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Wolbachia endosymbionts distort DNA barcoding in the parasitoid wasp genus *Diplazon* (Hymenoptera: Ichneumonidae)

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Molecular species delimitation has the potential to speed up both discovery and description rates for new species. However, several studies have shown a limited performance of the standard DNA barcoding approach which relies on a single mitochondrial gene, cytochrome oxidase 1 (*COI*). Besides incomplete lineage sorting or a low substitution rate, distortion of mitochondrial inheritance patterns by bacterial endosymbionts could explain the failure of barcoding. Numerous reviews have discussed this phenomenon, but only few empirical examples exist. In the present study, we examine the effect of *Wolbachia* bacteria on barcoding in the parasitoid wasp genus *Diplazon*. Although integrative taxonomy recognizes 16 species, *COI* only recovers up to ten. Adding multivariate morphometrics, genotyping a fast-evolving nuclear gene (*ITS2*) and screening the *Diplazon* species for *Wolbachia*, we show that the failure of DNA barcoding coincides with the presence of the endosymbiont. Two species even share identical *COI* haplotypes and *Wolbachia* strains, even though *ITS2* suggests that they are not closely related. This is one of very few examples of mitochondrial DNA introgression between well-established insect species, facilitated by *Wolbachia*. We review similar reports, provide a list of criteria to identify endosymbiont-mediated introgression, and discuss the prevalence and impact of this phenomenon on insect systematics and evolution.

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ADDITIONAL KEYWORDS: cytochrome oxidase 1 – hybridization – introgression – species delimitation.

INTRODUCTION

Molecular data have the potential to greatly speed up the taxonomic workflow and facilitate species discovery, as demonstrated in numerous studies (e.g., Blaxter, 2004; Butcher *et al.*, 2012; e.g., Tautz *et al.*, 2002; Vogler & Monaghan, 2007). Such an increase in the speed at which we describe the biodiversity on our planet is sorely needed because species are going extinct at unprecedented rates as a result of the destruction of natural habitats, the introduction of invasive species, and other consequences of human activities (Pimm *et al.*, 2014). For animals, the use of a single marker, part of the mitochondrial cytochrome *c* oxidase subunit 1 (COI), has been advocated by the Barcode of Life Consortium (www.barcodeoflife.org) (Hebert, Ratnasingham & deWaard, 2003b; Hebert *et al.*, 2003a). Especially in earlier studies, this 'barcoding' locus was reported to be sufficient to delimit the vast majority of species examined (Hajibabaei *et al.*, 2006; Gómez *et al.*, 2007; Derycke *et al.*, 2008; Smith *et al.*, 2008; Schmidt *et al.*, 2015).

Soon, however, reports on inconsistencies between DNA barcoding and established species hypotheses started to accumulate; the prevalence of species non-monophyly and failures of threshold-based delimitation methods were estimated in different studies to range between approximately 10% and 30% (Funk & Omland, 2003; Meier *et al.*, 2006;

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Bergsten *et al.*, 2012). The most commonly invoked explanations for such discordances are inadequate taxonomy, rapid speciation rates relative to the substitution rates of the marker, incomplete lineage sorting, and hybrid introgression. The first issue, inadequate taxonomy, either in the form of misidentifications or poorly supported species hypotheses, is especially important when unverified data are retrieved from databases, or in poorly studied and highly diverse taxa (Collins & Cruickshank, 2012). This issue can only be resolved by careful taxonomic work. The second issue, insufficient variability, can be ameliorated by informed marker choice; variability has to be sufficiently high to allow distinguishing between very closely-related species, and some statistical approaches for species delimitation even require some variation at the intraspecific level (Pons *et al.*, 2006; Zhang *et al.*, 2013). Mitochondrial DNA (mtDNA) is advantageous in this respect because of comparatively high evolutionary rates and a corresponding high per-base-pair information content at or around the species boundary (Brown *et al.*, 1982; Mindell & Thacker, 1996; Lin & Danforth, 2004; Mueller, 2006; Simon *et al.*, 2006; Zink & Barrowclough, 2008); on the other hand, nuclear markers (especially introns) often still need to be established for non-model taxa. The third issue, incomplete lineage sorting, leads to ancestral polymorphisms being retained in different, closely-related species, resulting in discordances between gene trees and species trees (Knowles & Carstens, 2007; Rosenberg & Tao, 2008; Edwards, 2009; Prufer *et al.*, 2012). Once more, mtDNA is at an advantage here compared to nuclear DNA because it will attain complete lineage sorting and thus reciprocal species monophyly more quickly as a result of the four-fold smaller effective population size of the mitochondrial compared to the nuclear genome.

By contrast, the last issue (i.e., a higher susceptibility of mtDNA to hybrid introgression) has been discussed as a major drawback of mtDNA (Ballard & Rand, 2005; Hurst & Jiggins, 2005; Bachtrog *et al.*, 2006; Rubinoff, Cameron & Will, 2006). After a hybridization event, the recombining nuclear genes are continuously eliminated with successive backcrossing, but recombination does not or only very rarely occur in mtDNA. Hybridization thus sometimes leads to the complete introgression of a foreign mitochondrial genome into another species (Paquin & Hedin, 2004; Berthier, Excoffier & Ruedi, 2006; Edwards & Bensch, 2009; Galtier *et al.*, 2009; Petit & Excoffier, 2009; Nicholls *et al.*, 2012). Besides the lack of recombination, selective sweeps caused by hitchhiking with selfish genetic elements such as endosymbiotic bacteria have been shown to greatly influence the population biology of mtDNA in a number of species (Heath *et al.*, 1999; Hurst & Jiggins, 2005; Raychoudhury *et al.*, 2009); the success of mtDNA-based species delimitation could thus be dependent on the infection history of the species under consideration.

The impact of intracellular endosymbionts can be manifold. In the best-case scenario with respect to DNA barcoding, the endosymbionts remain within one host species (or are transferred between species only infectiously, i.e., not via hybridization; see below). By manipulating the reproductive biology of their hosts to maximize their own transmission, as demonstrated, for example, in the α-proteobacterium *Wolbachia*, they cause a decrease in the intraspecific mtDNA variability, in the extreme case causing the spread of a single haplotype within a whole population or even species (Turelli, Hoffmann & McKechnie, 1992; Jiggins, 2003; Charlat *et al.*, 2009). They might thus contribute to the 'barcoding gap' (i.e., the difference between intraspecific and interspecific variation) and thus even improve the performance of DNA barcoding. Endosymbionts only cause problems if an infection is passed on between species through a (potentially very rare) hybridization event; under such a scenario, the bacteria are transferred alongside a foreign mitochondrium and, if the bacterium manages to successfully spread through the new host species through vertical transmission, it will drag the mtDNA with it.

The numerous reviews that discuss this mechanism of endosymbiont-mediated mtDNA introgression (Johnstone & Hurst, 1996; Jiggins, 2003; Ballard & Rand, 2005; Hurst & Jiggins, 2005; Galtier *et al.*, 2009) draw on very few convincing empirical examples. For a study to provide plausible evidence for the role of an endosymbiont in facilitating mtDNA introgression, it needs to include both donor and recipient species and demonstrate a strict association of both their mtDNA and endosymbiont strains. To our knowledge, there are currently only six studies that fulfil these requirements (Ballard, 2000; Jiggins, 2003; Narita *et al.*, 2006; Whitworth *et al.*, 2007; Gompert *et al.*, 2008; Raychoudhury *et al.*, 2009).

In the present study, we provide evidence for endosymbiont-mediated mtDNA introgression from a group of parasitoid wasps. The genus *Diplazon* has recently been revised on a mainly morphological basis, with the discovery of four new species in Europe (Klopfstein, 2014). Twenty Western Palaearctic species are currently recognized. We analyzed several specimens from 15 species from Switzerland and Sweden and one North American species with DNA barcoding and found a very poor recovery rate of the morphologically defined species. Parasitoid wasps might be even more prone to *Wolbachia* infections

than other insects because of the additional infection pathway via their arthropod hosts (Cook & Butcher, 1999; Heath *et al.*, 1999). We thus screened the wasps for *Wolbachia* and found that infections usually coincide with a failure of DNA barcoding and vice versa. To examine the potential role of *Wolbachia* in distorting mtDNA inheritance through hybrid introgression, we compiled a morphometrics dataset to support the morphological species concepts, studied three nuclear and one additional mitochondrial marker for the wasps, and performed multilocus strain typing for the endosymbiotic bacteria.

MATERIAL AND METHODS

Taxon sampling and identification

We identified 140 specimens of 15 European species of *Diplazon* parasitoid wasps from Switzerland and Sweden (Table 1; see also Supporting information, Table S1) using the keys provided in a recent morphological revision of the group (Klopfstein, 2014). A North American species, *Diplazon bradleyi*, was added together with five outgroup species of the same genus group as *Diplazon* (Klopfstein, Kropf & Quicke, 2010; Klopfstein *et al.*, 2011). Because of the importance of the sculpture of the tergites, mesoscutum and mesopleuron for delimiting species in this genus, we examined all specimens under the same lighting, a 23-W energy saving lamp. Whenever a specimen did not entirely match the morphological species concepts in the revision, identifications were marked with a 'cf.'. This was also the case for the specimens of *Diplazon tibiatorius* with dark hind coxae, which are considered to belong to this species despite this deviation from the common morphotype of *D. tibiatorius* with orange hind coxae (Klopfstein, 2014). Scientific names and morphological terminology are employed sensu Klopfstein (2014).

Morphometric analysis

The morphological differentiation of *Diplazon* species is largely based on colour and sculpture of the integument (Klopfstein, 2014). To some extent, such features are subjective and depend on taxonomic expertise. We thus conducted a shape principle component analysis (PCA) using an independent set of measurements to establish how well the qualitative morphological differentiation is supported by the measurements. We measured 14 characters covering most body parts (see Supporting information, Tables S2, S3). The selection corresponds to the measurements used for calculating some of the typically used body ratios in Ichneumonidae (Townes, 1969; Klopfstein, 2014).

We applied the multivariate ratio analysis (MRA) of Baur & Leuenberger (2011) to our data. MRA is a relatively new approach that is especially suited for analyzing body measurements in a taxonomy context because it offers several tools for the analysis of size

Table 1. Summary of taxon and gene sampling

Diplazon species	Specimens*	Countries†	*COI*	*ITS2*	*28S*	*EF1α*	*NADH1*
D. albotibialis	2f	CH, SE	2	2	2	2	2
D. annulatus	4f, 1 m	CH, SF	5	5			
D. bradleyi	1f	US	1	1	1	1	1
D. deletus	8f	CH, SE	8	8	2	1	1
D. flixi	11f, 3 m	CH	14	14	2	1	1
D. hyperboreus	2f	SE	2	2	1	1	1
D. laetatorius	8f	CH, ES, SE, US, ZM	8	8	4	2	2
D. orientalis	3f, 1 m	TH	4	4	1	1	1
D. pallicoxa	3f	CH, SE	3	3	1	1	1
D. parvus	9f	CH, SE	9	6			
D. pectoratorius	7f	CH, SE, SF	7	7	2	1	1
D. scutatorius	11f	CH, SE	11	11	2	2	2
D. tetragonus	19f, 3 m	CH, SE	22	21	3	1	1
D. tibiatorius	5f	CH, SE	5	5	4	2	2
D. varicoxa	21f, 6 m	CH, SE	27	25	3	3	3
D. zetteli	9f, 4 m	CH	13	12	1	1	1

*Number of female (f) and male (m) specimens included in the analysis.
†Country of origin of the specimens. CH = Switzerland; ES = Spain; SE = Sweden; SF = Finland; TH = Thailand; US = United States of America; ZM = Zambia.

and shape in the multivariate geometrical framework (Baur *et al.*, 2014; Schweizer, Hertwig & Seehausen, 2014). Here, we computed a shape PCA and plotted the first shape PC against isometric size, defined as the geometric mean of all variables (see Supporting information, Fig. S1). Graphic visualization of the correlation of size with shape allowed us to estimate the amount of allometry in the data (Klingenberg, 1998) (i.e., to judge the impact of an indirect size effect on the separation of some taxa). Morphometric analyses were calculated with R, version 3.1.2 (R Core Team, 2014), using slightly modified R-scripts provided by Baur *et al.* (2014). Scatterplots were generated with the package 'ggplot2' (Wickham, 2009).

Molecular methods

Genomic DNA was extracted from whole specimens preserved in 80% ethanol using the Promega Wizard kit for blood and tissue extractions. Vouchers and DNA samples are kept at the Natural History Museum in Bern and the Swedish Museum of Natural History in Stockholm (Table 1; see also Supporting information, Table S1). Approximately 700 bp from the 5′ end of the mitochondrial *COI* were amplified using the primers LCO and HCO designed by Folmer *et al.* (1994). To obtain approximately 800 bp of the nuclear ribosomal RNA (rRNA) internal transcribed spacer 2 (*ITS2*), we used the primers designed by Quicke *et al.* (2006). Three additional markers, part of the nuclear 28S rRNA (*28S*), the F2 copy of elongation factor 1-α (*EF1α*) (Klopfstein & Ronquist, 2013), and the mitochondrial NADH 1 gene (*ND1*), were taken from previous studies (see Supporting information, Table S1) (Klopfstein *et al.*, 2010, 2011).

Polymerase chain reactions (PCR) were carried out in 20-μl final volumes using Promega GoTaq Flexi DNA Polymerase kits. Final volumes contained 30 pmol of $MgCl_2$, 16 pmol of both primers, 4 pmol of each dNTP, 0.3 U Taq polymerase and 2 μl of genomic DNA. PCR conditions were: 94 °C for 2 min, 35 cycles of 30 s at 94 °C, 30 s at the respective annealing temperature (51 °C for *COI* and 49 °C for *ITS2*), and 1 min at 72 °C, followed by a final extension at 72 °C for 10 min. PCR products were either purified with the GFXTM DNA and Gel Purification kit (Amersham Biosciences) or by the purification service of Macrogen Korea. The PCR products were sequenced on an ABI 377 automated sequencer using Big Dye Terminator technology (Applied Biosystems). All new sequences (112 new sequences of *COI* and 134 of *ITS2*) have been deposited in GenBank under accession numbers KR230498 to KR230743 (see Supporting information, Table S1).

Endosymbiont screening and strain typing

To test whether diplazontine wasps are infected with endosymbionts, we performed a preliminary PCR screening of 32 female specimens from a variety of species, including eight *Diplazon* species (*Diplazon deletus*, *D. flixi*, *D. laetatorius*, *D. parvus*, *D. pectoratorius*, *D. tetragonus*, *D. varicoxa*, and *D. zetteli*), for *Cardinium hertigii*, *Rickettsia* sp., *Spiroplasma ixodetis*, *S. poulsonii*, and *Wolbachia pipientis*, using the primers listed in Duron *et al.* (2008). We obtained positive results only in twelve cases for *Wolbachia*, and none for any of the other endosymbionts. We thus focussed on *Wolbachia* for further analyses within the genus *Diplazon*.

We performed PCR screening on one to ten female specimens per species using primers that amplify a fragment of the *Wolbachia* surface protein gene (*wsp*) (*wsp81F* and *wsp691R*; (Braig *et al.*, 1998). These primers have been used successfully in the past to test for *Wolbachia* infections in Hymenotpera (Beukeboom & Pijnacker, 2000) and showed a good performance, especially for supergroup A and B Wolbachia, the two groups previously found in hymenopterans (Simões *et al.*, 2011). As a control for the quality of the extracted DNA, we amplified the ribosomal *28S* gene, which is specific to eukaryotes, alongside the *Wolbachia* screening, using the forward primer designed by Belshaw & Quicke (1997) and the reverse primer from Mardulyn & Whitfield (1999) and the PCR conditions described above. Specimens tested positive for *Wolbachia* in the first round were used as positive controls in a second round of screening.

At least one infected individual per species was then typed using a multilocus sequence typing approach relying on five house-keeping genes (Baldo *et al.*, 2006), complemented by the faster-evolving *wsp* gene; the general primers were used as listed in Baldo *et al.* (2006) (http://pubmlst.org/wolbachia). Because multiple infections were detected in several species, we used molecular cloning in *Escherichia coli* with the Topo Ta Cloning kit (Life Technologies) to separate the different products of the *wsp* gene in some specimens (Table 2). Four clones were sequenced per specimen. Because multilocus sequence typing is not possible for multiple infections without making assumptions about the prevalence of different allele combinations at the five loci (Arthofer *et al.*, 2011), we only resolved the full sequence typing for the single infections. Nevertheless, we studied some of the double-peaked sequences of the multiple infections and found that they were in part in accordance with multilocus sequence typing (MLST) strain types already found as single infections in other specimens (Table 2); however, as a

result of the highly speculative nature of these associations, we only submitted the strain information from single infections to the *Wolbachia* MLST database and to Genbank (accession numbers KR230444 to KR230477 for the MLST loci and KR230478 to KR230497 for the *wsp* gene).

Alignment and calculation of pairwise distances

The sequences of the protein-coding *COI* were aligned with MUSCLE (Edgar, 2004) after translation into amino acids using MEGA, version 6.06 (Tamura *et al.*, 2013). Alignment was straightforward because no indels were detected. In *ITS2*, the alignment posed more problems because of numerous indels of varying length. We thus only aligned the ingroup taxa and rooted the tree according to previous multigene phylogenetic analyses (Klopfstein *et al.*, 2010, 2011). Alignments can be downloaded from TreeBASE (http://purl.org/phylo/treebase/phylows/study/TB2:S17676).

Uncorrected pairwise distances (*p* distances) were calculated in MEGA with pairwise deletion. Plots were produced in R, version 3.1.2 (R Core Team, 2014). To measure the performance of barcoding, we used the threshold approach because it does not require strict reciprocal monophyly of all the species. We tried thresholds of 2%, 1%, and 0.5% uncorrected *p* distance.

Phylogenetic analysis

Phylogenetic analyses of the *COI* and *ITS2* genes were performed under a Bayesian and a maximum likelihood (ML) approach. For the Bayesian analyses, we used MrBayes version 3.2.2 (Ronquist *et al.*, 2012) with a 'mixed' substitution model (integrating over the space of possible submodels of the general time-reversible model; Huelsenbeck, Larget & Alfaro, 2004) and gamma-distributed among-site rate variation, including a proportion of invariant sites. *COI* was run with the combined first and second codon position in one and the third codon position in a second partition, *ITS2* was unpartitioned. The four independent Markov chain Monte Carlo (MCMC) runs with one cold and three heated chains were run for 10 000 000 generations and sampled every 1000th generation. As a conservative burn-in, we used half of the samples. Convergence was good as judged from the average standard deviation of split frequencies (ASDSF) for the topology parameter and the potential scale reduction factor (PSRF) for the scalar parameters (ASDSF = 0.0064 for *COI* and 0.0043 for *ITS2*, PSRF < 1.002 for all parameters). For the ML analyses, we used RAXML version 8.1 (Stamatakis, 2014), with the GTR model and partitioning as above. Clade support was assessed by 1000 bootstrap replications.

Single-gene trees were also obtained for three additional markers (*28S*, *EF1α*, *ND1*) but for a reduced set of specimens (see Supporting information, Table S1). For the single-gene analyses, we ran MrBayes as specified before but with two independent runs only. To obtain a good estimate of the species tree needed to test for a correlation between *Wolbachia* infections and a failure of barcoding in a phylogenetic contest, a concatenated analysis of all five genes was performed using a single sequence sampled randomly for each species. Settings were chosen as for the *COI* and the *ITS2* analyses, with the protein-coding genes partitioned into combined first and second versus third positions. All analyses were run on the University of Bern Linux Cluster UBELIX.

Correlation analysis

To determine whether the *Wolbachia* infection status showed any correlation with the performance of the mitochondrial COI in species delimitation, we scored each species as being infected or not and as being recovered in the most sensitive analysis with the threshold set at 0.5% uncorrected *p* distance. Because the *Wolbachia* infections did not appear to be independent from the phylogeny, we had to use a comparative statistical method that corrects for the phylogenetic relationships. We used BayesDiscrete from the BayesTraits, version 2.0 (Pagel & Meade, 2006) under an independent and a dependent model of evolution. We run both a ML and a Bayesian approach. For ML, we used the best-scoring tree found during the ML search on the five-gene dataset in RaxML as a test phylogeny. Likelihoods obtained under the two models with 1000 ML attempts per tree were compared by a likelihood-ratio test. For the Bayesian approach, we evenly sampled 1000 trees from the post-burn-in phase of the four independent runs in MrBayes to represent the posterior distribution of topologies. The dependent and independent models were compared using a Bayes factor test, running both in standard mode (i.e., assuming a fixed number of four rates under the independent and eight under the dependent model) and in the reversible-jump mode (which tries all combinations of equal and zero rates).

RESULTS

COI barcoding

The sequences of the barcoding portion of *COI* (5′ portion of the gene) of 140 specimens of *Diplazon*

Table 2. *Wolbachia* infection status, barcoding success, and *Wolbachia* MLST and *wsp* profiles in *Diplazon* and related species

Species	*Wolbachia* infection	Individuals inf/test*	Success barcoding†	Ind #‡	ST§	*CoxA*	*FbpA*	*FtsZ*	*GatB*	*HpcA*	*wsp*¶	*HVR1*	*HVR2*	*HVR3*	*HVR4*
Diplazon albotibialis	None	0/2	0												
Diplazon annulatus	Multi	1/2	0												
Diplazon bradleyi	Multi	1/1	1								?				
Diplazon deletus	Single	1/4	0	706	92	59	17	3	54	68	442	51	55	15	25
Diplazon flixi	Single	1/3	0	832	92	59	17	3	54	68	688**	51	269**	15	25
Diplazon hyperboreus	None	0/2	1												
Diplazon laetatorius	None	0/4	1												
Diplazon orientalis	None	0/3	1												
Diplazon pallicoxa	None	0/3	1												
Diplazon parvus	Triple or more	3/5	0	625							23/338/?	1/1/17	12/12/9	21/21/?	19/269/18
Diplazon pectoratorius	None	0/3	0												
Diplazon scutatorius	None	0/3	1												
Diplazon tetragonus	Single	1/3	0	843	432**	1	408**	3	88	257**	23	1	12	21	19
Diplazon tibiatorius	Double or more	2/2	0	1B2, 1B4	432**	1	408**	3	88	257**	23/?	1/1	12/12	21/?	19/19
Diplazon varicoxa	Triple or more	10/10	0	825		(1/7)	(120/249?)	(3/6)	(88/8?)	(185?)	23/?	1/1	12/56	21/15	19/272
Diplazon zetteli	None	0/4	1												
Campocraspedon caudatus	None	0/1	N/A												
Syrphophilus asperatus	Single	1/1	N/A	514	433**	84	120	200**	234**	257**	689**	11	9	267**	302**
Tymmophorus obscuripes	Single	2/2	N/A	818		52		201**	54	41	75	11	9	15	25
Tymmophorus suspiciosus	None	0/1	N/A												
Xestopelta gracilima	None	0/1	N/A												

*Number of infected/tested individuals.

†Success of DNA barcoding: species delimitation unsuccessful (0) or successfull (1) at the most sensitive threshold of 0.5%.

‡Specimen identifier.

§*Wolbachia* strain type from multilocus sequence typing (MLST), based on the five loci *CoxA*, *FbpA*, *FtsZ*, *GatB*, and *HpcA*.

¶*Wolbachia* surface protein gene (*wsp*) allele number, and typing of its four hypervariable regions (*HVR*).

**New alleles found in the present study.

?No sequence obtained.

showed surprisingly little divergence between species of which some were described more than 100 years ago and whose status has subsequently remained unchallenged in the taxonomic literature. Of the 15 species included with more than one specimen, nine were not recovered as monophyletic on the majority-rule consensus tree obtained from the Bayesian analysis (Fig. 1). Even more notable, several species shared identical haplotypes: *D. deletus* with *D. flixi*, *D. annulatus* with *D. tetragonus*, and *D. parvus* with *D. varicoxa*.

Because gene-tree monophyly is not a necessary condition for certain species-delimitation methods to work, we used the threshold method as promoted by many barcoding proponents to assess the extent of discordance between morphologically defined species and those recovered by DNA barcoding. The species recovered when using the threshold method are shown in Figure 2. At 2% uncorrected *p* distance, a value often used for species delimitation in insects (Hebert *et al.*, 2004), only six species are recovered, eight at 1%, and ten at the very low value of 0.5%.

Figure 3 shows that the intra- and interspecific distances overlap broadly in several species pairs. The use of a relative threshold as sometimes proposed (e.g., ten times the intraspecific variability) (Hebert *et al.*, 2004) would thus not have improved the situation. We here use the distance to the closest other species (i.e., the minimum interspecific distance) because reporting the average instead of the smallest interspecific distances exaggerates the barcoding gap (Meier, Zhang & Ali, 2008).

Morphometry

The morphometric analyses supported most of the species hypotheses derived from discrete morphological characters. For all analyses, only the first shape PC was informative, which was then plotted against isometric size. A scatterplot confined to the three species pairs of special interest in the present study (*D. deletus–D. flixi*, *D. annulatus–D. tetragonus*, *D. parvus–D. varicoxa*) (Fig. 4; see also Supporting information, Fig. S1) revealed that some of them cannot be separated based on quantitative morphology. For example, *D. annulatus* is nested within *D. tetragonus*, and *D. parvus* within *D. varicoxa*. However, *D. deletus* is clearly distinct from *D. flixi* by the first shape PC (Fig. 4). The two species are also entirely overlapping in size; hence, the shape difference cannot be attributed to an allometric size effect.

Nuclear *ITS2*

As morphometry did not provide sufficient support for all the morphological species hypotheses, we sequenced a nuclear gene that is known to evolve at a relatively fast rate, the rRNA spacer *ITS2*. The resulting gene tree (Fig. 5) corresponds well to *COI* concerning the deeper nodes but shows a very different pattern for some of the species. *Diplazon deletus* and *D. flixi* do not appear as closely related species in *ITS2* but, instead, the former clusters with a North American species, *D. bradleyi*. There is very high support for this grouping and, in addition to the information in the nucleotide sequence, there are three indels in the *ITS2* sequence that support this relationship (of length 1, 2, and 4 bp, respectively). These indels are not present in *D. flixi* or any other species grouped with *D. deletus* in the *COI* analyses. *Diplazon flixi* now clusters with specimens of the two morphologically very similar species: *Diplazon hyperboreus* and *D. zetteli*. Neither *D. parvus*, nor *D. varicoxa* are recovered by *ITS2* as monophyletic, although they appear as clearly separated on the current gene tree, with *D. parvus* now sharing identical sequences with *D. tibiatorius*. This grouping again is in better accordance with the morphology of the species (Klopfstein, 2014), and suggests that these are good species after all. The species *annulatus* and *tetragonus*, however, are not recovered by *ITS2* either but, instead, show a pattern similar to the *COI* tree. Finally, several species that are monophyletic on the *COI* tree are not supported here (e.g., the aforementioned *D. tibiatorius* and the species pair *D. scutatorius* and *D. orientalis*, which share identical *ITS2* sequences). The two specimens of *D. hyperboreus* do not cluster together; one of them showed some deviations from the morphological diagnosis of the species (see below).

The gene trees obtained from the single-gene analyses of two additional nuclear markers (*28S* which is in close proximity to *ITS2* in the genome, and *EF1α*; see Supporting information, Fig. S2) show that these markers both evolve too slowly to contain much information about the species in question; although the *D. albotibialis* and *D. pectoratorius* pair and the group around *D. laetatorius* are recovered, there is little or no resolution among the questionable species. Not surprisingly, the second mitochondrial gene sequenced, *ND1*, largely confirms the picture recovered in the *COI* tree but with less resolution. *Diplazon deletus* and *D. flixi* once more have identical sequences, as have *D. bradleyi* and *D. varicoxa* (*D. parvus* and *D. annulatus* were not sequenced for this locus).

Wolbachia infections and correlation analysis

Twenty out of 54 *Diplazon* individuals or eight of the 16 species tested positive for *Wolbachia* infections, (Table 2). To test whether the infections coincided with a failure in DNA barcoding, we used a method

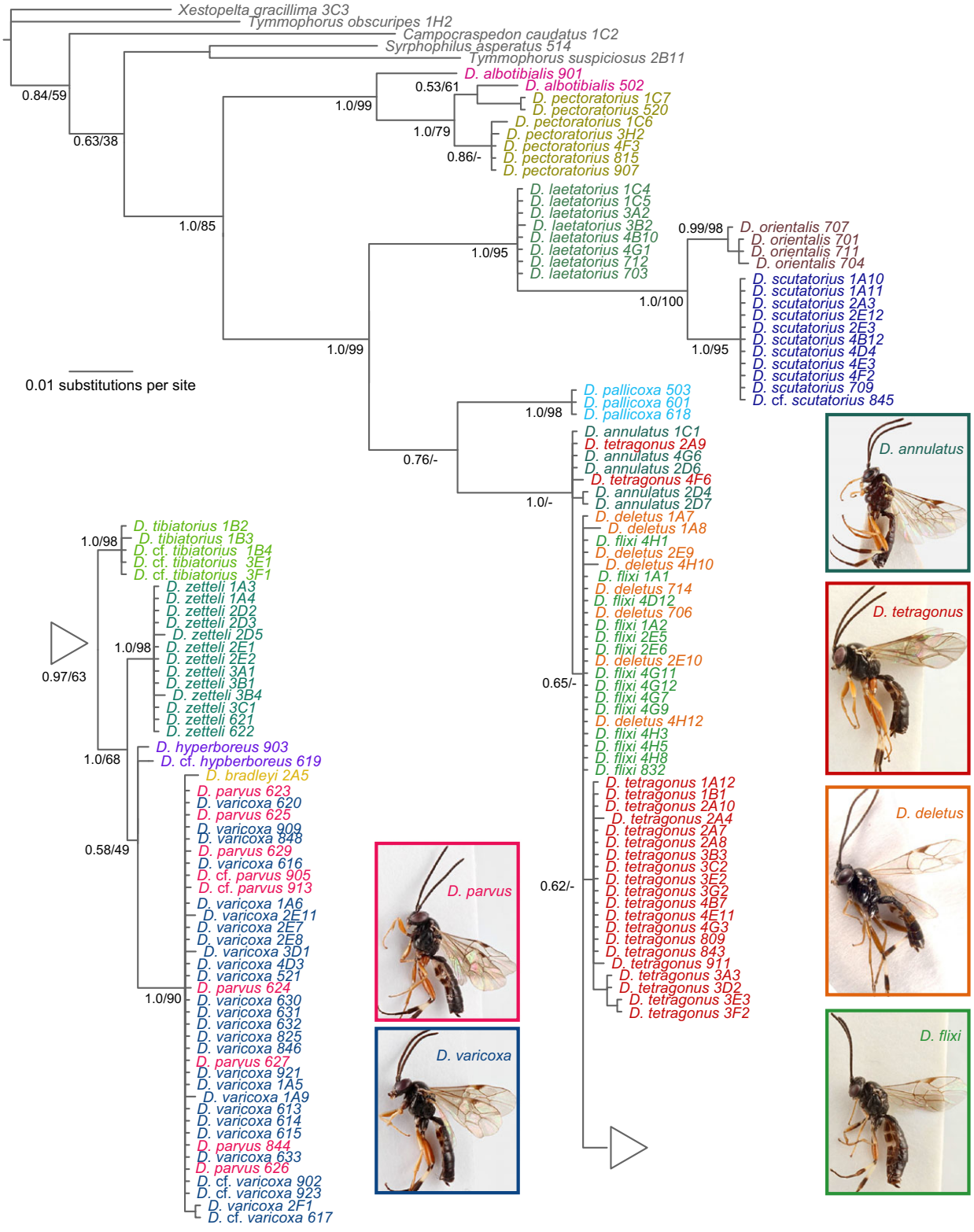

Figure 1. Bayesian majority-rule consensus tree as retrieved from the barcoding fragment of *COI* mtDNA. Support values close to the nodes represent Bayesian posterior probabilites and bootstrap support based on 1000 replicates. Inlaid photographs show specimens of some of the unresolved species. Part of the tree was cut at the triangle and moved to the left to fit on a single page.

Threshold	*albotibialis*	*pectoratorius*	*laetatorius*	*orientalis*	*scutatorius*	*pallicoxa*	*annulatus*	*tetragonus*	*deletus*	*flixi*	*tibiatorius*	*zetteli*	*hyperboreus*	*bradleyi*	*parvus*	*varicoxa*
2%	1		2	3	4	5	6									
1%	1		2	3	4	5	6					7		8		
0.5%	1		2	3	4	5	6			7	8	9		10		

Figure 2. Numbers of species recovered and identity of lumped species as obtained by the threshold method for three different threshold values. Distances are uncorrected pairwise distances in the CO1 barcoding locus.

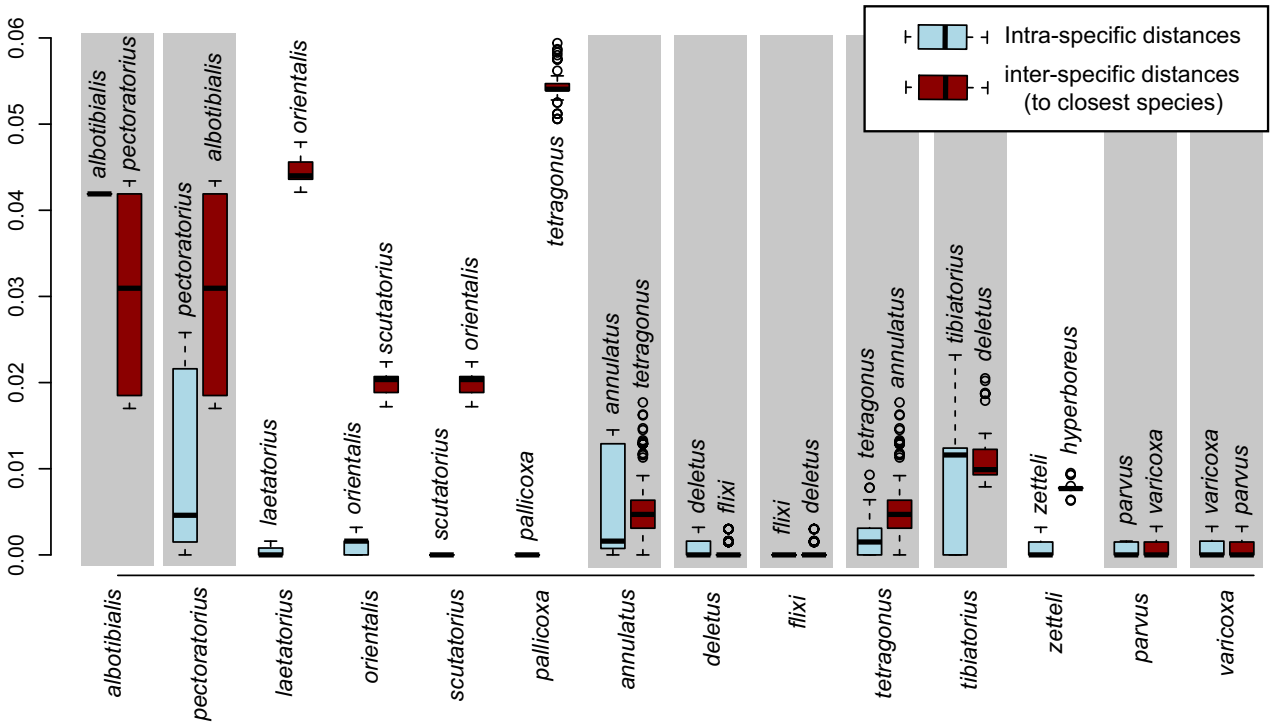

Figure 3. Intra- and interspecific uncorrected *p* distances for each species. The minimum interspecific distances are shown (i.e., those to the closest species included in our dataset; species identities are indicated above the respective boxplot). Grey bars indicate species that do not show any barcoding gap (i.e., for which intra- and interspecific distances overlap).

correcting for phylogenetic relationships by comparing an evolutionary model assuming independent with one based on dependent evolution. Barcoding was considered successful if the species were recovered at the most sensitive threshold of 0.5%. Both the ML approach and the Bayesian approach significantly preferred the dependent over the independent model of evolution in both ML model testing (likelihood ratio test statistic = 10.29, $P < 0.0358$) and Bayesian testing [Bayes factor as 2 × (difference in the logarithm of the harmonic mean) from standard MCMC = 5.14, from reversible-jump MCMC = 2.84; Bayes factors are considered significant from a value of 2 and highly signficiant from a value of 5; Kass & Raftery, 1995].

To examine the extent to which this method was sensitive to taxon sampling, we also analyzed the dataset under the assumption that the aberrant specimen of *D. hyperboreus*, which was retrieved apart from the specimen with the typical morphology in the *ITS2* tree, actually represents a different species. This change had a drastic impact on the

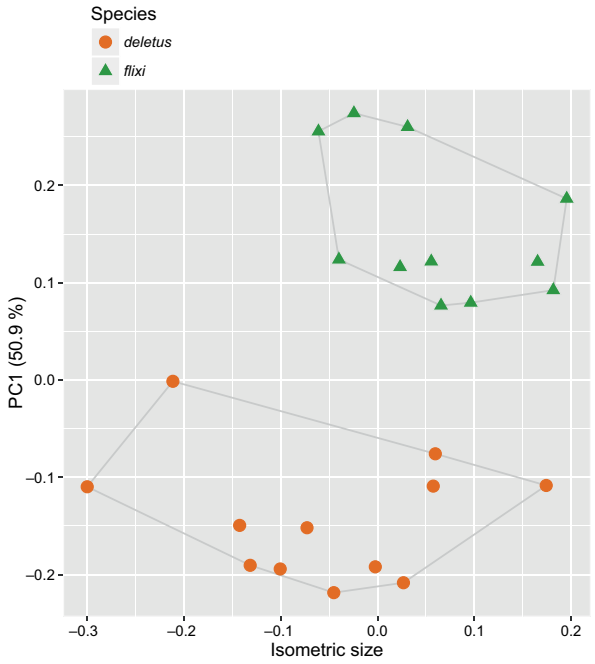

Figure 4. Scatterplot of isometric size versus the first shape principle component of the species pair *Diplazon deletus–Diplazon flixi*. PC, principal component.

result, which thus has to be considered with caution. With this new dataset, the significance disappeared, with the likelihood ratio test statistic dropping to 5.47 and the P value increasing to 0.24.

Wolbachia diversity

The *Wolbachia* surface protein gene (*wsp*) sequences indicated single infections in *D. deletus*, *D. flixi*, *D. tetragonus*, and the outgroup species *Syrphophilus asperatus* and *Tymmophorus obscuripes*. Multiple infections, as evident from polymorphic peaks and often also length variation in the *wsp* sequences, were detected for *D. annulatus*, *D. bradleyi*, *D. parvus*, *D. tibiatorius*, and *D. varicoxa*. For the latter three, we performed molecular cloning of the *wsp* gene to assess how many infections were present and whether they were similar to single infections already detected in the present study (Table 2).

Multilocus sequence typing of the singly-infected species recovered one known and three unknown *Wolbachia* strains (Table 1). *Diplazon deletus* and *D. flixi* are both infected with the same strain #92 (http://pubmlst.org/wolbachia). The sequences of the fast-evolving *wsp* gene differs only by a single mutation in the second hypervariable region of the gene, which is further evidence for the very close relationship between those *Wolbachia* infections. The same strain has also been found in two lepidopteran species from the USA (Wolbachia MLST database, http://pubmlst.org/wolbachia; accessed 20 May 2015). *Diplazon tetragonus* has two previously unknown alleles for the genes *FbpA* and *HpcA* and thus harbours a new strain. Interestingly, the *wsp* allele found in this species has previously been found in species as diverse as the parasitic wasp *Nasonia longicornis* (Darling) (Hymenoptera, Pteromalidae), the fruit fly *Rhagoletis cerasi* (Linnaeus) (Diptera, Tephritidae), and the vinegar fly *Leucophenga maculosa* (Coquillett) (Diptera, Drosophilidae) (http://pubmlst.org/wolbachia). *Diplazon tibiatorius* carries multiple though closely related *wsp* alleles, although MLST sequence typing only recovered a single strain, which is identical to the one found in *D. tetragonus*. Finally, the infections of *D. varicoxa* and *D. parvus* could not be typed by the MLST approach because of multiple infections (triple or more), although one of the cloned *wsp* sequences corresponds to the same allele (#23) as the infections in *D. tetragonus* and *D. tibiatorius* (Table 2). The phylogeny of the *wsp* sequences of the single-infected species and the successfully cloned multiple infections (Fig. 6) confirms the strain typing results.

DISCUSSION

Failure of DNA barcoding in *Diplazon*

We found a rather poor performance of the standard DNA barcoding approach in delimiting species in the parasitoid wasp genus *Diplazon*. Using a 2% sequence divergence threshold as often advocated (Hebert *et al.*, 2004), only six of the 16 species could be recovered, making this approach clearly insufficient in this genus. This result is somewhat in contrast to other studies that have reported good success of barcoding in other groups of parasitoids, where *COI* typically recovered many more species than morphology (Smith *et al.*, 2008; Stigenberg & Ronquist, 2011; Butcher *et al.*, 2012). However, morphological examinations were often not very detailed in the past and most studies covered only limited geographical regions. Further studies will show whether the poor performance in *Diplazon* is just an exception for the group. A simple DNA-based method for species delimitation would be urgently needed in parasitoid wasps, given their enormous and highly understudied diversity (Quicke, 2012). On the other hand, parasitoids might have extreme population dynamics because of their high trophic level, show unusually biased sex-ratios and even high levels of inbreeding in some groups, and potentially have very fast speciation rates through host switching and ecological speciation (Feder & Forbes, 2010; König *et al.*, 2015). Furthermore, parasitoids might be more prone to *Wolbachia* infections as a result of their

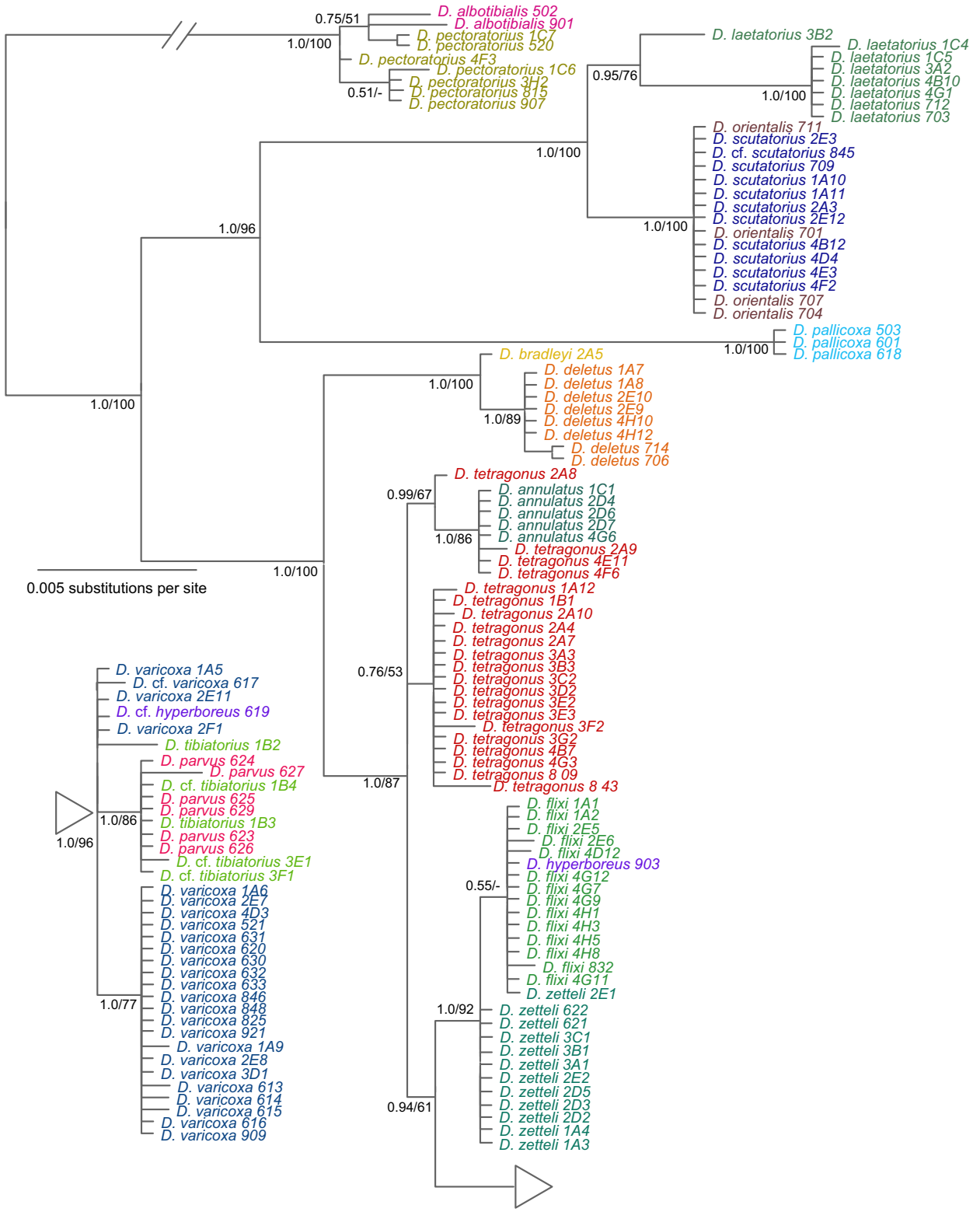

Figure 5. Bayesian majority-rule consensus tree as retrieved from the internal transcribed spacer 2 rRNA. The *Diplazon* species are shown in different colours. Support values close to the nodes represent Bayesian posterior probabilites and the bootstrap support based on 1000 replicates. The branch leading to *Diplazon albotibialis* and *Diplazon pectoratorius* has been shortened to fit on a single page.

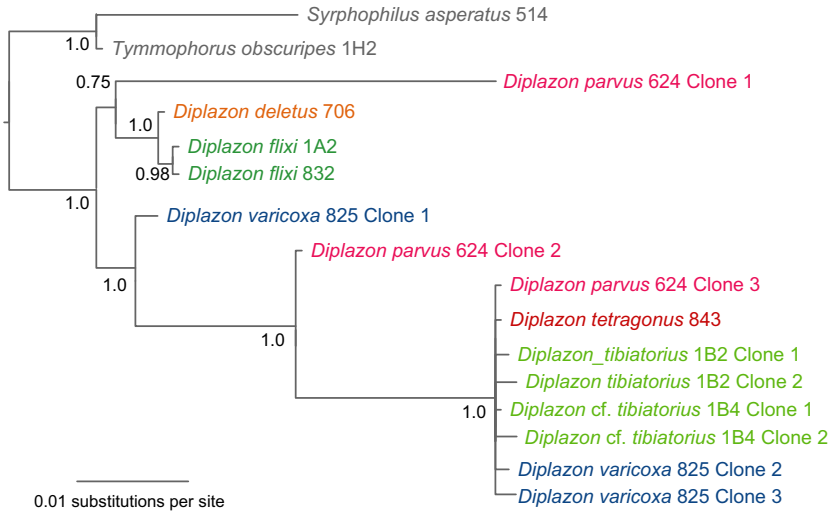

Figure 6. Bayesian majority-rule consensus tree of the *Wolbachia* surface protein (*wsp*) sequences of isolates from nine species of Diplazontinae parasitic wasps. Strains separated by molecular cloning in *Escherichia coli* were given arbitrary numbers. Values next to nodes represent Bayesian posterior probabilities.

intimate relationships with their hosts that might act as a potent transmission pathway (Cook & Butcher, 1999; Vavre *et al.*, 1999). All of these factors might complicate the population biology of their mtDNA and thus impede DNA barcoding.

In *Diplazon*, only a combination of the two markers *COI* and *ITS2* recovered most of the morphologically defined species; in some cases, for example, the recently described *D. parvus*, species status is only supported by the combined information because this species was polyphyletic in both markers but with respect to two different species (Figs 1,4). Given the limited use of *COI* and *ITS2* as markers for species delimitation, the establishment of additional markers is necessary. A recent bioinformatics approach using comparative genomics (Hartig *et al.*, 2012) has already established a plethora of candidate loci for the order Hymenoptera, some of which contain introns that might provide sufficient variability to resolve questions at the species level, and could be analyzed in multispecies coalescent approaches that make use of the information inherent in independently segregating markers to identify reproductively isolated units (Yang & Rannala, 2010). In any case, the failure of a single-marker identification system in this genus suggests that caution is necessary when using DNA taxonomy in parasitic wasps.

Correlation between *Wolbachia* infections and a failure of barcoding

We found a significant correlation between the *Wolbachia* infection status and the failure of barcoding. However, this result should be treated with caution because it was very sensitive to the species hypotheses. This was exemplified by our exercise of assuming species status for the aberrant individual of *D. hyperboreus*, which was sufficient to annihilate our result. The test is probably not very stable because of the very small number of taxa sampled (eight infected versus eight non-infected putative species) and very short branch-lengths in the crown group of the tree. Furthermore, the infection status of a species might have changed recently or not be detected correctly in our few-specimen assay.

A recent critique of phylogenetic comparative methods in general highlighted a basic shortcoming in that they often even retrieve a significant correlation if the character histories involve only a single origin (Maddison & FitzJohn, 2015). Currently, there is no way to resolve this issue except for the careful interpretation of such a result. In our case, a single origin of both characters can be excluded from the phylogenetic distribution, although the species with both a failure of barcoding and *Wolbachia* infections are certainly concentrated among the crown group of the tree. The failure of barcoding might be tightly linked to lowered evolutionary rates or rapid speciation rates, and a phylogenetic component is likely for both. By contrast, the observed *Wolbachia* infections certainly do not go back to a single infection in an ancestral species because some strains are very divergent and several species harbour multiple strains. Furthermore, the retention of *Wolbachia* over such time scales and that numbers of species boundaries is highly unlikely for this endosymbiont, which normally shows much faster infection dynamics (Werren,

Baldo & Clark, 2008; Raychoudhury *et al.*, 2009). Even when accepting the correlation as true, there might still be other causal explanations than endosymbiont-mediated hybrid introgression (e.g., a role of *Wolbachia* in increasing speciation rates, which would at the same time decrease the success of DNA barcoding) (Werren *et al.*, 2008; Raychoudhury *et al.*, 2009). We thus need additional evidence to support the introgression scenario.

A strong case for *Wolbachia*-mediated mtDNA transfer

Additional evidence for *Wolbachia*-mediated mtDNA introgression comes from several sources. Under a scenario of hybrid introgression, we expect the following patterns: (1) very low mtDNA diversity indicative of a recent selective sweep; (2) mtDNA haplotypes that are much more similar than likely given the species relationships; (3) identical or at least very similar *Wolbachia* infections; and (4) a likely opportunity for hybridization (Hurst & Jiggins, 2005).

For the species pair *D. deletus–D. flixi*, without any doubt two good biological species that were also clearly distinct in our morphometric analysis (Fig. 4), all four of the above points are fulfilled. Both *COI* and *ND1* show identical haplotypes between the species and the Swiss and Swedish populations of *D. deletus*. There is strong evidence in the nuclear *ITS2* marker that *D. deletus* is more closely related to the North American *D. bradleyi*; the sharing of an mtDNA haplotype in *D. deletus* and *D. flixi* through incomplete lineage sorting is thus highly unlikely. The single *Wolbachia* infections in both species are of the same strain type and only differ by a single mutation in the highly variable *wsp* gene, an observation in accordance with transmission through hybridization. Finally, *D. deletus* and *D. flixi* have been collected in the same Malaise traps in the Swiss Alps which suggests geographical and phenological opportunity for hybridization. The case is similar for the species *D. parvus* and *D. varicoxa* that share identical *COI* sequences both in Switzerland and Sweden, whereas *D. parvus* clusters with *D. tibiatorius* in the *ITS2* tree. Their multiple *Wolbachia* infections appear to partly overlap as well (because both species carry multiple infections, the strains could not be fully typed). *Wolbachia*-mediated mtDNA transfer is thus the likely cause for the failure of barcoding in these cases, but insufficient variability or incomplete lineage sorting probably also played a role (e.g., in the sister species *D. albotibialis* and *D. pectoratorius*, which do not harbour *Wolbachia* infections).

There are very few other convincing examples for endosymbiont-mediated mtDNA introgression in the literature. Ballard (2000) could best explain the discordance between nuclear and mitochondrial gene trees in the vinegar fly genus *Drosophila* (Diptera, Drosophilidae) with an introgression event from *Drosphila simulans* Sturtevant to *Drosphila mauritiana* Tsacas and David, and such a transfer could even be repeated experimentally (Aubert & Solignac, 1990). Jiggins (2003) found a shared mtDNA haplotype in those individuals of two species of *Acraea* butterflies (Lepidoptera, Nymphalidae) that were infected with the same *Wolbachia* strain but not in the uninfected individuals, and such a pattern is best explained by endosymbiont-mediated hybrid introgression. Narita *et al.* (2006) recovered the same pattern in two recently discovered sibling species of the butterfly genus *Eurema* (Lepidoptera, Pieridae). Whitworth *et al.* (2007) examined twelve species of the blowfly genus *Protocalliphora* (Diptera, Calliphoridae) and found that four species shared *COI* haplotypes and *Wolbachia* strains as judged from the *wsp* gene, whereas AFLP markers suggested that these species were not closely related. Similar results were obtained by Gompert *et al.* (2008) in *Lycaeides* butterflies (Lepidoptera, Lycaenidae). Finally, Raychoudhury *et al.* (2009) examined the *Wolbachia* infections in the parasitoid wasp genus *Nasonia* (Hymenoptera, Pteromalidae) and found the likely co-transmission of *Wolbachia* and mtDNA from *Nasonia giraulti* Darling to *Nasonia oneida* Raychoudhury & Desjardins. Unusually large intraspecific mtDNA variation that coincides with infections by different *Wolbachia* strains has been found in several studies (Ballard, Chernoff & James, 2002; Marshall, 2004; Riegler *et al.*, 2005; Charlat *et al.*, 2009; Atyame *et al.*, 2011; Xiao *et al.*, 2012; Ritter *et al.*, 2013) but, without a clear hybridization scenario including the species of origin, it might be that the bacterial strains became associated with divergent mtDNA haplotypes long after a transfer event (or incomplete lineage sorting).

CONCLUSIONS

Reports of endosymbiont-mediated mtDNA introgression are very rare, with to our knowledge only six convincing cases currently found in the literature. It is difficult to estimate the prevalence and thus importance of this phenomenon. A recent study by the Consortium for the Barcode of Life (Smith *et al.*, 2012) addressed *Wolbachia* and barcoding but mainly focussed on whether the *CoxA* gene (the bacterial counterpart of *COI*) of the endosymbionts had been amplified instead of the sequences of the host insects in recent barcoding initiatives; no conclusive data was provided about hybrid introgression. Recent estimates of the prevalence of *Wolbachia* infections on the one hand (20–65% of all insect spe-

cies; Hilgenboeker *et al.*, 2008; Werren & Windsor, 2000) and of hybridization on the other (approximately 10% of animal species; Mallet, 2005) suggest that endosymbiont-mediated mtDNA introgression might not be as rare as previously assumed. The scarcity of empirical studies could simply be the result of an inherent difficulty to confirm this scenario; most studies that include *Wolbachia* bacteria are conducted within species or they are part of a broad screening study that does not assess mtDNA patterns in the hosts. Financial considerations and favourable reports on the success of DNA barcoding have led to many biodiversity studies including only a single mtDNA marker and consequently not detecting any mito-nuclear discordances. Decreasing sequencing costs, the establishment of additional nuclear markers with sufficient variability, and the broad realization of the limitation of a single-marker species delimitation system will improve this situation and lead to a more precise assessment of the prevalence of this phenomenon.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Figure S1. Scatterplot of the first shape principle component versus isometric size in selected species pairs of the genus *Diplazon*.

Figure S2. Bayesian consensus trees of the concatenate dataset of five genes, and single-gene trees for the nuclear 28S rDNA and EF1a genes and the mitochondrial NADH1.

Table S1. List of specimens and Genbank accession numbers.

Table S2. Description of characters used in morphometrics of *Diplazon*.

Table S3. Measurement data from morphometric analysis.