

A first demonstration of interspecific hybridization in *Myrmica* ants by geometric morphometrics (Hymenoptera: Formicidae)

Ali BAGHERIAN YAZDI, Wolfgang MÜNCH & Bernhard SEIFERT



Abstract

A case of hybridization between *Myrmica scabrinodis* NYLANDER, 1846 and *M. vandeli* BONDROIT, 1920 is demonstrated by means of geometric morphometrics in a nest sample found in Baden-Württemberg / Germany. 41 landmarks and 252 semilandmarks were fixed in four anatomical aspects in 299 worker ants – dorsal head, frontodorsal clypeus, dorsal mesosoma and lateral petiole. 316 relative warps (RWs) were extracted from the shape variables. Shape differences between these very similar species are visualized by mean deformation grids exaggerated by the factor of three. Among a number of differences already known, geometric morphometrics revealed also differences not discovered so far by conventional morphometrics or subjective character assessment. A full separation of the parental species was provided by the first two RWs of the head and clypeus aspect but this approach was not sufficient to reliably demonstrate hybrid identities on individual level. A stepwise linear discriminant analysis (LDA) using the RWs as characters and reducing character number from 316 to 25, offered very powerful separation of the 291 workers of the parental species ($F = 6735.1$, ANOVA) and placed the eight workers of the hybrid sample in a coherent cluster exactly in the empty space between the parental species. Basically similar results were achieved with conventional linear morphometrics considering 16 characters but this system was less powerful ($F = 3108.0$, ANOVA) and placed two specimens of the parental species close to the hybrids. There was no directional asymmetry detectable in the whole material and the hybrid sample did not show increased fluctuating asymmetry. With the current methodology applied, data acquisition time was 48 minutes per specimen in conventional linear morphometrics but as much as 160 minutes in geometric morphometrics. The main problems of the latter method are the slowness of the automatic z-stack imaging methods and the absent software assisting landmark fixation. A considerable reduction of total data acquisition time to 84 (one-fold digitizing) or to 108 minutes (two-fold digitizing) per specimen is predicted if a grid-and-sector system assisting landmark fixation is automatically projected on the screen. The overall hybridization frequency for the region of Baden-Württemberg is estimated as 0.44% from the side of *M. vandeli* and 0.03% from the side of *M. scabrinodis*. Despite apparently strong reproductive barriers between *M. scabrinodis* and *M. vandeli*, rare hybridization may occur because of the constant and extremely close spatial association, largely overlapping swarming times and some aspects of male mating behavior. *Myrmica vandeli* is very likely a facultative temporary social parasite of *M. scabrinodis*. As much as 17% of the 225 *M. vandeli* nests found in Baden-Württemberg still contained *M. scabrinodis* workers.

Key words: *Myrmica*, hybridization, geometric morphometrics, relative warps analysis, fluctuating asymmetry, directional asymmetry, temporary social parasitism.

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Introduction

Interspecific hybridization is likely to be very common in ants but strongly underestimated. The true dimension of this phenomenon became apparent by intensive and detailed research in the fauna of Central Europe: Interspecific hybridization is known in $\geq 18\%$ of the 176 free-living ant species and 14% of the 37 genera of this area (SEIFERT 1999, SEIFERT 2006, KULMUNI & al. 2010, SEIFERT & al. 2010, STEINER & al. 2010, BERNASCONI & al. 2011). The

combination of haplo-diploid sex determination, eusociality and very long colony life is perhaps the deciding factor of increased hybridization frequency in ants. While hybridization is frequent on the per-species level, it is rare on the individual level. Excluding species with Social Cleptogamy, in which up to 30% of all successful matings may be heterospecific (SEIFERT 2006, UMPHREY 2006), the frequency of hybridization is estimated to vary normally be-

tween 0.1 and 2%. Realistic estimates of interspecific hybridization frequencies based upon thorough studies are not available for other insect groups with the exception of butterflies: DESCIMON & MALLET (2009) reported 16% hybridizing species within 440 European butterfly species and MALLET & al. (2007) gave a ratio of 28% for some 70 South American Heliconiinae species.

The genus *Myrmica* LATREILLE, 1804 probably includes more than 200 species (B. Seifert, unpubl.) which occur in high abundance in many ecosystems of the Holarctic but not a single case of hybridization is known so far in this ant group. Twenty species are identified in Central Europe and mixed mating swarms of several species have been repeatedly observed by several authors (e.g., STITZ 1939, WOYCIECHOWSKI 1990, RADCHENKO & ELMES 2010) and one of us (BS). RADCHENKO & ELMES (2010) reported up to six species in a single swarm but when they checked pairs in copulation on a few occasions these always were conspecific. This suggests strong barriers to hybridization in *Myrmica* the nature of which is not investigated so far. On the other hand, the lack of extensive genetical studies of nuclear DNA, the difficult species delimitation and the dominance of subjective assessment of characters in *Myrmica* taxonomy very likely could lead to a failure to identify hybrids.

Investigating ant populations in 26 localities of Baden-Württemberg in which *M. vandeli* BONDROIT, 1920 was found, Wolfgang Münch collected as many as 225 nest samples of this species and more than 3500 of *M. scabrinodis* NYLANDER, 1846. *Myrmica vandeli* always occurred syntopic with *M. scabrinodis* in bogs and moist mountain heath or grassland and often in very high densities – up to 49 nests / 100 m² of *M. vandeli* and 42 nests / 100 m² of *M. scabrinodis* were recorded at the same site. As much as 16.9% of the *M. vandeli* nest populations also contained *M. scabrinodis* workers – not rarely the latter were present in high proportions. Such mixed nests have also been found in Great Britain, Denmark, Finland, East Germany, Poland, and the Ukraine (ELMES & al. 2003, SEIFERT 2007, RADCHENKO & ELMES 2010). The all-area mean of 17% found for Baden-Württemberg is higher than frequencies reported from other regions and in a local patch near Münsingen 11 out of 16 *M. vandeli* nests contained *M. scabrinodis*. It is not the aim of this paper to investigate and discuss the sociobiological background of these data thoroughly. We only state that *M. vandeli* is apparently a facultative temporary social parasite of *M. scabrinodis* with a weak tendency for early disabling or killing the host queens.

It must be emphasized that all these mixed nests mentioned above were intranidal associations of two species with workers of apparently pure phenotypes and not suspicious of containing hybrid specimens. Wolfgang Münch found only two samples possibly representing hybrids *M. scabrinodis* x *vandeli* in his huge material and Bernhard Seifert identified only one worker with putative hybrid characters (associated in the same sample with a normal *M. scabrinodis* worker) in the collection of Senckenberg Museum of Natural History. The status of these three samples will be investigated in this paper by means of geometric morphometrics and conventional linear morphometrics. All conditions for credible phenotypic identification of hybrids (SEIFERT 1999) are provided: Character recording followed reproducible stan-

dards and was accurate enough to depict interspecific differences, the parental species were separable in worker individuals with a high confidence and there was sufficient knowledge about which other congeneric species occur at the collecting site of a putative hybrid sample.

Ant material investigated

The study was confined to material largely from the sympatric European range of both species and contained 99 nest samples with 299 workers. All samples are now in the collection of Senckenberg Museum of Natural History Görlitz. Anticipating the final determinations, these are in detail:

Myrmica scabrinodis: 63 nest samples with 187 workers from: A n d o r r a : Andorra City - 9 km NNW, 17.V.1991, samples No 5, 19, 25, 50, 77 [42.68°, 1.47°, 1850 m]. F r a n c e : Briançon, VI.1955 [44.90°, 6.63°, 1300 m]; Fointebleau, VI.1955 [48.39°, 2.66°, 110 m]; Frasne, 22.VIII.2010 [46.831°, 6.154°, 840 m]; Tourettes-sur-Loup, [43.72°, 7.06°, 400 m]. G e r m a n y : Baintdt, Kiesgrube, 12.IX.2006, No ANA2 [46.8364°, 9.6611°, 495 m]; Baruth, 27.VIII.1965 [51.23°, 14.59°, 150 m]; Baruth, Schafberg, 9.V.2010, samples No 782, 915 [51.2316°, 14.6006°, 165 m]; Baruth, Schafberg, 23.V.2010, No 6 [51.232°, 14.601°, 171 m]; Baruth, Schafberg, 5.VIII.2010, samples No 2, 3 and 4 [51.2316°, 14.6006°, 165 m]; Berzdorf, Hutberg, VIII / IX. 1982, No 819 [51.055°, 14.886°, 280 m]; Bilzingsleben, Steinrinne, 10.IX.1981 [51.271°, 11.061°, 170 m]; Gundholzen-W, Graues Ried, 8.X.2005, No 240 [47.699°, 8.963°, 443 m]; Breitlohms, 5.V.1990 [48.695°, 8.419°, 970 m]; Dubringer Moor, 9.VIII.1982, No 12-1, [51.398°, 14.163°, 135]; Grambach, 3.V.1991, No 225 [50.006°, 9.774°, 280 m]; Königshain - 1 km N, 1.VIII.1989 [51.198°, 14.851°, 300 m]; Heldrungen, 8.IX.1981 [51.30°, 11.19°, 130 m]; Kunnersdorf - 1 km SE, 21.V.1983 [51.197°, 14.947°, 258 m]; Löbauer Berg, Südrand, 10.V.1983, No 1 + 2 [51.088°, 14.699°, 360 m]; Löbauer Berg, 10.V.1983, Basaltklippen [51.0895°, 14.6927°, 420 m]; NSG Dellenhäule, 21. / 22.V.2006, samples No 1, 2, 3, 4, 5, 6, 8 [48.816°, 10.241°, 582 m]; Kreba, Spisk, 6.VIII.1989 [51.361°, 14.714°, 144 m]; Oppitz, Initialmoor, 15.VII.2003, samples No 1, 2, 3 [51.296°, 14.423°, 131 m]; Reichental - 2 km ENE, 5.V.1990 [48.735°, 8.415°, 660 m]; Reinheim-Zeilhard, 2.VI.1991, samples No 13, 35 [49.85°, 8.78°, 230 m]; Schmölln, FND Roter Berg, 1982 [50.93°, 12.45°, 230 m]; Hinterzarten-E, Hirschenmoor, 21.VI. 2005, No 234 [47.908°, 8.110°, 880 m]; Hinterzarten-S, Erlbruckmoor, 2.XI.2005, No 396 [47.983°, 8.105°, 940 m]; Lenzkirch-W, Hochmoor Ursee, 20.IX.2005, No 122 [47.865°, 8.170°, 844 m]; Sondershausen, 29.V.1983, No 1-3 [51.374°, 10.834°, 356 m]; Utzenfeld, Ungendwiedener Weidfeld, 28.VII.2005, No 92 [47.841°, 7.863°, 1100 m]; Wolmatinger Ried, 17.VII.2006, No 6673WOL [47.6742°, 9.1433°, 393 m]; Wolmatinger Ried, 10.XI.2006, No 7776WOL [47.7028°, 9.1033°, 395 m]; Würzburg, Dürrbach, 27.V.1991, No 068 [49.816°, 9.915°, 250 m]. P o l a n d : Izbica, T-Cross, 22.VIII.2006, samples No 68, 78 [54.658°, 17.419°, 2 m]. R u s s i a : Odinsk - 4 km WSW, 13.VII.2008, No 3 [52.400°, 103.700°, 466 m]. S p a i n : Espot - 1 km W, 6.V.1991, No 131 [42.588°, 1.071°, 1400 m]; Soria, 27.V.1987 [41.76°, -2.460°, 1100 m]. S w e d e n : Krokstrand, 1986 [59.000°, 11.433°, 60 m]. S w i t z e r l a n d : Salategnas, 12.VII.2010, samples No 31, 35, 37 [46.516°, 9.651°, 1960 m]; Sur - 1.0 km S, Mäh-

wiese, 15.VII.2010, samples No 17, 26 [46.514°, 9.631°, 1635 m]. Serbia: Jaile (= Jajce?), 3.VII.1985 [? 44.33°, 17.27°, 1100 m].

Myrmica scabrinodis x vandeli: One nest sample with eight workers from Germany: Unterhölzer Wald, 16.VII.2004, No 264-4 [47.933°, 8.593°, 679 m].

Myrmica vandeli: 35 nest samples with 104 workers from: Austria: Bangser Ried, 24.IX.1994 [47.276°, 9.557°, 439 m]. Czech Republic: Kvilda, 16.VI.1977, [49.014°, 13.577°, 1050 m]; Soumarsky most, 3.VIII.1982, samples No G45, E46 [48.902°, 13.837°, 780 m]. Denmark: Ganlose, 7.VIII.2010, samples No 536, 546, 548 [55.79°, 12.26°, 25 m]. France: "France" (probably Rhone Alpes, leg. Collingwood) [45°, 5°, 400 m?]; Le Bizot - 1 km SE, 11.VII.1990 [47.117°, 6.665°, 890 m]; Massignieu-de-Rives, 1982 [45.75°, 5.77°, 250 m]; Ambert, 24.VIII.1984, [45.55°, 3.75°, 530 m]. Germany: Bad Brambach, 20.VI.1986 [50.2006°, 12.2844°, 665 m], Bad Brambach, 23.VIII.1987, samples No 1, 2 [50.2006°, 12.2844°, 665 m]; Enzenstetten, 8.VIII.2003, No E22 [47.6295°, 10.5973°, 818 m]; Erda - 1 km NE, 24.IX.1997, samples No X, C23 [50.681°, 8.536°, 292 m]; Federsee, 26.IX.1980 [48.08°, 9.62°, 579 m]; Grasleiten, Hintere Schaar, 12.VIII.2003, No G21 [47.7426°, 11.0860°, 694 m]; Harz, Benneckenstein, 28.VIII.1953 [51.672°, 10.708°, 528 m]; Hengen - 2.05 km SSE, 2.VII.2010, samples No 491-2, 493-2 [48.468°, 9.478°, 723 m]; Hengen - 2.5 km SE, 7.VI.2010, No 2 [48.466°, 9.486°, 715 m]; Kempten - 14 km E, Görisried, Röhrenmoos, VIII.1994 [47.700°, 10.463°, 870 m]; Pfrontener Wasenmoos, 8.VIII.2003, No W10 [47.4925°, 10.5917°, 889 m]; Reichental - 2 km ENE, 5.V.1990 [48.735°, 8.415°, 660 m]; Rothenrain, 15.VIII.2003, No K25 [47.800°, 11.515°, 659 m]; Zainingen - 2.95 km SSE, 20.IX.2010, samples No 134-3, 135-3, 136-3, 138-3 [48.460°, 9.567°, 809 m]. Poland: Krakow: Tyniec, 20.VII.1984 [50.01°, 19.81°, 250 m]. Sweden: Andrarum, 6.VIII.2011 [55.668°, 13.947°, 112 m]; Stens-torp, 8.VI.2010 [58.27°, 13.71°, 173 m].

Methods

Character recording: An average of three mounted workers per sample was investigated by two-dimensional geometric morphometrics and conventional linear morphometrics. The number of investigated workers was increased to eight in samples hypothesized to contain hybrids.

Linear morphometrics: Linear morphometrics of 16 morphometric characters was performed according to the methods and character definitions given by SEIFERT (2011) but the following additional character was introduced:

ClyEx – depth of excision on anteromedian clypeal margin in a position in which the upper and lower margins of the excision superimpose. This is usually given after tilting the head by $\pm 45^\circ$ from dorsal towards the frontal viewing position (dorsofrontal view).

Geometric morphometrics and digitizing: The spatial positioning of specimens for imaging the anatomical aspects was according to the following rules:

Head aspect: Maximum head width and maximum head length are at the same level in visual plane.

Clypeus aspect: The head is tilted until the posterior clypeal margin (i.e., its border line with frontal triangle) is in the same level of the visual plane as anterior clypeal margin – this is more or less a 45° tilt.

Mesosoma aspect: Mesosoma in dorsal view with an anterior and two posterior reference points in visual plane (i.e., at same horizontal level). The anterior reference is the transition point between anterior pronotal slope and anterior pronotal shield. The posterior reference is the horizontal portion of the upper margin of propodeal lobes just before they ascend to subspinal excavation.

Petiole aspect: petiole in lateral view.

Z-stack photographs were made with a Leica Z6 APO photomicroscope equipped with $2.0\times$ planapochromatic objective and the automontage software Leica application suite version 3. Twenty to fifty z-stacks (depending upon the depth of the anatomical field and magnification) were integrated in the final automontage picture, which was subject to landmark analysis without prior manipulation of contrast, contour sharpness or other parameters. Landmarks were fixed in unambiguously defined surface points and semilandmarks were determined by the curve-tracing method implemented in the tpsDig software package (ROHLF 2010c). Semilandmarks are points evenly spaced on an outline delimited by two fixed points. 14 landmarks and 95 semilandmarks were determined in head aspect. These figures are 3 and 62 in clypeus aspect, 15 and 63 in mesosoma aspect, 3 and 32 in petiole aspect.

Symmetrizing: Correlations between the Procrustes and tangent shape distances were calculated using tpsSmall software version 1.20 (ROHLF 2003). Symmetrizing was done under use of the tpsRelw software version 1.49 (ROHLF 2010b) by Procrustes superimposition of the landmark configurations of the left and right aspects of petiole and by superimposing reflections in head, clypeus and dorsal aspect of mesosoma. For reduction of excess data, subsequent analyses of the head, clypeus and mesosoma aspects were conducted on the half configurations. Symmetric landmarks and semilandmarks were removed in each outline using the tpsUtil 1.46 software application (ROHLF 2010a). However, for better visualization of shape changes, deformation grids are presented for the total shape configurations.

The size: As a measure of overall size variation, the centroid size (BOOKSTEIN 1991) was calculated for each shape in both species, by using tpsRelw version 1.49 (ROHLF 2010b) and tested for normality using the Shapiro-Wilk test. The Independent-Samples T-Test procedure was conducted on whole data set to test significant centroid size differences between species for each anatomical field.

Relative warps (RWs): The landmarks and semilandmarks were subject to a Generalized Procrustes Analysis. The semilandmarks were then slid along the outline curve until they matched as well as possible the positions of corresponding points along an outline in a reference configuration (BOOKSTEIN 1997). We used tpsRelw version 1.49 (ROHLF 2010b) for sliding semilandmarks based on minimizing the bending energy of the thin-plate spline describing the deformation of the target relative to the reference (median shape) (ZELDITCH & al. 2004). Partial warp (shape variables) and relative warp scores (which is a PCA of shape variables with $\alpha = 0$, ROHLF 1995) were calculated by using tpsRelw version 1.49. The RWs were computed to summarize the variation among the specimens in as few dimensions as possible.

Deformation grids: Deformation grids were generated by using RWs as characters in a linear discriminant ana-

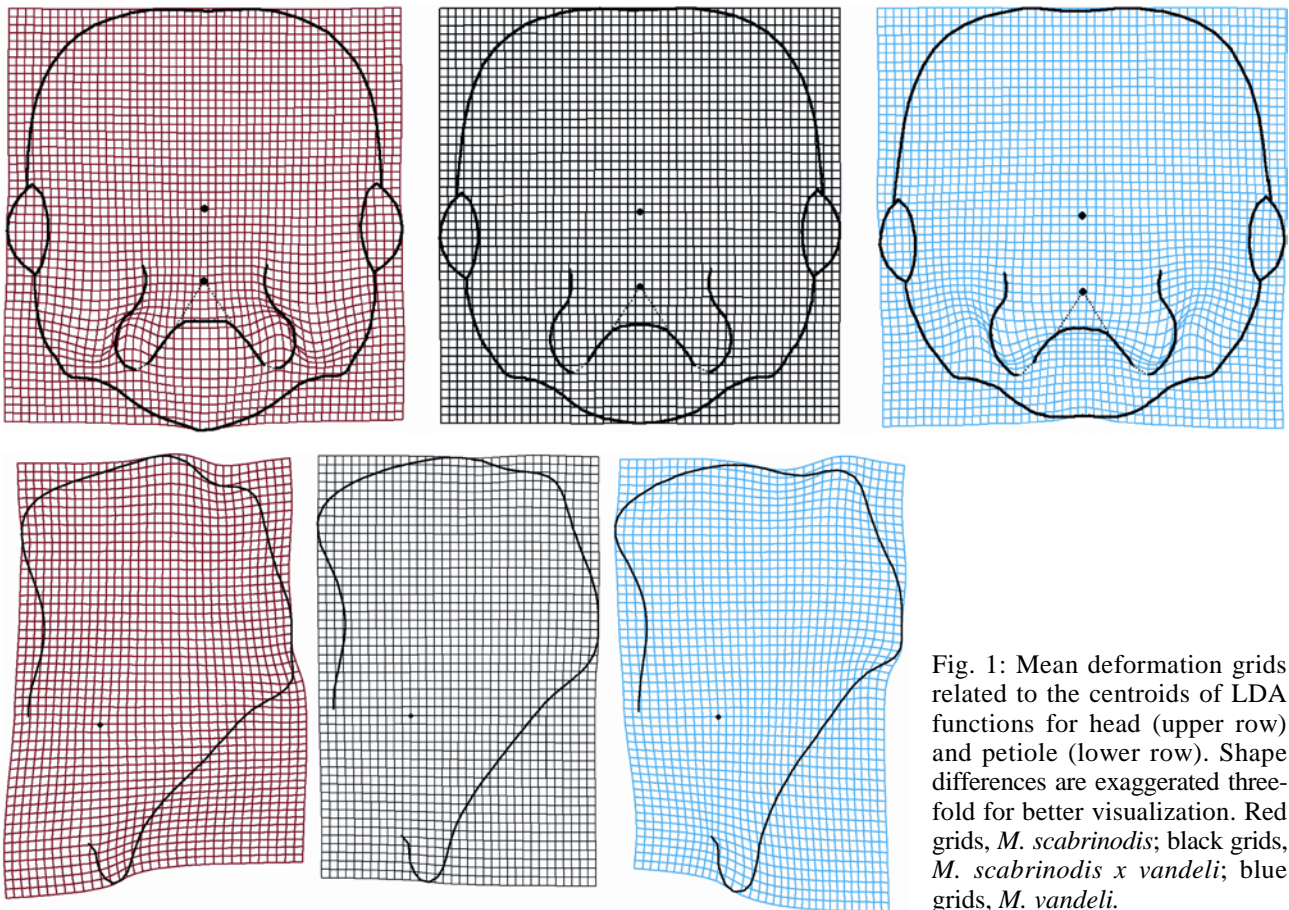


Fig. 1: Mean deformation grids related to the centroids of LDA functions for head (upper row) and petiole (lower row). Shape differences are exaggerated three-fold for better visualization. Red grids, *M. scabrinodis*; black grids, *M. scabrinodis x vandeli*; blue grids, *M. vandeli*.

lysis (LDA) with stepwise character reduction run with the SPSS 16.0 software package. The tpsRegr software program (ROHLF 2009) then calculated a multivariate regression analysis with the input variables being the LDA scores and x-y-shape coordinates of the landmarks and semilandmarks. The software transforms the coordinate information to deformation grids related to the discriminant scores. The resulting mean deformation grids (Figs. 1, 2, 7) are then related to the centroid of the LDA scores and exaggerated deformation grids were formed by multiplying the parameters of LDA with the factor three.

The procedure to identify hybrids: The pre-analysis separation of the parental species was based on stereomicroscopic investigation of the eight discriminative characters presented in the determination key of SEIFERT (2007). The first step of exploratory data analysis was finding a reliable system of separating the parental species using the data of those 281 workers from nest samples not suspected to contain hybrid individuals. The procedure combined a RWs analysis (as explorative data analysis) with a LDA run with the SPSS 16.0 software package (as a hypothesis-driven data analysis). The error of the LDA was checked by leave-one-out cross-validation (LACHENBRUCH & MICKEY 1968, LESAFFRE & al. 1989). RWs analysis was done for the head, clypeus, mesosoma and petiole aspects separately and in an analysis pooling the PWs of all four anatomical aspects. All 18 individuals from the supposedly problematic samples (Baruth – 1965, Zainingen – 138-3, Unterhölzer Wald – 264-4) were then run in this character-reduced LDA as wild cards (i.e., without having imposed

a hypothesis). Individuals, having a discriminant score after this run clearly inside the cluster of a parental species, were now hypothesized of belonging to this cluster. The LDA was repeated until only unclear specimens remained in the wild card group. Basically the same stepwise procedure was also performed with data from the linear morphometrics.

Results and discussion

The basic requirements for geometric analysis are fulfilled: Variation of the specimens in shape spaces was perfectly correlated with tangent space (ZELDITCH & al. 2004) for all anatomical aspects. This allows the use of the tangent plane approximation in further statistical analyses and interpretation of results.

Sizes: Centroid sizes of both species were normally distributed in all anatomical aspects (Asymp. Sig. > 0.05) and means of centroid sizes were significantly different between the species in all anatomical aspects ($p < 0.001$).

Asymmetries: We excluded asymmetric shape variation from hybrid analysis as there was no directional asymmetry demonstrable in the investigated ants with any test system applied and because the later identified hybrid ants did not show significantly larger fluctuating asymmetries (FA) than the parental species in a ANOVA of subtracted Procrustes distances of left and right body sides. We are aware that FA might be an additional source of information as just ant hybrids may show significantly increased FA (SEIFERT 2006). The result in our study ants is not very different from that of preliminary studies on ant hybrids in

Tab. 1: RAV-corrected morphometric data of the ten most-discriminative characters of workers of *M. scabrinodis* and *M. vandeli* and of hybrids *M. scabrinodis x vandeli*.

	<i>Myrmica vandeli</i> (n = 104)	<i>Myrmica scabrinodis</i> <i>x vandeli</i> (n = 8)	<i>Myrmica scabrinodis</i> (n = 187)	F of ANOVA of parental species
ClyEx / CS [%] (1100)	1.81 ± 0.22 [1.27, 2.36]	1.30 ± 0.12 [1.12, 1.55]	0.77 ± 0.25 [0.13, 1.52]	1119.1
FR / CS (1100)	0.350 ± 0.012 [0.327, 0.390]	0.332 ± 0.008 [0.324, 0.346]	0.320 ± 0.011 [0.287, 0.350]	458.5
SL / CS (1100)	0.759 ± 0.017 [0.711, 0.792]	0.786 ± 0.011 [0.764, 0.797]	0.791 ± 0.015 [0.764, 0.838]	285.8
PPHL / CS (1100)	0.188 ± 0.009 [0.169, 0.208]	0.190 ± 0.005 [0.182, 0.199]	0.170 ± 0.010 [0.146, 0.197]	230.3
PoOc / CL (1100)	0.443 ± 0.006 [0.42300, 457]	0.436 ± 0.006 [0.425, 0.442]	0.428 ± 0.010 [0.404, 0.460]	205.6
CL / CW (1100)	1.019 ± 0.016 0.980, 1.057]	1.024 ± 0.012 [1.002, 1.040]	1.040 ± 0.015 [1.002, 1.081]	117.7
CS [µm]	1139 ± 49 [1012, 1260]	1104 ± 38 [968, 1167]	1073 ± 64 [919, 1228]	83.9
PEH / CS (1100)	0.319 ± 0.009 [0.297, 0.355]	0.333 ± 0.005 [0.327, 0.342]	0.330 ± 0.010 [0.308, 0.369]	74.8
PEW / CS (1100)	0.261 ± 0.009 [0.247, 0.288]	0.273 ± 0.008 [0.265, 0.289]	0.275 ± 0.012 [0.245, 0.317]	57.0
PPW / CS (1100)	0.385 ± 0.013 [0.355, 0.419]	0.388 ± 0.009 [0.376, 0.403]	0.397 ± 0.014 [0.356, 0.458]	45.5

Lasius, *Formica* and *Temnothorax* (SEIFERT 2006; B. Seifert, unpubl.). These studies showed a significant increase of FA in hybrid individuals but the indicative value of FA compared to other characters was small. This weak contribution of FA in hybrid analyses is thought to be caused by factors such as poor environmental quality, different forms of stress during ontogenetic development or strong inbreeding which may lead to increased asymmetries also in non-hybrid individuals (MARKOW 1994, HOELZEL & al. 2002).

Deformation grids and interspecific differences: In order to better visualize the very small interspecific shape differences of *M. scabrinodis* and *M. vandeli*, mean deformation grids were exaggerated by a factor of three (Figs. 1, 2). It becomes apparent in head aspect that *M. vandeli* has a relatively wider head, relatively smaller clypeal length, stronger convergence of head sides before and behind eyes, wider frontal lobes, and larger minimum distance of frontal carinae. In clypeal aspect *M. vandeli* shows much larger excavation of anteromedian clypeal margin and wider frontal lobes. The petiole of *M. vandeli* shows a longer ventral profile and a shorter, less straight dorsal profile, not abruptly passing on to the dorsocaudal petiolar slope (i.e., the distinct dorsocaudal petiolar corner typically seen in *M. scabrinodis* is frequently missing). The mesosoma of *M. vandeli* shows straighter, more diverging propodeal spines, and a bigger mesosomal width at the level of the Bulla glandulae metapleuralis. The intermediate position of the sample later identified as hybrid is indicated by its regular and almost rectangular grid in all four anatomical aspects. Summing up, geometric morphometrics exposed, among a number of differences already known, also differences

not discovered to date by conventional morphometrics or subjective character assessment.

Table 1 shows the RAV-corrected data of the ten most discriminative characters of linear morphometrics. Hybrids are intermediate in four of the characters but approach either *M. scabrinodis* or *M. vandeli* in six other characters. This mixture of intermediate and one-parent dominated characters is quite a typical situation for ant hybrids (SEIFERT 1999, 2006; SEIFERT & al. 2010). The best single character is the depth of clypeal excision but measuring this sensitive character requires much care.

Hybrid identification by explorative and hypothesis-driven data analysis: We apparently have clear interspecific differences of the mean shapes for many characters but are these characters sufficient for complete species discrimination on the individual level and a reliable identification of hybrids? *Myrmica scabrinodis* and *M. vandeli* are already separable through an explorative analysis of single anatomical aspects: The RW plots of both head and clypeus aspect (Figs. 3, 4) show separate clusters. RW1 of the petiole aspect (describing 41.7% of total variation) ordinated the ants to species but with considerable overlap and there was almost no separation in the mesosoma aspect (data not shown). A LDA with stepwise character reduction and leave-one-out cross-validation classified 100% of specimens correctly both in the head (28 RWs considered) as well as the clypeus aspect (17 RWs considered). 99.6% correct classification was achieved in petiole aspect (21 RWs) and 98.9% in mesosoma aspect (23 RWs).

In order to amplify the discriminative power for hybrid identification, the PWs of all four anatomical aspects were pooled and 316 RWs were extracted. These 316 RWs were

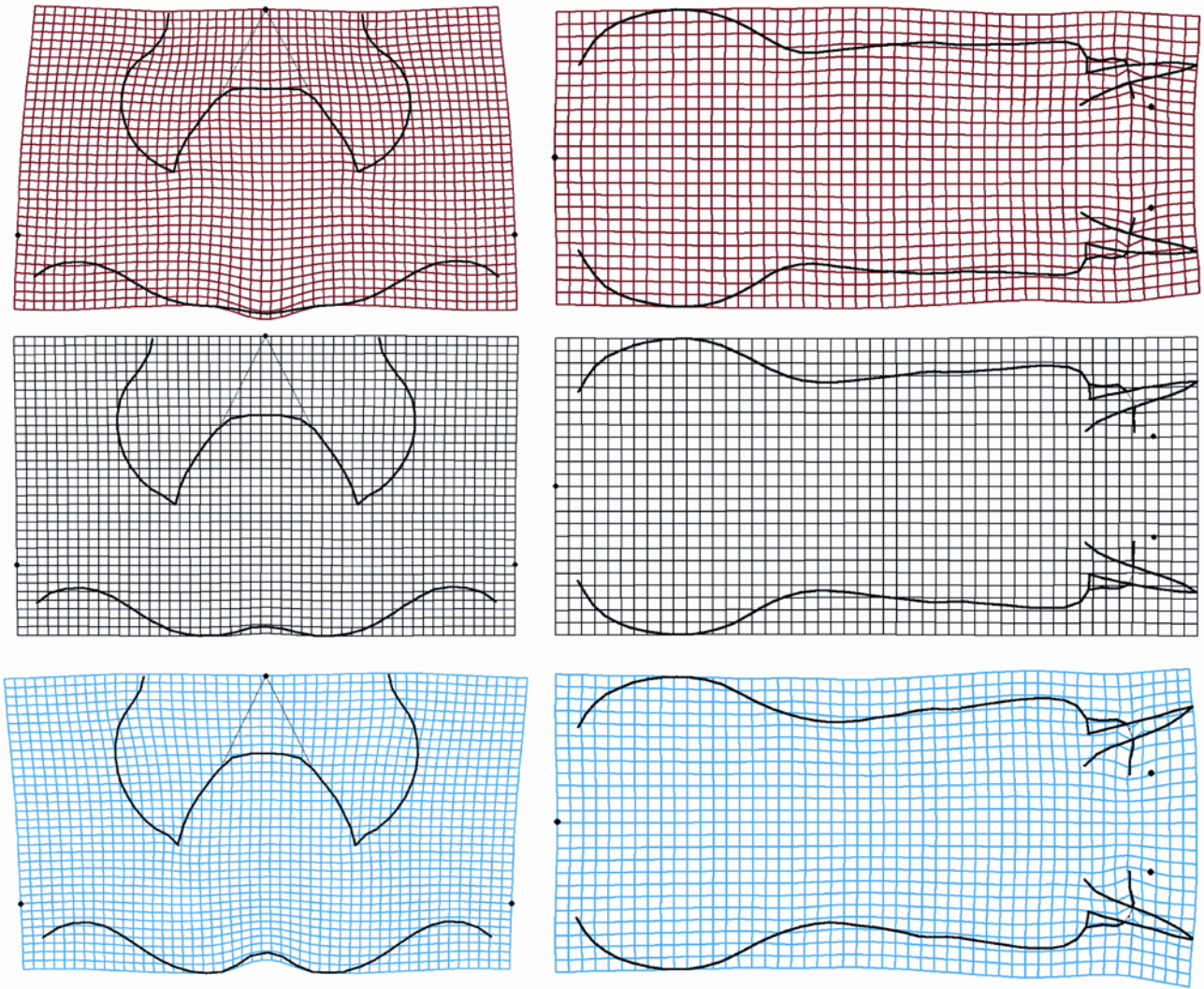


Fig. 2: Mean deformation grids related to the centroids of LDA functions for clypeus (left) and mesosoma (right). Shape differences have been exaggerated three-fold for better visualization. Red grids, *M. scabrinodis*; black grids, *M. scabrinodis x vandeli*; blue grids, *M. vandeli*.

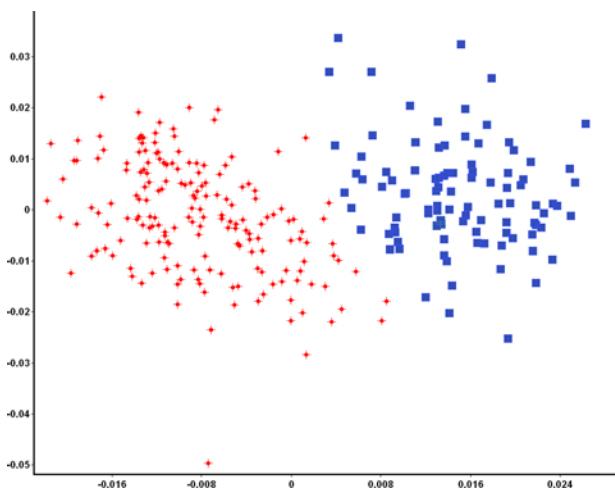


Fig. 3: Scatter plot of the first (abscissa) and second (ordinate) relative warps of the head aspect of *M. scabrinodis* (red cross-dots) and *M. vandeli* (blue squares) which represent 47% of total variance.

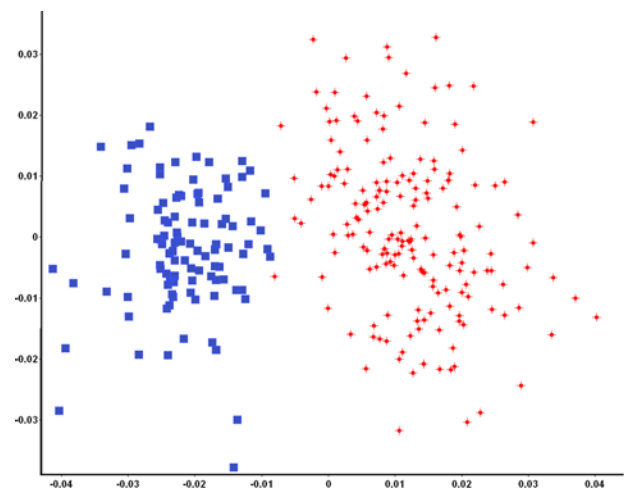


Fig. 4: Scatter plot of the first (abscissa) and second (ordinate) relative warps of the clypeus aspect of *M. scabrinodis* (red cross-dots) and *M. vandeli* (blue squares) which represent 51% of total variance.

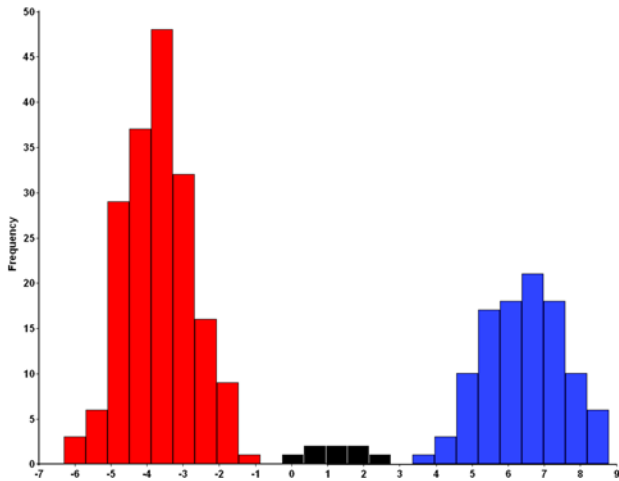


Fig. 5: Histogram of the LDA scores calculated from 25 relative warps in a pooled analysis of all four anatomical aspects in workers of *M. scabrinodis* (red, $n = 187$), hybrids *M. scabrinodis x vandeli* (black, $n = 8$), and *M. vandeli* (blue, $n = 104$).

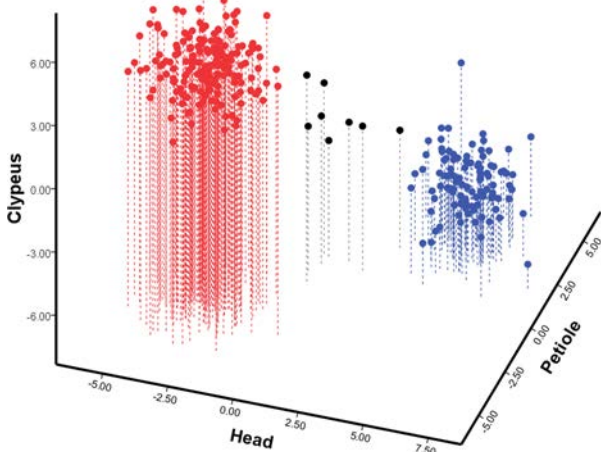


Fig. 6: Three-dimensional plot of the independent LDA scores of the head (28 relative warps considered), clypeus (17 RWs considered) and of petiole aspect (21 RWs considered) in workers of *M. scabrinodis* (red, $n = 187$), hybrids *M. scabrinodis x vandeli* (black, $n = 8$), and *M. vandeli* (blue, $n = 104$).

introduced as characters in a stepwise LDA reducing the number of considered RWs to 25 – this is one fourth of the number of individuals in the smallest class (*M. vandeli*). This condition implies a very low risk of the LDA to confirm a wrong prejudice. The result of the LDA was very clear: for 289 degrees of freedom, the LDA scores of the parental species differed with $F = 6735.1$ if tested in an ANOVA. The three problematic samples, on which no hypothesis has been imposed when running the LDA, were also clearly allocated. The two workers of sample Baruth – 1965 were placed deeply within the *M. scabrinodis* cluster, all eight workers of sample Zainingen – 138-3 were clearly allocated to *M. vandeli* and all eight workers of the sample Unterhölzer Wald – 264-4 were nicely placed in the empty space between the very separate clusters of the parental species (Fig. 5). Basically the same result is achieved when the independent LDA scores of the head, clypeus

and petiole aspects are shown in a three-dimensional plot (Fig. 6).

The two-class LDA applied above was necessary to avoid prejudice of the investigator in the treatment of problematic samples but it showed only the vector separating the parental species and exposing the intermediacy of the hybrids. A three-class LDA (or Canonical Variates Analysis – CVA) adds a second vector possibly describing features of the hybrid in which it deviates from both parents. We ran this CVA as stepwise method, which reduced the number of characters from 316 to 38. The 1st axis, separating the parental species, described 95.9% of variance and had a canonical correlation of $r = 0.983$. The centroids along this vector were situated at -4.099 for the *M. scabrinodis* and at 7.043 for the *M. vandeli* cluster. The centroid of the hybrid at 1.191 shows their almost exactly intermediate position as it was already found in the two-class LDA. The 2nd axis of the CVA described 4.1% of variance and had a significant canonical correlation of $r = 0.741$. The centroids along this vector were at 0.152 for *M. scabrinodis*, at 0.239 for *M. vandeli* and at -6.553 for the hybrids. Accordingly, there should be at least one unique feature of the hybrid sample. Figure 7 shows the mean deformation grids in four body aspects describing the deviation of hybrids from both parents. Unique features of the hybrid are exposed in the petiole aspect only whereas the grids in the other three body aspects are regular and nearly rectangular.

The hybrid phenotypes are not caused by environmental modification or infection: Is it possible that factors other than interspecific hybridization – namely unusual environmental conditions or infections – could have produced a nest population in which all workers show weakly variable hybrid-like phenotypes? The answer is no. The nest Unterhölzer Wald – 264-4 was by no means a founding nest but contained a mature population of large workers in the first, second and probably also third year of life (for age structure and demography of mature *Myrmica* nests see ELMES 1975, ELMES & WARDLAW 1982, ELMES 1987). If environmental conditions were responsible for the intermediate phenotype of this nest population, these must have acted constantly with one and the same result over a minimum of two years – i.e., they must have caused intermediates only and must have inhibited the production of normal phenotypes. Such an outcome is extremely unlikely for a modifying environmental factor which always should show some fluctuation. Another argument against environmental modification is that this sample was found on a small 29 m^2 plot together with four normal *M. scabrinodis* and three normal *M. vandeli* nests which did not contain a fraction of intermediate phenotypes. Furthermore, all published studies on ant hybridization with parallel morphological and genetic documentation (KULMUNI & al. 2010, SEIFERT & al. 2010, STEINER & al. 2010) provide no suggestion of environmental conditions to produce hybrid-feigning phenotypes. Such an outcome is also not known to be caused by infections. Different types of infections, independent if caused by fungi, bacteria, viruses or metazoan parasites (e.g., ELTON 1991, HEINZE & al. 1998, BUSCHINGER & al. 2004, CsÖSZ 2012) never modify the morphology of the whole nest population in a comparable way and there are no parasite-induced ant morphs known with phenotypes reminiscent of interspecific hybrids.

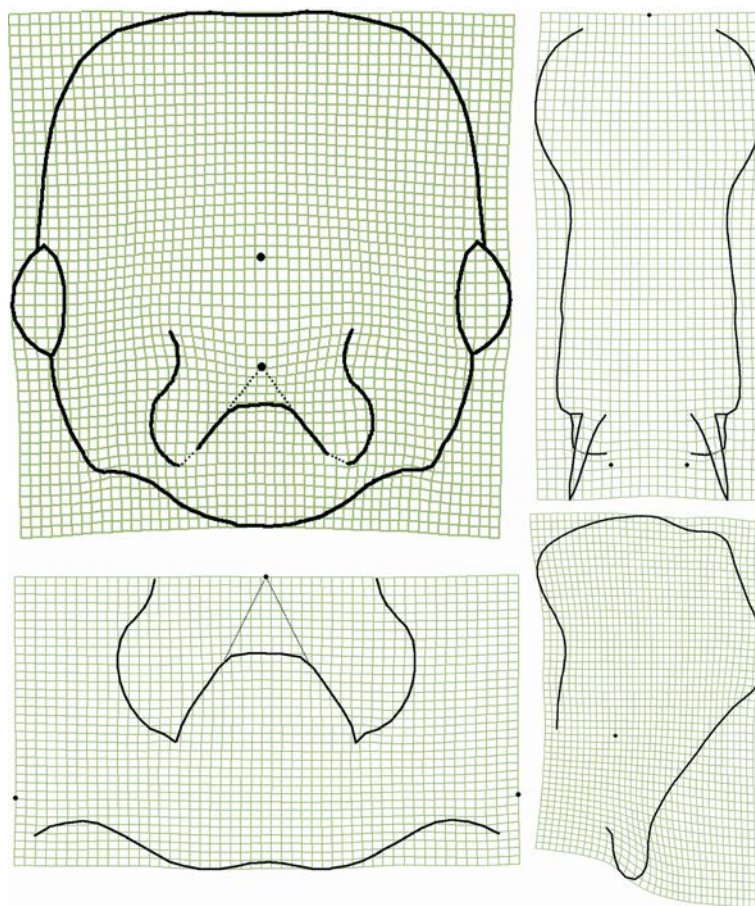


Fig. 7: Mean deformation grids of head, mesosoma, clypeus, and petiole of the hybrid sample of *M. scabrinodis* \times *vandeli* related to the 2nd canonical vector of a three-class canonical variates analysis which describes only 4.1% of total variation. The grids show in which shapes the hybrid differs from both parental species. Shape differences are exaggerated three-fold for better visualization. A significant deviation from a rectangular grid (i.e., unique feature of the sample) is exposed in the petiole aspect only.

Comparing the performance of geometric and conventional morphometrics: Conventional linear morphometrics achieved a basically similar but as a whole less clear result with about 0.5% misidentification. A stepwise LDA, considering absolute size (CS) and 15 RAV-corrected shape characters (SEIFERT 2011) and reducing the number of characters to nine, clearly separated the parental species: for 287 degrees of freedom, the LDA scores of the parental species differed with $F = 3108.0$ if tested in a ANOVA. All eight workers of the sample Unterhölzer Wald – 264-4 were placed between the clusters of the parental species, which is good confirmation of the hybrid identification by geometric morphometrics. The first worker of sample Baruth – 1965 was clearly allocated to *M. scabrinodis* but the other worker was placed in the transition zone between hybrids and *M. scabrinodis*. Seven of the eight workers of the sample Zainingen – 138-3 were clearly allocated to *M. vandeli* but one worker was placed among the sample of the hybrids of Unterhölzer Wald – 264-4.

We conclude that linear morphometrics achieved a smaller discriminative power than geometric morphometrics. For basically the same set of individuals investigated, linear morphometrics separated the parental species with an F value of 3108.0 against one of 6735.1 in the geometric approach. The linear morphometrics made one clear misidentification in a specimen from Zainingen – 138-3 and one ambiguous placement of the second specimen from Baruth – 1965. In a subjective side-by-side comparison of the sample Zainingen – 138-3 with three syntopic *M. vandeli* samples no difference was noted. The problematic specimen from Zainingen (which is in sculpture, overall shape and hairiness fully comparable to its seven *M. vandeli*

nest mates) has a longer scape, the petiole is distorted and the whole waist is thicker. As result, RAV-corrected scape length, petiole width, postpetiole width, and petiole height ratios are clearly within the *M. scabrinodis* range and the LDA score of linear morphometrics places this abnormal specimen erroneously within the true hybrids. This indicates that accidental morphological aberration in particular body parts is more likely to affect the results in linear morphometrics – in other words: Geometric morphometrics with its much more complex character system should lead to more robust conclusions.

It remains unclear if geometric morphometrics was closer to the truth than linear morphometrics in the second worker from the sample Baruth – 1965. This specimen showed a mesosomal sculpture and clypeal excision reminiscent of *M. vandeli*. Hence, it might possibly represent a *M. scabrinodis* with introgression of some *M. vandeli* alleles. However, as no *M. vandeli* material is known so far from the whole landscape around Baruth and because its nest mate is a clear *M. scabrinodis*, a hybrid hypothesis seems less probable. On the other hand, this sample represents the oldest available material from the region (year 1965) and a sympatric population of *M. vandeli* might well have existed at this time. *Myrmica vandeli* is strongly endangered in Saxony by habitat destruction which may have been caused early in the region by altered management of moist and wet grassland after the finishing of traditional peasant cultures due to socialist collectivization in 1961.

Summing up, geometric morphometrics basically offers a higher potential than conventional morphometrics but it is, with the currently achieved speed of image recording and in the absence of a software assisting landmark / semi-

landmark fixation, slower than linear stereomicroscopic morphometrics. The efficiency of both approaches is compared in the next section.

Comparing the expenditure of time in geometric and conventional morphometrics: To provide a realistic picture of the efficiency of the methods, we give a more detailed account of the time required for different working steps in conventional linear morphometrics and geometric morphometrics. Recording 16 characters of linear morphometrics by conventional stereomicroscopic investigation, assisted by simple input software, 23 measurements and ± 48 minutes are needed per specimen. Working time for data acquisition in geometric morphometrics was much larger. Z-stack imaging of four anatomical aspects for geometric morphometrics needed 60 minutes. This perhaps cannot be reduced significantly by improving working routines. Manually fixing 293 landmarks and semilandmarks on the screen required another 120 minutes per specimen (30 in head, 30 in clypeus, 40 in mesosoma and 20 in petiole) because each aspect was digitized twice. Two-fold digitizing was required by the tpsRelw software for the bilaterally symmetric body parts. This methodology improves the accuracy and allows determining the measuring error of the subject but this mode of data acquisition took 375% of the time needed in conventional linear morphometrics. Applying the software program BigFlip of the IMP series by H.D. Sheets (ZELDITCH & al. 2004) – which does not require two-fold manual fixing of the landmarks on the screen in the bilaterally symmetric body parts head, clypeus and mesosoma – reduces the time for fixing landmarks / semilandmarks from 120 to 70 minutes (15 in head, 15 in clypeus, 20 in mesosoma and 10 in petiole). This gives a total of 120 minutes per specimen or 260% of the data acquisition time needed for conventional linear morphometrics. We have no data at hand if the loss in accuracy compared to the two-fold landmark fixation is really relevant for the final result. A further acceleration of the procedure can be achieved when software for automatic projection or superimposition of a combined fan-and-comb frame which assists landmark / semilandmark fixation will be available. This frame, anchoring on six landmark points, allows for equal linear and angular spacing of semilandmarks. We tested this on the screen with a frame dummy and found the time for fixing landmarks / semilandmarks to be reduced to 40%. Such software can be programmed with moderate costs and reduces the time for fixing landmarks / semilandmarks from 70 minutes in the last mentioned procedure to 24 minutes (6 in head, 6 in clypeus, 8 in mesosoma, and 4 in petiole). This gives a total of $60 + 24 = 84$ minutes for one-fold and $60 + 48 = 108$ minutes per specimen for two-fold digitizing or 175 - 225% of the data acquisition time for conventional linear morphometrics. This figure describes the efficiency currently achievable without spending much money for software programming.

A further acceleration could be achieved by software capable of automatically recognizing landmarks. According to the information of a German company specialized in microscopic image recognition such software is expected to recognize about 70% of the landmarks automatically if some 30,000 € of development costs are spent. This would mean a reduction of data acquisition time to $60 + 8 = 68$ minutes per specimen. These figures show that the main efficiency problem is the first working step – 60 minutes of

imaging time. Modern Laser-Scanning-Microscopes, the luxury alternative to conventional z-stack photomicroscopes, provide images of small insects with a supreme optical resolution and x-y-z coordinates with an error $< 2 \mu\text{m}$ but the time of image generation is once again much larger and these LSMs have 3 - 6 times higher acquisition costs.

Considering the currently available 84 - 108 minutes per specimen, this is probably not the system for taxonomic routine investigation of small insects or a method of which practitioners of ecosystem surveys dream but its application in particular, very difficult problems is fully justified. Much developmental work has to be done and much money to be spent until geometric morphometrics becomes the tool of which ant taxonomists dream.

What advanced morphometrics can perform but modern molecular genetics not: One may ask why applying such laborious morphological methods is necessary in the era of whole-genome scans and always faster operating molecular genetics. We argue that morphology-based investigation methods will remain the backbone of taxonomy and biodiversity research, not least for practical reasons. Why do several authors consider morphology as the core element within integrative taxonomy and biodiversity research (SEIFERT 2009, BOERO 2010, SCHLICK-STEINER & al. 2010, STEINER & al. 2009) and what is its irreplaceable function? For taxonomic work, comprehensive comparison of any (potentially) new species with established species is indispensable. The only genuine references of an established species and testable link to Zoological Nomenclature are (a) its original description and (b) its primary type specimen. These two references have absolute priority in the taxonomic decision making process. The original descriptions of 99% of the 1.2 million zootaxa described since LINNAEUS (1758) are based on morphology and other phenotypic characters and lack any genetic information. Hence, genetics cannot establish a link to nomenclature via original descriptions but morphology can. Very often, however, advanced morphologists cannot decide on the basis of original descriptions when these do not contain diagnostic information. Then, investigation of the unique type specimen (the holo-, lecto- or neotype) is the only way to find a testable and clear decision. Though extraction of DNA from freshly collected arthropod material may be possible with only minor damage of external characters (e.g., ROWLEY & al. 2007, HUNTER & al. 2008), the problem of non-destructive and yielding extraction from dried, older arthropod vouchers (i.e., from the vast majority of existing type specimens) remains frequently impossible. To sacrifice a small spot of foot epithelium of a large-bodied bird type specimen represents no problem. However, small-bodied, often tiny arthropods comprise 84% of worldwide eumetazoan biodiversity and no responsible curator of an arthropod collection, would agree to the (often yield-less) destructive sampling or chemical extraction procedures in a unique and irreplaceable primary type specimen. Morphological methods, light microscopy in particular, allow non-destructive investigation of even the oldest insect specimens, which have often experienced complete DNA degradation due to environmental conditions, storage medium or preparation.

The problem also extends beyond types: an immeasurable number of other, partly historical or palaeontological vouchers need to be identified at any one time, for a wide

range of biological fields (cf. KÜHNELT 1974, FRANCOEUR 1976, ALBERCH 1993, BROOKE 2000, SCHLICK-STEINER & al. 2003, WHEELER 2003, SUAREZ & al. 2005, STEINER & al. 2006). Most of these vouchers can only be identified by advanced morphological methods. If there are, for instance, only head capsules of ants conserved in a 10000-year-old deposit of a peat bog or a lake, geometric morphometrics, as it is presented in this paper, offers a good chance for correct species identification but DNA analysis would likely fail.

Biological background of the reported hybridization: Interspecific hybridization in *Myrmica* is assumed to occur rarely and it may be asked which biological background allowed the reported hybridization. The first two factors to be considered are the very close spatial association of the species and the most probable, facultative, temporary social parasitism of *M. vandeli*. There is no site of *M. vandeli* known without syntopically occurring *M. scabrinodis* and the frequency of *M. vandeli* nests containing *M. scabrinodis* workers is 17% in the all-area mean in Baden-Württemberg and up to 69% for a particular patch. This constant spatial confrontation or coexistence should have caused the build-up of strong reproductive barriers. Even if the queens of the *M. scabrinodis* colonies taken over by *M. vandeli* would die very soon or would be inhibited in their production of sexuals, *M. scabrinodis* worker oviposition should not be inhibited fully. Hence, mixed nests should produce at least some *M. scabrinodis* males in addition to the *M. vandeli* sexuals. The time of sexual production of the two species is largely overlapping in Central Europe (SEIFERT 2007) and there is a high likelihood that mature males of *M. scabrinodis* meet mature *M. vandeli* gynes at least inside or in front of the nests if not joining mixed mating flights. Initiation of mating behavior in males, also of species performing an ordinary nuptial flight, does not necessarily require a flight to have taken place before: it is known from outdoor observations in *M. rugulosa* and *M. ruginodis* and a laboratory crossbreeding experiment between *M. rugulosa* and *M. speciosoides* (SEIFERT 2007; B. Seifert, unpubl.) that males try to mount by force any female (including their worker nest mates) when these walk on ground in front of the nest entrances before take-off. Hence, there could be plenty of possible places and occasions in which interspecific mating between *M. scabrinodis* and *M. vandeli* might occur. Under these assumptions, the observed hybrid frequency of 0.44% (from *M. vandeli*'s point of view) seems low and indicates quite effective reproductive barriers.

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