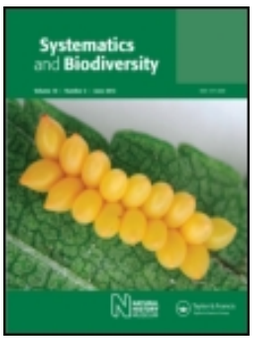

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Julie K. Stahlhut , Jason Gibbs , Cory S. Sheffield , M. Alex Smith & Laurence Packer

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Perspective

Wolbachia (Rickettsiales) infections and bee (Apoidea) barcoding: a response to Gerth *et al.*

JULIE K. STAHLHUT[1], JASON GIBBS[2], CORY S. SHEFFIELD[3], M. ALEX SMITH[1] & LAURENCE PACKER[4]

[1]Biodiversity Institute of Ontario, University of Guelph, 50 Stone Road East, Guelph, Ontario, Canada
[2]Department of Entomology, Cornell University, Ithaca, New York, USA
[3]Royal Saskatchewan Museum, 2340 Albert Street, Regina, Saskatchewan, Canada
[4]Department of Biology, York University, 4700 Keele Street, Toronto, Ontario, Canada

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In a recent Perspective, Gerth *et al.* (2011) expressed concern over how *Wolbachia* (*Wolbachia pipientis* Hertig) infections may affect the success of DNA barcoding efforts in bees. The potential and realized effects of endosymbiont-induced selective sweeps on host mitochondrial DNA diversity have been noted repeatedly – and rightly so – in the literature for some years. However, we are equally concerned with other misconceptions, including (a) presuming that a positive *Wolbachia* test indicates a stable infection, (b) presuming that *Wolbachia*-infected hosts cannot be identified with a single-locus barcode, and (c) inferring specific *Wolbachia*–mtDNA interactions based only on incomplete genotyping of *Wolbachia* strains. We address these issues in the context of the Gerth *et al.* (2011) survey of *Wolbachia* prevalence among the German bee fauna. We also clarify some of the context-dependent strengths and limitations of DNA barcoding when it is used as a research tool by taxonomists and ecologists.

Key words: bees, DNA barcodes, horizontal transmission, hybrid introgression, Hymenoptera, mitochondria, multi-locus strain typing, selective sweeps, vertical transmission, *Wolbachia*

Introduction

In a recent Perspective article, Gerth *et al.* (2011) reported the widespread presence of the intracellular reproductive symbiont *Wolbachia* among the bees and apoid wasps of Germany. Of the species screened, 49 of 74 bee species (66%) and six of 13 wasp species (46%) tested positive for *Wolbachia*, based on PCR amplification of one or more *Wolbachia* loci. These results are not surprising, since estimates of worldwide *Wolbachia* prevalence among arthropod species range from 19% to 76%, depending on the screening method used (Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000; Werren *et al.*, 2008; Simões *et al.*, 2011). *Wolbachia* can infect both diplodiploid and haplodiploid host species, though its range of phenotypes may differ depending on the underlying genetic background and sex determination system of the host (Bordenstein *et al.*, 2003; van Wilgenburg *et al.*, 2006).

Wolbachia effects on host reproduction have been thoroughly addressed elsewhere (Werren *et al.*, 2008; Saridaki & Bourtzis, 2010), but in summary: *Wolbachia* is normally vertically transmitted via egg cytoplasm, and can persist in host populations by biasing reproductive success in favour of infected females. This means that *Wolbachia* can sweep through a population, carrying along the mitochondria of the infected matriline. Depending on the source of the *Wolbachia* and the mechanism of the sweep, this process may vastly reduce mtDNA variation throughout an entire species (Jiggins, 2003), split intraspecific matrilines by infection status (Xiao *et al.*, 2012), or cause an mtDNA haplotype from one species to invade and sweep through another via hybrid introgression (Raychoudhury *et al.*, 2009). The latter two possibilities are of the most concern, since intraspecific lineage splitting and interspecific introgression are the most likely *Wolbachia*-related confounders of DNA barcode data. Alternatively, *Wolbachia* may have little or no discernible effect on mtDNA variation in some host species (Shoemaker *et al.*, 2003).

Gerth *et al.* (2011) expressed concern about the ability of DNA barcoding to identify bee species when *Wolbachia* is present. This concern is reasonable, but not new; the possible effects of reproductive endosymbionts on mtDNA diversity have been discussed in the literature for some

Correspondence to: Julie K. Stahlhut. E-mail: juliestahlhutmobile@gmail.com

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years (Jiggins, 2003; Hurst & Jiggins, 2005), observed in natural systems (Narita *et al.*, 2006; Whitworth *et al.*, 2007) and further addressed in several barcoding studies of Hymenoptera (Smith & Fisher, 2009; Smith *et al.*, 2012).

However, as researchers with experience in bee taxonomy, *Wolbachia* ecology and genotyping, DNA barcode library construction, and barcode-based ecological surveys (Gibbs, 2009*a*, 2009*b*, 2010; Packer *et al.*, 2009; Sheffield *et al.*, 2009; Stahlhut *et al.*, 2010; Smith *et al.*, 2011), we are equally concerned when assumptions about sporadic ecological events needlessly dissuade researchers from applying a useful method. We address these concerns via our perspectives on host–*Wolbachia* interactions, the strengths and limitations of DNA barcoding and the different ways in which barcoding is used as a research tool.

What does a positive *Wolbachia* test mean?

PCR-based *Wolbachia* screens show whether *Wolbachia* DNA is present in the specimen, but say nothing about either its transmission routes or its effects on host mtDNA. A positive screen may indicate an established association with the host, but it may also reveal a transient or rare infection (Keeling *et al.*, 2003), an infection harboured by an associate of the specimen (Hughes *et al.*, 2004), or even transfer of *Wolbachia* DNA to the host nucleus (Dunning Hotopp *et al.*, 2007). In cases where only a single specimen was tested (e.g. the Colletinae and Melittinae species in Gerth *et al*., 2011), we cannot be sure whether the species or population hosts a stable infection.

Can *Wolbachia*-infected hosts be barcoded using only the standard COI sequence?

In general, yes. As of July 2012, the Barcode of Life Data System (BOLD, http://www.boldsystems.org; Ratnasingham & Hebert, 2007), contains more than 137 000 published records for Hymenoptera specimens. Over 42 000 have species names, representing more than 6800 species. Nearly 1300 of the named species are members of the superfamily Apoidea, and because of their status as pollinators, bees have been heavily targeted for barcoding by taxonomic specialists (see http://ibol.org/wg-1–6-pollinators/ for a brief history of this effort). Based on previously cited estimates of prevalence, we may presume that between one-fifth and three-quarters of these species include at least some *Wolbachia*-positive individuals.

Taxon-specific studies that include *Wolbachia*-infected arthropods usually show little or no effect of *Wolbachia* infection on species delimitation via DNA barcodes (Smith *et al.*, 2007, 2012; Linares *et al.*, 2009). For example, in the Smith *et al.* (2012) study, 18 of 57 barcoded and *Wolbachia*-screened ant species contained some *Wolbachia*-positive individuals, but only one putative species had a barcoding anomaly associated with *Wolbachia* infection. This was a *Pristomyrmex* morphospecies whose two deeply divergent barcode clusters were associated with different *Wolbachia* strains, but the study design did not exclude the possibility that these two mitochondrial haplogroups were actually separate species.

It is important to note that barcoded specimens are not routinely screened for *Wolbachia* infection via PCR with *Wolbachia* primers. However, a BOLD user who is concerned about unrecognized endosymbiont effects has the option to examine published barcode data. Through the BOLD public data portal and workbench, the user can obtain collection information, download sequences and trace files for further analysis, build trees, view possible mismatches and barcode gap distributions, and even annotate records that raise any questions. This means that the user's judgement is part of the specimen identification process, as it is when comparing visible characters via morphological keys. If named species do not cluster as expected, the user can decide and document whether an identification match is sufficiently reliable to be useful, and whether the anomaly is itself an interesting research topic.

As noted by Gerth *et al.* (2011), *Wolbachia* DNA itself is not a barrier to barcoding. Inadvertent amplification of bacterial sequence with arthropod barcoding primers is uncommon; when it occurs, it is easy to discover and correct (Smith *et al.*, 2012). And, if a *Wolbachia* sequence is accidentally submitted to BOLD in place of its host barcode, BOLD automatically recognizes and flags the *Wolbachia* sequence as a non-target amplicon.

Can we presume that *Wolbachia* infection is a sign of interspecific mitochondrial introgression?

No, because *Wolbachia* transmission routes among host species are notoriously variable. Previous studies, based on multi-locus strain typing (MLST) (Maiden *et al.*, 1998; Baldo *et al.*, 2006) have revealed *Wolbachia* strain specializations associated with host biogeography, feeding ecology and/or host taxon (Baldo *et al.*, 2008; Russell *et al.*, 2009; Stahlhut *et al.*, 2010). All of these findings were consistent with horizontal transmission as the main mechanism of *Wolbachia* spread in arthropod communities, though each pointed to a different pattern of spread. None of these findings rule out sporadic occurrences of introgression, but neither do they suggest that introgression is a common route of infection, even when similar *Wolbachia* strains are pre-adapted to infect congeneric hosts (Baldo *et al.*, 2008; Watanabe *et al.*, 2012). The distinction is important. Both horizontal transmission and introgression

transfer a *Wolbachia* strain from a donor to a recipient species, but their expected effects on DNA barcoding are completely different.

Horizontal transmission is simply the transfer of an infection between hosts which contact each other in their shared environment; the host species need not be closely related (Russell *et al.*, 2009; Stahlhut *et al.*, 2010). The bacteria move from one host to the other, but mitochondria do not. If a horizontally transmitted *Wolbachia* infection persists and favours infected matrilines in both hosts, then both hosts will undergo mitochondrial sweeps that reduce only intraspecific mtDNA variation. Barcode clusters for the two species will not become more similar to each other than we would expect in the absence of *Wolbachia*.

Unlike horizontal transmission, introgression can transfer both *Wolbachia* and mitochondria between species. The donor and recipient species must be such close relatives that a fertile hybrid cross is possible between a *Wolbachia*-infected female of the donor species and a male of the recipient species (Jiggins, 2003; Jaenike, 2007). *Wolbachia*-infected daughters from this cross must be able to backcross with the paternal species; since both *Wolbachia* and mtDNA are vertically transmitted through females, this introduces both elements into the paternal population. Both the *Wolbachia* strain and the mitochondrial haplotype must then persist in the genetic background of the previously uninfected species, and the infection must induce a phenotype that favours the reproductive success of infected females. Only if all of these conditions are satisfied will mitochondria from the donor species supplant those of the recipient species, homogenizing the DNA barcodes even if the nuclear genomes remain distinctive (Jiggins, 2003; Raychoudhury *et al.*, 2009). For simplicity, pedigrees depict full diploids, but we would expect similar results in haplodiploids (Vavre *et al.*, 2000; Reumer *et al.*, 2010).

Can *Wolbachia* interfere with specimen identification and species delimitation via DNA barcoding?

Yes; this has been both predicted in the literature (Hurst & Jiggins, 2005) and observed in barcoding studies. For example, some morphologically distinctive members of the calliphorid fly genus *Protocalliphora* are strikingly resistant to barcode-based species delimitation, with evidence that *Wolbachia*-mediated mtDNA introgression is responsible (Baudry *et al.*, 2003; Whitworth *et al.*, 2007). In such cases, *Wolbachia* would cause unwarranted lumping if the barcode alone were used to assign species names.

Wolbachia infections can also cause unusually high mtDNA divergence between infected and uninfected populations of presumed conspecifics, which could cause unwarranted splitting (Hurst & Jiggins, 2005). But, trade-offs among *Wolbachia* effects and other conditions can stabilize *Wolbachia* infections at below 100% prevalence without splitting host mtDNA matrilines by infection status (Dyer & Jaenike, 2004; Stahlhut *et al.*, 2006). And, because *Wolbachia* infection can reinforce reproductive isolation between populations, *Wolbachia*-associated splitting of mtDNA lineages may even be a clue to a speciation event in progress (Shoemaker *et al.*, 1999; Telschow *et al.*, 2005, 2007).

Interpretation of problematic barcode data depends on the intended application. For example, if the goal is specimen identification, a shared barcode will narrow the possible choices to a small number but not give a definitive result. This can also happen with the use of morphological keys, and the consequences in either case depend on whether some uncertainty is tolerable. If uncertainty is not tolerable, additional study will be necessary – as it is when morphology alone is not sufficient to distinguish between species (i.e. when 'the morphological equivalent of the 'barcode gap' is zero . . ..'; Packer *et al.*, 2009).

Is *Wolbachia* affecting host mtDNA dynamics in my community or taxon of interest?

This is an interesting question in evolutionary ecology, and the only way to answer it is to explicitly test for discordances among mitochondrial, nuclear and endosymbiont genes. In the case described by Gerth *et al.* (2011), this question could be explored by first barcoding the bees, and then comparing the barcodes to those from morphologically determined conspecifics and congenerics. If barcodes are discordant with other evidence for species delimitation, it would then be informative to test for associations among mtDNA haplotypes, nuclear genes and *Wolbachia* strains. These combined approaches can reveal previously unknown details of endosymbiont dynamics within and between species (Raychoudhury *et al.*, 2009; Dyer *et al.*, 2011).

This is not a question that can be answered via PCR screening of a small collection. Additionally, sequencing only one or two *Wolbachia* genes is unlikely to reveal much about *Wolbachia* transmission between host species. In particular, the *wsp* gene is prone to recombination, and is under directional selection (Schulenburg *et al.*, 2000; Jiggins *et al.*, 2001; Baldo *et al.*, 2005). Its resulting variability makes *wsp* an excellent single marker for distinguishing among different *Wolbachia* strains, but a poor one for tracing strain genealogy (Stahlhut *et al.*, 2010). In other words, *wsp* performs similarly to the DNA barcode sequence of a metazoan, since DNA barcodes usually form monophyletic clusters within species but do not necessarily show which species are most closely related (Blaxter *et al.*, 2005; Rubinoff & Holland, 2005). Unless full genome sequencing is practical, MLST is a good compromise for revealing true strain similarities, requiring sequences from only five

housekeeping genes (Maiden *et al.*, 1998; Baldo *et al.*, 2006). With the exception of the *Wolbachia* endosymbiont of *Osmia caerulescens* (Linnaeus), the only true MLST locus sequenced by Gerth *et al.* (2011) is *ftsZ*, the least variable of the five MLST loci and therefore the least sensitive to resolving multiple strains (Baldo *et al.*, 2006; Stahlhut *et al.*, 2010). Thus, any estimation of sequence similarity drawn from *ftsZ* alone will almost certainly be too high.

Do taxonomists and ecologists draw conclusions from DNA barcode data without regard to other evidence?

No, and we believe that much confusion arises from conflating the multiple uses of DNA barcoding, each of which comes with its own assumptions and its own standards of evidence. At one extreme, some users of DNA barcodes (and morphological keys) are interested only in comparing individual specimens to reference material and obtaining a tentative match. The needs of taxonomists and ecologists go well beyond this level of detail.

By definition, integrative taxonomic methods require multiple approaches (Padial *et al.*, 2010), but taxonomy was both integrative and iterative even before the invention of DNA barcoding or the coining of the term 'integrative taxonomy'. Taxonomists have always considered multiple lines of evidence, such as morphology, biogeography and behaviour, when describing new species, and have always compared decisions made via one group of characters with those based on others (DeSalle *et al.*, 2005). With molecular methods now almost universally available, mitochondrial and nuclear gene sequences provide supporting or contrary evidence for taxonomic hypotheses just as other characters do (Gibbs, 2009*a*, 2009*b*; Droege *et al.*, 2010; Fernández-Triana, 2010; Smith *et al.*, 2011). DNA barcodes and other sequences thus function as independent tests of decisions based on morphology, geography and behavioural ecology (Packer *et al.*, 2009; see also Rehan & Sheffield, 2011; Vickruck *et al.*, 2011 for a recent application of these methods to bee taxonomy).

Similarly, ecologists who use barcode data in biodiversity surveys understand the vast difference between estimating the magnitude of diversity and naming every species that contributes to it (Smith & Fisher, 2009; Smith *et al.*, 2009). Few ecologists have the expertise, the time, or even the need to identify every specimen collected in a regional survey, even if the study is restricted to specific higher taxa. So, barcode clusters provide a quantitative, inclusive, efficient and reproducible basis for accumulation curves and species richness estimates when morphological identification of all specimens is impractical. At this scale, sporadic mtDNA lumping or splitting events are usually of little interest and should have little effect.

Conclusion

DNA barcodes can both answer existing research questions and generate new ones. This is also true of morphological studies, phylogenetic reconstructions and species richness estimates. Like these other methods, barcoding comes with assumptions and limitations that must always be considered when interpreting results. There are two concepts that we believe are especially important to address in this context, given the concerns about endosymbiont effects on host mtDNA dynamics.

The first issue is the meaning of a barcode gap, or a discontinuity between intraspecific and interspecific levels of variation. A barcode gap is often a clue to species delimitation, but the existence of a universally applicable gap size between sibling species was never meant to be a 'central dogma' of barcoding (Gerth *et al*., 2011). The commonly used 2–3% cutoffs are often empirically useful for biodiversity estimates, and for provisional comparison of a specimen to a reference barcode, but variation among interspecific barcode gap sizes has been noted in the literature by the optimistic, the pessimistic and the undecided alike (Hebert *et al.*, 2003*b*; Meier *et al.*, 2006; Wiemers & Fiedler, 2007). However, unexpected barcode gap magnitudes are not insurmountable barriers to barcode-based identifications, because distance measures, tree topology and diagnostic nucleotides are also informative (Steinke *et al.*, 2005; Rach *et al.*, 2008; Gibbs, 2009*a*, 2009*b*; Kuksa & Pavlovic, 2009; Buck *et al.*, 2012; Williams *et al.*, 2012).

The second issue is that there is a substantial difference between barcode-based specimen identification and barcode-inclusive taxonomic and ecological research. DNA barcoding was developed as a tool for specimen identification (Hebert *et al.*, 2003*a*). By definition, it is a standardized method, linked to supporting evidence from both voucher specimens and sequencing trace files (Hebert & Gregory, 2005). Barcode-based identification using a validated reference library is analogous to using a morphological key developed by an expert taxonomist: it usually works well, it takes time for a researcher to learn both its power and its limitations, the associated reference material sometimes needs revision, and when the method does not work as expected, it may be revealing something potentially interesting about the biology of the research subjects.

Phylogenetics, taxonomy and population biology all address broader and more complex questions than specimen identification. In these contexts, DNA barcode data can reveal interesting questions for future research, but barcoding methods were not designed to definitively answer them. This is true in the same sense that a single gene tree is insufficient to reconstruct the evolutionary history of a species (Degnan & Rosenberg, 2009), a single morphological character is insufficient evidence for a species diagnosis (Hermes & Melo, 2008; Packer *et al.*, 2009), and a mitochondrial sweep alone is not definitive evidence of a true population bottleneck (Hurst & Jiggins, 2005).

Wolbachia and other reproductive endosymbionts should be recognized as both markers of and contributors to host mitochondrial diversity (Moran *et al.*, 2008; Gueguen *et al.*, 2010; Stahlhut, 2010). When considered in this way, endosymbionts are not underminers of DNA barcoding success, even though they sometimes complicate data interpretation. Rather, endosymbionts are potentially major players in the actual generation of host diversity. Meanwhile, the appropriateness and sufficiency of DNA barcoding as a research tool depends on the nature of the question and on the analysis and interpretation of possible outcomes in the appropriate biological context.

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