinidae on the basis of five model species of two genera, using 16 morphometric characters.

Chemometric methodology, outside insect taxonomy, was also develop by us and was used for identification of molluscs (*Patella*) (Hernández-Borges *et al.*, 2004).

Molecular analyses of tachinids

A wide survey of DNA analyses used within the genus *Tachina* was published in a parallel paper (Novotná *et al.*, 2009).

Identification within Tachina

The present taxonomy of *Tachina* was established by Herting (1984), who recognized four subgenera; but the existence of a new one was discussed by Novotná *et al.* (2009). See also the diverse opinions published by O'Hara *et al.* (2009). Novotná *et al.* (2009) included an identification key based on the male terminalia, illustrated by original pen drawings and deep focus micrographs, some of them used for the first time. (The results were confirmed by mitochondrial markers CO I, Cyt b, 12S and 16S rDNA).

This paper also resolved some old taxonomic discrepancies, as: (i) the taxonomic concept of the genus was evaluated, see above; (ii) the position of the present subgenus *Tachina* s. str. seemed to be untenable; while *T. grossa* (Linnaeus, 1758) remained within the existing subgenus *Tachina* s. str., a new subgenus could be created for *T. magna* (Giglio-Tos, 1890); (iii) and an expected new species from subgenus *Eudoromyia* was confirmed within European boreo-alpine material, although it has not been described formally; (iv) *T. nigrohirta* (Stein, 1924) was resurrected from synonymy and confirmed as a valid species; (v) some differences, possibly of a specific nature, between central European and Japanese specimens of *T. nupta* (Rondani, 1859) were found.

In our study, we compare ANN with previous results concerning molecular analyses and morphology of the male postabdomen (Novotná *et al.*, 2009) to test if we can synthesize the results of all three methods.

Table 1. List of characters selected for creation of ANN sub-databases.

No. of characters/ used for:	Abbr. used	Measured and verbal characters (for figures of characters 1–16 see Vaňhara *et al*. (2007); for characters 25–30 see Novotná *et al*. (2009))
1	cs_1	length of costal **section cs_1**
2	cs_2	length of costal **section cs_2**
3	cs_3	length of costal **section cs_3**
4	cs_4	length of costal **section cs_4**
5	cs_5	length of costal **section cs_5**
6	R_1	length of radius R_1
7	R_{2+3}	length of **radius R_{2+3}**
8	R_{4+5}	length of **radius R_{4+5}**
9	M part 1	length of the **basal part of medial vein**, i.e. between cross-vein bm-cu and bend of M
10	M part 2	length of **post angular vein**, i.e. medial vein M from its bend to the end
11	CuA_1 part	length of **anterior branch CuA_1** of cubital vein, its part between bm-cu and dm-cu
12	dm-cu	length of discal medial-cubital **cross-vein dm-cu**
13	r-m	length of radial-medial **cross-vein r-m**
14	bm-cu	length of basal medial-cubital **cross-vein bm-cu**
15	2nd	the **width of antennal segment 2** in its widest part
16	3rd	the **width of antennal segment 3** in its widest part
17	M, F	male, female
18/ *Eudoromyia*		**colour of fore tarsus,** dark/ yellow/red
19/ *Eudoromyia*		**colour of postpronotum,** dark/red
20/ *Eudoromyia*		**length of the male** claws compared with a length of **5th fore tarsomere**, claws longer, yes/no
21/ *Eudoromyia*		**width of 4th semi-final fore tarsomere** to its **length** for females, tarsomere wider than longer, yes/no
22/ *Servillia*	A1	the length of **anal vein A1**
23/ *Servillia*		colour of **thoracic hairs** reflection dark/pale
24/ *Servillia*		thickness of **apical bristles**: strong/weak
25/ *Euroromyia*		**syncercus laterally**: stronger proximal part longer/shorter than narrow distal one, or both parts are indistinguishable
26/ *Eudoromyia*		**dilation on syncercus**: absent/present
27/ *all subgenera*		**surstylus**: straight/distinctly curved towards syncercus
28/ *Eudoromyia*		**surstylus**: without/with outer incision
29/ *all subgenera*		**apex of surstylus**: simple/ bicuspidate
30/ *all subgenera*		**bacilliform sclerite**: upper apical projection wide/slender rounded/slender pointed

Materials and methods

Examination of taxa

For a model study of a polyphasic approach in this work, the taxonomically problematic genus *Tachina* with 12 West Palaearctic species, including potential new taxa suggested by Novotná *et al*. (2009) was chosen. For initial nomenclature of the *Tachina* species, the catalogues of Herting & Dely-Draskovits (1993) and Herting (1984) were used.

The tachinids studied were identified by C. Bergström, P. Cerretti, J. Čepelák, B. Herting, L.P. Mesnil, H. Novotná, R. Rozkošný, H.-P. Tschorsnig, J. Vaňhara and J. Ziegler.

Examination of material

For ANN evaluation, 227 dry-mounted, mostly pinned, specimens were used. Specimens of *Tachina* species were given by the following institutions and dipterologists:

BAR – M. Barták, Czech University of Agriculture, Praha, Czech Republic
BER – Ch. Bergström, Uppsala, Sweden
ČEP – J. Čepelák (late), collection deposited partly with J. Vaňhara (corresponding author)
CER – P. Cerretti, Università degli Studi di Roma 'La Sapienza', Roma, Italy
ICH – R.T. Ichiki; Japan International Research Center for Agricultural Sciences, Tsukuba, Japan
MIH – F. Mihályi (late), National Museum, Budapest, Hungary (arranged by L. Papp)
TSCH – H.-P. Tschorsnig, Staatliches Museum für Naturkunde, Stuttgart, Germany
VAŇ – J. Vaňhara, Masaryk University, Brno, Czech Republic
ZIE – J. Ziegler, Museum fuer Naturkunde, Humboldt University, Berlin, Germany

Specimens were photographed (using a stereomicroscope Olympus SZX 12 with attached Camedia C-5050 digital camera); the digitalized images were scaled (in μm) by means of an image analyser using the software M.I.S QuickPhoto Micro Olympus (Japan).

DNA species analyses

Methods for molecular analyses based on mitochondrial markers CO I, Cyt b, 12S and 16S rDNA are described fully in Novotná *et al*. (2009). DNA was extracted following the protocol according to Tóthová *et al*. (2008).

Examination of characters and ANN data management

Preferentially, we selected 16 morphometric characters in *Tachina*, 14 for the wing, which included the length of different wing veins or their sections and two for the antenna (table 1; see also Vaňhara *et al*., 2007). All linear distances were measured by manually selecting landmarks with a mouse on a computer screen (with the image file opened in the QuickPhoto programme), which automatically provided the value of the measurement. Sex of the studied specimens was recorded as an additional character. The right wing was measured; for four specimens with the right wing missing, the left one was measured (see Vaňhara *et al*., 2007).

For application of ANN, the wide basic database (227 specimens) was divided into smaller sub-databases according
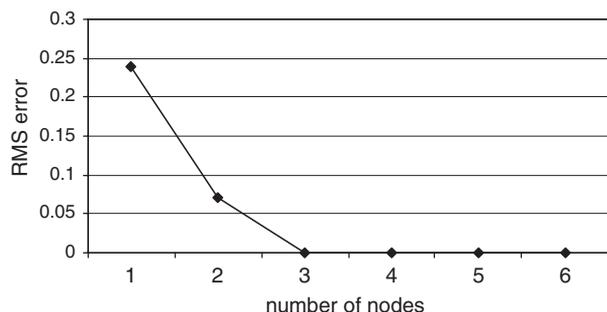
Fig. 1. Dependence of root mean square error (RMS) on the number of nodes in the hidden layer of ANN for a three-layered Multilayer perceptrons network architecture (MLP), five subgenera of *Tachina* trained. For ANN analyses *n* is usually recommended to be slightly higher (one or two nodes more) than the optimum found.

to logical taxonomic units needed for each case studied, i.e. according to subgenera or to species groups within the subgenus *Eudoromyia.* For sub-databases, specific characters (additional to the basic 16 shared characters) were sought and added in order for ANN to reach a better result (see table 1). To construct and achieve really reliable and 'clean' sub-databases (i.e. based on really well identified specimens), all evidently damaged, incomplete or atypical specimens were eliminated.

Tachina ANN computational strategy

ANN computation was performed using T RAJAN Neural Network Simulator, Release 3.0 D. (T RAJAN Software Ltd 1996–1998, UK). All computation was performed on a standard PC computer with operating system Microsoft Windows Professional XP 2003. The ANN strategy applied in this study is based on ANN methodology by Vaňhara *et al*. (2010) with background formed by Fedor *et al*. (2008, 2009).

The use of ANN consists of six distinct steps (commented on in detail in case 1 only and briefly summarised in further cases):

  (i) sub-database creation as a fundamental step for every case under study;
 (ii) multilayer perceptrons networks (MLP) architecture construction, which consists of input, one or more hidden layers and output;
(iii) number of nodes assessment in a hidden layer for optimalization of ANN using dependence of root mean square (RMS) error value, *n* is usually recommended to be slightly higher (one or two nodes) than the optimum found (see fig. 1);
(iv) training of neural network;
 (v) verification using cross-validation; and
(vi) identification of unknown specimens.

Results

Case 1: The recognition hitherto of four subgenera versus proposal of an additional new subgenus

Status quo

The present taxonomy of the genus *Tachina* was established by Herting (1984), who recognised four subgenera. The

recognition of a new subgenus was recently suggested by Novotná *et al*. (2009). The newly proposed subgenus would comprise *T. magna*, which has been treated as belonging to the subgenus *Tachina* s.str. The type species of that subgenus is *T. grossa*. However, results of our analyses confirmed a hypothesis that *T. magna* is more closely related to the species of the subgenus *Servillia* than to *T. grossa*. According to Novotná *et al*. (2009), establishment of a new subgenus for *T. magna* is the best solution to this problem.

Hypothesis for ANN

Are we able to use ANN to recognize a new subgenus?

ANN case 1 process

  (i) Sub-database: selected specimens of all 13 species (including a potential sp.n. (see also case 2) were used for subgeneric analysis, 81 specimens in total. Only males were analyzed due to the use of several additional characters on the male postabdomen (on surstylus, its apex and bacilliform sclerite) together with the basic 16 characters on the wing and antenna, which were applied to ANN computation, see characters of all subgenera in table 1.
 (ii) Multilayer perceptrons networks: as the number of taxa is not high (five subgenera) and ANN architecture should be conventionally the simplest, MLP was constructed as three-layered (24, *n*, 4 or 5), where 24 is a number of characters in the input layer, *n* is a number of nodes in the hidden layer, and 4 or 5 are the numbers of subgenera in the output layer (with or without a new subgenus in the training process).
(iii) Number of nodes: five was chosen for the single hidden layer (24, 5, 4 or 5) (fig. 2).
(iv) Training: of neural network on the specialized sub-database for case 1 was completely successful (100%), see table 2.
 (v) Verification: from all males of *T. magna*, two specimens were randomly selected and five different combinations tested by cross-validation. Only one male in one combination was marked as wrong, i.e. 98.7% success was achieved.
(vi) Identification: based on a training set with 71 samples of four subgenera, ten additional samples (of *T. magna*) were examined (see table 2; 'without training'). From these ten samples, 100% were classified as wrong specimens. This means that *T. magna* specimens were not placed within any of the four hitherto known subgenera (as a correct identification), thus supporting the proposal in Novotná *et al*. (2009) that they should belong to another (new) subgenus.

Additionally to this analysis, we put in the training process all the above mentioned ten males of *T. magna* as a fifth subgenus, i.e. 81 samples (see table 2; 'with training') were trained, using ANN architecture (24, 5, 5). As none of the examined specimens overlapped with any of the other trained sub-genera, we obtained confirmation of the above result.

Analysis of morphological data

See Novotná *et al*. (2009) for conclusions based on postabdominal characters. A potential new subgenus was

Table 2. ANN analysis supports new subgenus. Two methods of training were used, 'without' and 'with' specimens of a new subgenus. Wrong identification of ten specimens shows a good result for case 1; no specimen identified was put into known subgenera. It was supported also by DNA analysis and morphological study of male postabdomen, see case 1.

Explanation: '–' no material for identification.

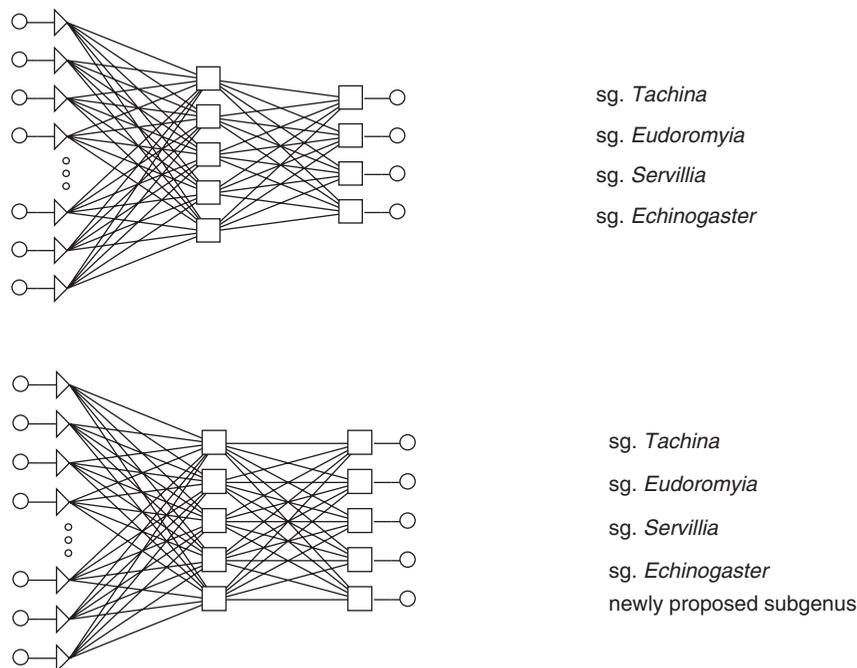| Subgenus	Training			Identification		
	Total	Correct	Wrong	Total	Correct	Wrong
T. (Eudoromyia)	39	39	0	–	–	–
T. (new subgenus proposed) (for *T. magna*) (without/with training)	0/10	0/10	0	10/0	0/0	10/0
T. (Servillia)	12	12	0	–	–	–
T. (Tachina) s.str. (for *T. grossa*)	13	13	0	–	–	–
T. (Echinogaster)	7	7	0	–	–	–
Total (without/with training)	71/81	71/81	0	10/0	0/0	10/0
%	100/100	100/100	0	100/0	0	100/0

Fig. 2. Used ANN architecture for resolution of subgenera, training without a new proposed subgenus (above) and trained with it (below). Three-layered Multilayer perceptrons network (MLP 24, 5, 4 or 5).

recommended for *T. magna*, as its current inclusion in the subgenus *Tachina* seemed to be problematic. Also, its position in the phylogenetic trees published by Novotná *et al*. (2009: see figs 18–20) proved that this species could hardly belong to any current subgenus. A new subgenus is not yet formally established because a wider revision of the species of subgenus *Tachina* is needed.

Analysis of molecular data

Parallel analyses based on up to four mitochondrial DNA markers with partial sequences of genes CO I, Cyt b, 12S and 16S rDNA of total length about 1500 bp have been used for validation of the new subgeneric position. Also, a combination of molecular (12S and 16S rDNA) and morphological (male postabdomen) characters has been discussed, and the

principle of recognising five subgenera was independently verified as well. Phylogenetic trees and their resolution are good and the reliability of branches is high. Both trees have been published in Novotná *et al*. (2009: see figs 5 and 6). The convincing and strong results obtained using ANN, as described above, are supported here by a quite decisive method, i.e. by molecular analysis; and a clear conclusion, at an interspecific level within the subgenus *Tachina*, is recommended.

Discussion of case 1

ANN applied as a further method yielded the same results as other polyphasic taxonomic approaches. The above mentioned ANN results validated the proposal of a new subgenus.

Table 3. ANN analysis supports proposed *T.* sp.n. (case 2). This species was identified among not trained material from French Alps. Also, two further not trained specimens of *T. fera* (det. Čepelák and Herting) were classified by ANN as wrong and identified in parallel as *T.* sp.n. This species was also supported by DNA analysis and morphological study of the male postabdomen, see case 2.

Explanation: '–' no material for identification.

Spp.	Training			Identification		
	Total	Correct	Wrong	Total	Correct	Wrong
T. casta	8	8	0	–	–	–
T. fera	16	16	0	1	0	1
T. fera (mountain Slovakia, ČEP)	0	0	0	1	0	1
T. magnicornis	9	9	0	–	–	–
T. nupta (Japan)	6	6	0	–	–	–
T. sp.n. (Scandinavia, BER)	12	12	0	–	–	–
-do- (French Alps, TSCH)	0	0	0	40	40	0
Total	51	51	0	42	40	2
%	100	100		100	95	5

This proposition is also underlain in the process of ANN by two different computational methods. In addition, this ANN process was also checked by consequent cross-validation. The results also dovetail into the context based on our parallel research of the male postabdomen (see Novotná *et al.*, 2009) and molecular analysis. However, we are aware that even if such strong support for a new subgenus was obtained by ANN, a phylogenetic dimension is missing from the ANN approach.

Case 2: New species vs. known West Palaearctic species of the subgenus Eudoromyia

Status quo

Among the specimens examined of the subgenus *Eudoromyia* (BER, ČEP, TSCH and ZIE coll.) the existence of a new species was determined (see Novotná *et al.*, 2009).

Hypothesis for ANN

Are we able to recognize a new species using ANN?

ANN case 2 process

(i) Sub-database creation: five *Eudoromyia* species were used, 52 specimens in total. To the ANN computation, 16 basic characters (wing, antenna) and several additional (male postabdomen, tarsus and colour of some parts of the body) were used, see characters of *Eudoromyia* in table 1.
(ii) Multilayer perceptrons networks: three-layered architecture (21, n, 5).
(iii) Number of nodes: six nodes in a single hidden layer (21, 6, 5).
(iv) Training: completely successful, see table 3.
(v) Verification: 12 specimens of sp.n. (BER) were 12 times randomly selected and then tested. by cross-validation with 100% correctness.
(vi) Identification: 40 not trained specimens from the French Alps were identified as *T.* sp.n.

Also, one not trained Slovak specimen, identified formerly as *T. fera* (Linnaeus, 1761) (det. Herting), was re-identified by ANN as *T.* sp.n. and subsequently also by the male postabdominal structure (table 3).

Analysis of morphological data

Novotná *et al.* (2009) described a postabdominal character, namely a distinct callus on the syncercus, which characterises the *T. fera* species group among species of the subgenus *Eudoromyia* (viz. *T. fera*, *T. canariensis* (Macquart, 1839), *T. casta* (Rondani, 1859) and the proposed sp.n.). In parallel, a male postabdomen was successfully placed here using ANN (100%), and subsequently a Slovak specimen was also correctly re-identified using ANN.

Analysis of molecular data

The combined analysis of two mitochondrial markers, 12S and 16S rDNA, with male postabdominal characters for support of taxonomic recommendations, was utilized in Novotná *et al.* (2009). In this study, a further two specimens from the French Alps were also ranked as belonging to the sp.n.

Discussion of case 2

ANN yielded identical results to the postabdominal characters and DNA analyses, done by Novotná *et al.* (2009). In this paper, ANN recognized that 40 specimens could not be assigned to any known West Palaearctic species of the genus *Tachina* (separately in *T. fera* gr. and *T. magnicornis* (Zetterstedt, 1844) gr.), but all of them were assigned to the same species as the trained Scandinavian material (12 specimens). Our conclusion reached here was anticipated by some previous authors in recent years, they published it in their faunistic studies as an unnamed *Tachina* sp., e.g. Ziegler & Lange (2001, 2006) and Tschorsnig *et al.* (2003). This new boreo-alpine species has been recorded in Sweden, Finland, Denmark, Alpine France and from the Slovak mountains. This new species has not yet been formally described.

Case 3: Mediterranean T. casta vs. 'Central European T. casta' (= syn. T. lefebvrei sensu Čepelák)

Status quo

T. lefebvrei (Robineau-Desvoidy, 1830) was described on the basis of material from the type locality in Sicily (Herting & Dely-Draskovits, 1993). Bezzi & Stein (1907) accepted it as a valid species in their catalogue, which J. Čepelák followed for

Table 4. ANN analysis of *T. casta* sensu Čepelák (as syn. *T. lefebvrei*). J. Čepelak identified his *T. lefebvrei* correctly among southern European specimens. But, all specimens of his *T. lefebvrei* from central Europe were correctly identified as other *Eudoromyia* species. This result was also supported by DNA analysis and morphological study of the male postabdomen, see case 3.

Explanation: '–' no material for identification.

Taxa	Training			Identification		
	Total	Correct	Wrong	Total	Correct	Wrong
T. casta	8	8	0	–	–	–
T. casta sensu Čepelák (= syn. *T. lefebvrei*)	0	0	0	22	1(Serbia)	21
T. fera	16	16	0	–	–	–
T. magnicornis	9	9	0	–	–	–
T. nupta (Japan, ICH)	6	6	0	–	–	–
T. sp.n. (Scandinavia, BER)	12	12	0	–	–	–
Total	51	51	0	22	1	21
%	100	100		100	5	95

a long time and also identified it from Slovakia (Čepelák, 1986); *T. lefebvrei* was also accepted by Zimin *et al.* (1970). Herting (1984) synonymized *T. lefebvrei* with *T. casta* (Rondani), which is according to present knowledge a Mediterranean species only and it was, after re-identification of several of Čepelák´s specimens, excluded from the Slovak checklist (Vaňhara *et al.*, 2004; Vaňhara & Tschorsnig, 2006).

Hypothesis for ANN

Does *T. casta* occur in Central Europe (identified as *T. lefebvrei* by J. Čepelák)?

ANN case 3 process

(i) Sub-database: five species from subgenus *Eudoromyia* were used for the *Case 3* sub-database, see case 2.
(ii) Multilayer perceptrons networks: three-layered architecture with a single hidden layer was constructed (21, n, 5).
(iii) Number of nodes: (21, 6, 5).
(iv) Training: see table 4 (100%).
(v) Verification: eight specimens of *T. casta* were eight times randomly tested by cross-validation. Success of identification was 100%.
(vi) Identification: from 22 Slovak and Serbian specimens identified by J. Čepelák as *T. lefebvrei*, the single Serbian specimen of *T. casta* (from Golubac) was re-identified by ANN as correct, see table 4. The remaining specimens were re-identified, mostly as *T.* sp.n. and *T. magnicornis*. The one other problematic specimen of *T. casta* from Sicily (det. Cerretti) was also correctly re-identified by ANN. No specimens from Slovakia were identified as *T. casta* (table 4).

Analysis of morphological data

Tachina casta differs from other representatives of the subgenus in details of the male postabdomen. The male postabdomen of 14 of Čepelák´s specimens determined as *T. lefebrei* were analyzed and most of them were identified as *T.* sp.n. or *T. magnicornis*. Čepelák´s correct identification of *T. casta* from Serbia (see above) was also verified by the postabdomen. None of Čepelák´s specimens named as *T. lefebrei* from Slovakia were re-identified as *T. casta*.

Analysis of molecular data

None of the three specimens of Čepelák´s *T. lefebvrei* under analysis of two mitochondrial markers, 12S and 16S rDNA, were *T. casta*. Their position in the phylogenetic tree was very unclear but far from the *T. casta* clade. Other specimens could not be analyzed, as they are more than 20 years old.

Discussion of case 3

No specimens of *T. casta* sensu Čepelák (det. as *T. lefebvrei*) that originated from Slovakia were confirmed by ANN. Also, this was supported by molecular and postabdominal results. This confirms that its previous exclusion from the Diptera list of Slovakia (Vaňhara *et al.*, 2004) was correct. Čepelák´s identification of one southern specimen from Serbia as *T. casta* was confirmed.

Case 4: Problematic T. nupta *vs.* T. magnicornis

Status quo

Tachina nupta was described in 1859 from Italy (cf. Herting & Dely-Draskovits, 1993). Because there is a great variability in morphological characters in the *T. magnicornis* species group, the species can be considered as problematic. Bezzi & Stein (1907) did not accept this species and mentioned it as a synonym of *T. magnicornis*. Some later authors considered it as a valid species (Mesnil, 1966; Zimin & Kolomietz, 1984; Mihályi, 1986; Herting & Dely-Draskovits, 1993; Čepelák & Vaňhara, 1997; Chao *et al.*, 1998). According to Herting & Dely-Draskovits (1993) there are six synonyms of this species. Mesnil (1966) classified the East Palaearctic *T. micado* (Kirby, 1884) as a subspecies of *T. nupta*. This taxon was synonymized by Herting (1984). The unclear position of *T. nupta* was presented by Tschorsnig & Herting (1994).

Hypothesis for ANN

Is West Palaearctic *T. nupta* a valid species?

ANN case 4 process

(i) Sub-database: five species from subgenus *Eudoromyia* were used for the case 4 sub-database, see case 2. We have at our disposal not only material of *T. nupta* from Europe

Table 5. ANN analysis of *T. nupta* identifications. From rare European material, only one specimen correlated with trained Japanese material. This result was also supported by morphological study of the male postabdomen, see case 4.

Explanation: '–' no material for identification.

Taxa	Training			Identification		
	Total	Correct	Wrong	Total	Correct	Wrong
T. casta	8	8	0	–	–	–
T. fera	16	16	0	–	–	–
T. magnicornis	9	9	0	–	–	–
T. nupta	6 (Japan)	6	0	11(Europe)	1	10
T. sp.n. Scandinavia (BER)	12	12	0	–	–	–
Total	51	51	0	11	1	10
%	100	100	0	100	9	91

but also six specimens from Japan. Because identification of West Palaearctic material of *T. nupta* has been uncertain for a long time, for ANN analysis we purposely utilized only some specimens of *T. nupta* from Japan (*T. nupta* East), whereas West Palaearctic specimens (*T. nupta* West) were included in the identification process only.

(ii) Multilayer perceptrons networks: three-layered architecture (21, n, 5).

(iii) Number of nodes: (21, 6, 5).

(iv) Training: correct (100%), see table 5.

(v) Verification: each one of six specimens of *T. nupta* East was tested (100%). Random test by cross-validation: six specimens of *T. nupta* East were six times randomly tested; all specimens were analyzed as correct. Success of identification was 100%.

(vi) Identification: all 11 specimens of *T. nupta* West (all BAR, CEP-VAN, ZIE coll.) previously identified by several tachinologists, were re-identified by ANN. One of the 11 analyzed specimens (BAR coll.) was *T. nupta* according to the structure of the postabdomen, but its validation by ANN was impossible due to it having damaged fore legs. The other ten were identified as sp.n. or wrong species, but some of them are rather damaged and some characters are not usable, see table 5.

Analysis of morphological data

The postabdomen of four specimens of *T. nupta* East (CER, ICH coll.) and one specimen of *T. nupta* West (BAR, coll.) were analyzed. Novotná *et al*. (2009) recommended a revision of the West Palaearctic *T. nupta*, which differs from Japanese and Iranian specimens under study. On the basis of postabdominal characters, both forms of '*nupta*' are without a medial or submedial callus on the syncercus. In one West Palaearctic specimen of *T. nupta* (drawings in Novotná *et al*., 2009), the tip of the surstylus is stout and short, but in Japanese specimens the tip of surstylus is slender and elongate. In Japanese *T. nupta*, the inner margin of the syncercus in lateral view is almost straight in the distal half, the syncercus relatively broad in the basal half and both distal projections of the bacilliform sclerite are separated by a shallow emargination; while in the closely related *T. magnicornis*, the inner margin of the syncercus in lateral view is slightly arched in the distal half, the syncercus is relatively slender in the basal half and both distal projections of the bacilliform sclerite are separated by a deep emargination. The Japanese *T. nupta* is closer to *T. magnicornis* than to the West Palaearctic specimen.

Analysis of molecular data

Partial nucleotide sequences of the 12S and 16S rDNA mitochondrial genes were used in Novotná *et al*. (2009). DNA analysis of two specimens of *T. nupta* from Japan was consistent in the molecular trees obtained. The single West Palaearctic representative of *T. nupta* (BAR) could not be analyzed due to its preservation in formaldehyde. In this study, the three West Palaearctic specimens formerly identified as *T. nupta* could be analyzed, but no *T. nupta* was found among them.

Discussion of case 4

ANN gave us identical results to the postabdominal characters and DNA analyses, when these could be done (Novotná *et al*., 2009). It was confirmed that only one of the West Palaearctic specimens of *T. nupta* could be assigned to the same species as the specimens of *T. nupta* from the East. The remaining ten specimens, formerly identified as *T. nupta*, were re-identified here by ANN as not belonging to *T. nupta*, but some of them could not be determined due to damage that had been suffered by this very old material. Consequently, the problem persists. For future studies, more material and from intervening areas is needed for testing. Also, type material should be sought and evaluated. For further information, see Novotná *et al*. (2009).

Case 5: Re-establishment of T. nigrohirta *as a valid species in the European fauna vs. synonymy with* T. ursina

Status quo

Tachina nigrohirta was described in 1924 by Stein. The type locality is in Austria (Herting & Dely-Draskovits, 1993). Most authors have accepted it as only a synonym of *T. ursina* Meigen, 1824 (Mesnil, 1966; Herting & Dely-Draskovits, 1993). Also, Tschorsnig & Herting (1994) regarded this species as: "indistinct and a possible form of *T. ursine*". The species was accepted and repeatedly identified only by J. Čepelák (Čepelák, 1986; Čepelák & Vaňhara, 1997). The species was restored to the European fauna by Tschorsnig *et al*. (2004) on the basis of five specimens, which we have studied and which were used for the present analysis. The two discussed species (*T. nigrohirta* and *T. ursina*) have hitherto been differentiated from each other only by the dorsal hairs on the thorax, which are completely or predominantly black in *T. nigrohirta* and by the marginal bristles on tergite 4, which are longer than the

Table 6. ANN analysis of former identifications of *T. nigrohirta*. Five specimens used for resurrecting *T. nigrohirta* from synonymy were used for training. Among additional known not trained material only five specimens were re-identified correctly. This result was also supported by DNA analysis and morphological study of the male postabdomen, see case 5.

Explanation: '–' no material for identification.

Taxa	Training			Identification		
	Total	Correct	Wrong	Total	Correct	Wrong
T. lurida	40	40	0	–	–	–
T. ursina	17	17	0	–	–	–
T. nigrohirta	5	5	0	12	5	7
Total	62	62	0	12	5	7
%	100	100	0	100	42	58

segment. In *T. ursine*, the hairs are pale yellow or whitish and the marginal bristles are shorter (Tschorsnig & Herting, 1994).

Hypothesis for ANN

Was the re-establishment of *T. nigrohirta* as a valid species correct?

ANN case 5 process

(i) Sub-database: 64 specimens in total. Additional characters for this case (anal vein, thoracic hairs, apical bristles), see *Servillia* in table 1.
(ii) Multilayer perceptrons networks: (20, n, 3).
(iii) Number of nodes: (20, 4, 3).
(iv) Training: trained *T. nigrohirta* (5 specimens TSCH coll.) had been formerly studied in the connection with its re-establishment as a valid species (see above).
(v) Verification: cross-validation of five specimens of *T. nigrohirta*, from 64 trained specimens of *Servillia*, was randomly tested. In all five tests, 100% validation was found. Random test by cross-validation: five specimens of *T. nigrohirta* were five times randomly tested. Success of identification was 100%.
(vi) Identification: 12 not trained specimens, formerly identified as *T. nigrohirta*, were used (table 6). Five were correctly classified (ČEP, ZIE coll.) and seven were wrong (four were re-identified as *T. ursina*, two as *T. lurida* (Fabricius, 1781) and one specimen was ranked as wrong due to missing data. Two of the above 'correct' specimens were also re-identified by the postabdominal analysis as *T. nigrohirta* (ČEP).

Analysis of morphological data

Novotná *et al*. (2009) used postabdominal characters to distinguish *T. nigrohirta* from the closely related *T. ursina*. Four specimens of *T. nigrohirta* (ČEP, TSCH) were analyzed and two of them were also analyzed by molecular methods.

Analysis of molecular data

Two specimens from the ANN trained *T. nigrohirta* (TSCH) were analyzed. One of these, also identified on the basis of the postabdomen and correctly identified by ANN (from unknown specimens of ČEP coll.), was also validated by molecular analysis.

Discussion of case 5

Novotná *et al*. (2009) found important characters on the male postabdomen which unambiguously supported the validity of *T. nigrohirta*. Also, ANN analyses confirmed this, as did the molecular analyses. One specimen was validated by all three of the above mentioned polyphasic methods (TSCH). Inclusion of *T. nigrohirta* as a valid species in the Fauna Europaea database by Tschorsnig *et al*. (2004) was correct.

Discussion

This paper is part of our wider project concerning the genus *Tachina* from the West Palaearctic area. The first paper (Novotná *et al*., 2009) studied both the male postabdomen with the resulting phylogenetic consequences and the molecular background. The present paper practices polyphasic taxonomic principles on the same model taxa to add the results from ANN analyses. ANN were used here not only for identification, but also for solving longstanding taxonomic and faunistic problems and discrepancies. Three independent and parallel methods unambiguously supported the principles of polyphasic taxonomy. From this model project, it is concluded that polyphasic taxonomy could be applied to any other insect group in order to attain a consensus assessment based on genotypic and phenotypic inputs used in parallel.

Conclusions

The parallel tools of polyphasic taxonomy proposed in our study represent a useful method for solving long-standing taxonomic difficulties within the genus *Tachina*. ANN, molecular analysis of up to four mitochondrial markers and male postabdominal morphology, three parallel distinct methods, yielded consistent taxonomic results.

Solved taxonomic questions and uncertainties:

(i) ANN can be used as a parallel taxonomic tool, not only for specific and subgeneric identification, but also for solving longstanding taxonomic and faunistic problems and discrepancies.
(ii) Subdivision of *Tachina* into five subgenera was verified. A potential new subgenus should be created for *T. magna*, which is not closely related to *T. grossa* and cannot, thus, be included in the same subgenus *Tachina* s.str. A new subgenus is not established here formally because a subsequent wider revision of the genus *Tachina* is needed.
(iii) A new species preliminarily designated on the basis of discrete structures of the male terminalia was supported

by the DNA analysis as well as ANN. The description will be published separately.

(iv) The previously reported occurrence of *T. casta* in Central Europe was refuted by all three methods, and its previous elimination from the national checklist of Slovakia by us was confirmed to be correct.

(v) ANN analysis illustrates that *T. nupta* from Japan is consistent and differs from the West Palaearctic specimens. Status of West Palaearctic *T. nupta* is apparently ambiguous and must be solved by a revision and a study of material from additional geographical regions.

(vi) Based on the ANN, molecular analysis and male terminalia characters, *T. nigrohirta* is definitively resurrected from synonymy with *T. ursina*.

From the general point of view, multiple taxonomical re-analyses gave more accuracy to the results obtained. Reliability of ANN results obtained here and the power of ANN were confirmed by two independent non-numerical methods (molecular analysis, comparative morphology) for the first time. The utility of the polyphasic taxonomy approach was evidenced and could be applied in entomology generally.

Acknowledgements

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