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► **To cite this version:**

Marie Cariou, H el ene Henri, Sonia Martinez, Laurent Duret, Sylvain Charlat. How consistent is RAD-seq divergence with DNA-barcode based clustering in insects?. *Molecular Ecology Resources*, 2020, 20 (5), pp.1294-1298. 10.1111/1755-0998.13178 . hal-03017182

**HAL Id: hal-03017182**

**<https://cnrs.hal.science/hal-03017182v1>**

Submitted on 20 Nov 2020

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# How consistent is RAD-seq divergence with DNA-barcode based clustering in insects?

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**Running tittle:** Assessing DNA barcoding in insects

**Key words:** RAD-seq, divergence, barcode, mitochondria

## 21 Abstract

22

23 Promoted by the barcoding approach, mitochondrial DNA is more than ever used as a  
24 molecular marker to distinguish and identify species. Yet, it has been repeatedly argued that it  
25 may be poorly suited for this purpose, especially in insects where mitochondria are often  
26 associated with invasive intracellular bacteria that may promote their introgression. Here we  
27 inform this debate by assessing how divergent nuclear genomes can be when mitochondrial  
28 barcodes indicate very high proximity. To this end, we obtained RAD-seq data from 92  
29 barcode-based species-like units (that is Operational Taxonomic Units, OTU), spanning 4  
30 insect orders. In 100% of the cases, the observed median nuclear divergence was lower than  
31 2%, a value that was recently estimated as one below which nuclear gene flow is not  
32 uncommon. These results suggest that although mitochondria may occasionally leak between  
33 species, this process is rare enough in insects to make DNA barcoding a reliable tool for  
34 clustering specimens into species-like units.

35

36 Mitochondrial DNA is widely used to track population histories and identify species, with a  
37 number of pros and cons making this issue controversial (Galtier, Nabholz, Glémin, & Hurst,  
38 2009; Hebert, Hollingsworth, & Hajibabaei, 2016; Hebert, Stoeckle, Zemplak, & Francis, 2004;  
39 Hurst & Jiggins, 2005; Moritz & Cicero, 2004; Ratnasingham & Hebert, 2007; Sloan, Havird, &  
40 Sharbrough, 2017). Beyond their technical ease of use, mitochondrial sequences possess  
41 characteristics that make them particularly suitable for short evolutionary time scale  
42 inferences. Thanks to a high, albeit variable, mutation rate (Allio, Donega, Galtier, & Nabholz,  
43 2017), they tend to efficiently discriminate closely related lineages. Their short expected  
44 coalescence time, due to maternal inheritance, should also make them less susceptible than  
45 nuclear sequences to the persistence of ancestral polymorphism in diverging populations, that  
46 is, incomplete lineage sorting. On the other hand, because they lack recombination and are  
47 genetically linked with invasive cytoplasmic elements such as *Wolbachia* (Werren, Baldo, &  
48 Clark, 2008), mitochondria might be particularly prone to selective sweeps (Cariou, Duret, &  
49 Charlat, 2017). Such events may also facilitate introgression between incipient species  
50 following hybridizations, a process that introduces disagreements between demographic and  
51 genetic genealogies (Hurst & Jiggins 2005; Galtier et al. 2009). Several reports have indeed  
52 demonstrated cases of *Wolbachia*-driven mitochondrial introgressions (Charlat et al., 2009;  
53 Jiggins, 2003; Narita, Nomura, Kato, & Fukatsu, 2006), and *Wolbachia* infected species generally  
54 harbour a reduced mitochondrial polymorphism (Cariou et al., 2017). Mitochondrial DNA may  
55 also be subject to much wider variations in mutation rates and evolve much less neutrally, than  
56 has been traditionally acknowledged (Allio et al., 2017; Galtier et al., 2009; Sloan et al., 2017).  
57 Because of these problematic issues, cautions have been repeatedly raised regarding the use  
58 of mitochondrial markers to infer evolutionary histories (Chan & Levin, 2005; Galtier et al.,

59 2009; Hurst & Jiggins, 2005; Schmidt & Sperling, 2008; Sloan et al., 2017; Taylor & Harris, 2012;  
60 Tows & Brelsford, 2012). Should these valid criticisms be taken as good reasons for not relying  
61 on mtDNA to cluster unknown specimens into species-like units?

62           Whatever the markers considered, the frequency of gene flow is known to decrease  
63 with increasing divergence time. Roux et al. (2016) analysed this effect by exploring the  
64 prevalence of gene flow along the continuum of divergence leading to complete isolation. This  
65 study was based on transcriptome data from 61 pairs of populations (or closely related species),  
66 showing variable levels of divergence, and selected in order to sample evenly the phylogenetic  
67 and ecological diversity of animals. The analysis revealed a “grey zone” between 0.5% and 2%  
68 of net synonymous divergence (defined as the synonymous divergence measured from non-  
69 polymorphic sites), within which species definition is often controversial and where alleles can  
70 be exchanged at some but not all loci. However, above a threshold of about 2%, they observe  
71 that gene flow is suppressed and that species are indeed isolated. Are mitochondrial markers  
72 specific to this regard, often moving between lineages beyond the “grey zone”? Or is their bad  
73 reputation a caricature, as much as would be the idyllic picture of a “perfect” molecular marker  
74 ?

75           To address this question, we used RAD-seq, recognized as a robust “genome  
76 reduction approach” (Andrews, Good, Miller, Luikart, & Hohenlohe, 2016), to estimate of the  
77 genome-wide nuclear divergences in 92 pairs of insect specimens, each composed of two  
78 individuals that presumably belonged to the same species, or “Operational Taxonomic Units”  
79 (OTUs) as indicated by previously obtained DNA barcodes (figure 1). These specimens, spanning  
80 4 insects orders (figure 2) represent a phylogenetically diverse subset of a larger sample of  
81 Arthropods collected in Tahiti and surrounding islands (Ramage et al., 2017). In seven OTUs,

82 the RAD-seq experiment was repeated for one of the two specimens, in order to estimate the  
83 reliability of the data. In all cases, the global nuclear divergence, as estimated by the median  
84 RAD distance, were indeed very similar among replicates (S2 table).

85           The observed median RAD divergence ranged from 0% to 1.1% (Figure 3, S3 Table),  
86 that is, remained within the standard range of within species polymorphism. In 75% of the  
87 presumed species (69 cases) the median nuclear divergence was below 0.5%, that is, showed  
88 a typical value for specimens collected in randomly mixed populations (Roux et al., 2016). In  
89 the remaining 25% (23 cases), the median RAD distance remained well below 2%, that is, within  
90 the “grey zone of speciation”, suggesting the two specimens sampled likely belong to slightly  
91 isolated or large populations (Roux et al., 2016). Notably, this general picture of small nuclear  
92 distances is further reinforced by noting that, without polymorphism data in hands, we used  
93 here raw divergence, which is inflated in comparison with the “net divergence” used by Roux  
94 et al. (2016).

95           Arguably, mitochondrial DNA is not the ideal molecular marker. Even though it has a  
96 small effective population size, a high mutation rate, and usually doesn’t recombine, it is  
97 certainly not evolving as neutrally as would be required to make it suitable for any kind of  
98 evolutionary or demographic inferences. Some mitochondrial sites are likely under direct  
99 selection, and perhaps more importantly, mitochondria are genetically linked with maternally  
100 inherited elements, such as *Wolbachia* bacteria that tend to rapidly invade populations thanks  
101 to selfish drive strategies. For these reasons, some have argued that “mtDNA is perhaps  
102 intrinsically the worst population genetic and phylogenetic molecular marker we can think of”  
103 (Galtier et al., 2009). Yet, the Barcoding Of Life Database, mostly based on the mitochondrial  
104 locus CO1, hosts over 7 million records at the time of writing, from an estimated 600 000

105 species, suggesting it still has a future in the field, at least as a species assignment and  
106 delimitation tool.

107           How can we reconcile the above mentioned critical views with this massive usage of  
108 mitochondrial DNA? First, we may object that many of the criticisms would apply to any single  
109 locus, be it sampled from the nuclear or the cytoplasmic genome. Our data says that a very  
110 small mitochondrial distance is indicative of a very small global nuclear divergence in 92 out of  
111 92 cases, suggesting that in insects, mitochondrial DNA does not so often depart from the  
112 common genomic history. Of course, a deeper survey would likely reveal exceptions, but this  
113 would be true for any randomly picked nuclear locus that may sometimes fall at the edge of  
114 the overall distance distribution.

115           Second, it seems important to distinguish, among the many questions relevant to  
116 evolutionary biology and systematics, those that can be addressed at low risk with mtDNA from  
117 those that cannot. Here we answered one specific question: are very small mtDNA distances  
118 indicative of very small overall nuclear distances? In other words, how can we trust barcode-  
119 based clustering of specimens into species? “Very much” is the answer suggested by our  
120 random sample of 92 barcode-based OTUs. However, we did not assess all possible sources of  
121 cyto-nuclear discordance (Toews & Brelsford, 2012). For example, our data is not designed for  
122 testing if specimens harbouring distant mtDNA are always similarly distant at the nuclear level,  
123 which may not be the case in species subject to incomplete introgressions making them  
124 paraphyletic at the mtDNA level (Charlat et al., 2009; Funk & Omland, 2003; Hurst & Jiggins,  
125 2005). We did not either assess how much barcode-based genealogies are reliable, which is  
126 well known to depend on the timescale considered, giving more or less weight to signal  
127 saturation or introgression. Neither did we assess how often mtDNA may be subject to selective

128 sweeps, making them unreliable indicators of demographic histories. It may also be argued that  
129 our conclusion holds only for the taxonomic groups surveyed, that is, four orders of insects.  
130 However, insects are notoriously prone to *Wolbachia* infections, which may make them more  
131 prone than others to cytonuclear discordance, but likely not less.

132 In brief, there is no doubt that mtDNA is far from a perfect marker (Galtier et al., 2009).  
133 Yet, some specific objectives, such as the clustering of specimens into species-like units, can be  
134 attained at low cost and low risk with mitochondrial DNA barcodes. These markers are less  
135 appropriate for other categories of questions, that should generally be addressed not with a  
136 single locus, be it cytoplasmic or nuclear, but with genome wide data.

137

## 138 Material and Methods

139

### 140 *Samples and RAD-seq library preparation*

141

142 To compare mitochondrial and nuclear estimates of divergence, we selected 544 specimens  
143 distributed in 261 mitochondrial OTUs from the SymbioCode system, a large sample of  
144 Arthropods collected in French Polynesia, previously subject to DNA barcoding (Ramage et al.,  
145 2017). We obtained reliable data from 92 OTUs falling in 4 insect orders (Diptera, Hemiptera,  
146 Hymenoptera and Lepidoptera), as detailed in table S1. Importantly, the choice of OTUs and  
147 specimens was blind to the *Wolbachia* infection status, although it had been previously  
148 determined (Bailly-Bechet et al., 2017). In order to obtain RAD-seq data, DNA extracts were  
149 digested using Sbf1 restriction enzyme and distributed in 3 libraries (table S1). Seven samples



150 were analysed twice, in order to assess the accuracy of the average nuclear divergence  
151 estimates. Molecular Identifiers (MIDs) were designed using the barcrawl program (Frank,  
152 2009) and prepared according to Henri et al (2015). A mixture of 8 and 10 bp-MIDs was used  
153 to avoid sequence homogeneity at the restriction site that disturbs base calling (Krueger,  
154 Andrews, & Osborne, 2011) and 15% of phiX DNA were also added to mitigate low complexity  
155 issues. Sequence data was acquired in three experiments. The first library was sequenced using  
156 the HiSeq 2000 system at the « Génomique & Microgénomique » platform of Lyon1 University  
157 (ProfileXpert), producing 100 bp-long reads. The second and third libraries were sequenced  
158 using the HiSeq 3000 system at the Genotoul platform (Toulouse), producing 150 bp-long  
159 reads, with additional PCR amplification cycles to reach more suitable DNA quantities. We  
160 aimed at obtaining paired-end reads in all experiments, but only forward reads were obtained  
161 in one experiment, which was thus excluded from our analysis.

162

### 163 *RAD-seq data analysis*

164

165 Reads were demultiplexed using the process\_radtag program from the Stacks software pipeline  
166 (Catchen, Hohenlohe, Bassham, Amores, & Cresko, 2013), allowing for one mismatch in the  
167 molecular identifier or restriction site sequence. We used paired-end reads to identify and  
168 remove PCR duplicates, using a filterPCRdupl.pl script from the ConDeTri program (Smeds &  
169 Künstner, 2011). Forward reads were then clustered into loci using pyRAD (Eaton, 2014).  
170 Minimal identity (the threshold for inferring that two reads belong to the same locus) was set  
171 to 95%. Minimal coverage (the minimal number of reads in a cluster for it to be defined as a  
172 valid locus) was set to five, following preliminary analyses that showed lower thresholds

173 introduced errors that inflated the estimated genetic distances. Additional filters were used to  
174 identify spurious reads containing adaptors and MID sequences, as well as those showing  
175 significant blast hits with bacterial and archaeal genomes (as present in Ensembl Genome in  
176 October 2014) that represent potential contaminants. Notably, these additional filters removed  
177 only 4% of the data. Homologous loci shared between two specimens within an OTU were  
178 identified using SiLiX (Miele, Penel, & Duret, 2011), with clustering thresholds of 35% minimum  
179 identity and 80% minimum overlap. Clusters including more than one locus per species, that is,  
180 paralogous loci, were excluded.

181

## 182 Data accessibility

183

184 The mtDNA data used in this paper have been made accessible through earlier publications.

185 The RAD-seq will be made available upon on the pbil repository hosted by University of Lyon 1

186 at the following address: <ftp://pbil.univ-lyon1.fr/pub/datasets/>

187

## 188 Authors' contributions

189

190 MC designed and conducted the experiments, analyzed de the data and wrote the

191 manuscript; HH conducted the experimental setup and contributed to analyses and writing;

192 SM contributed to the experiments and writing; LD designed the experiments and analyses

193 and contributed to writing; SC designed the experiments and analyses and contributed to  
194 writing.

195

## 196 **Competing interest**

197

198 We have no competing interest.

199

## 200 **Funding**

201

202 This work was supported by the Centre National de la Recherche Scientifique (CNRS) (ATIP  
203 grant SymbioCode to S.C.).

204

## 205 **Acknowledgments**

206

207 This work benefitted from the computing facilities of the CC LBBE/PRABI. Sequencing  
208 reactions were performed in collaboration with the ProfileXpert (University Claude Bernard  
209 Lyon 1) and GeT core facilities (Toulouse, France, <http://get.genotoul.fr>) which is supported  
210 by the “France Génomique” National infrastructure and the Agence Nationale pour la  
211 Recherche (contract ANR-10-INBS-09).

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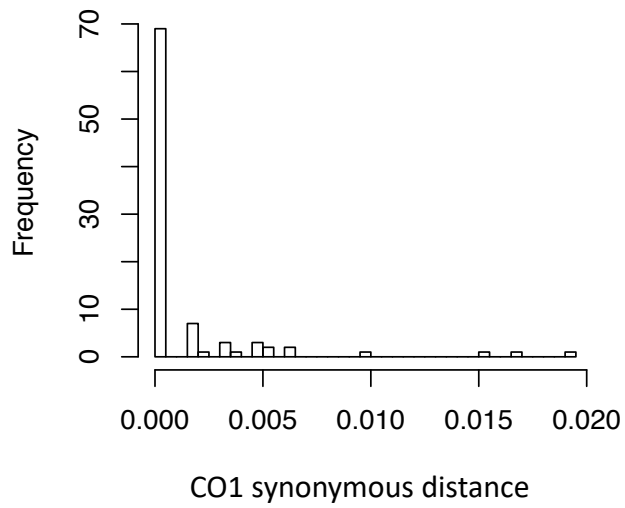
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380 **Figures**

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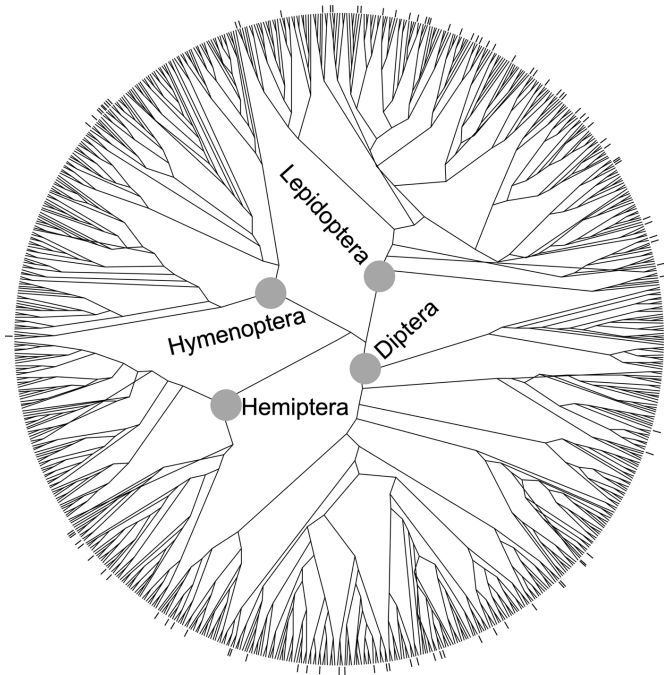


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383 **Figure 1.** Distribution of the CO1 distance within each of the 92 OTUs under study, computed  
384 from 3<sup>rd</sup> codon positions using data from Ramage et al (2017).

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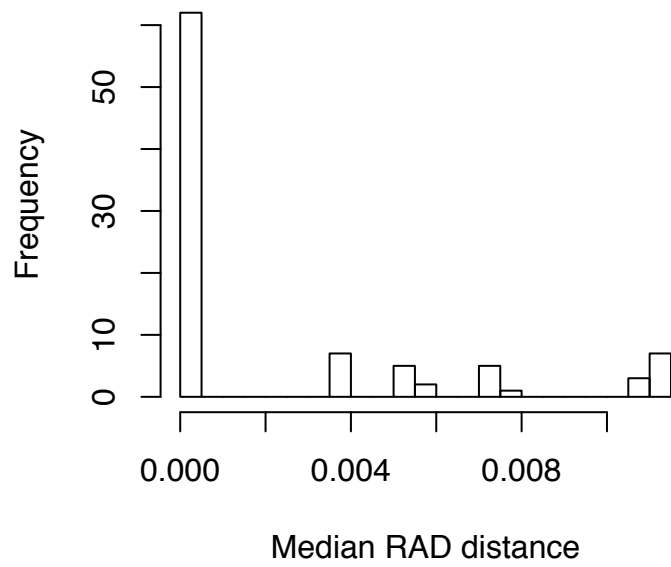
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389 **Figure 2.** Phylogenetic diversity of our sample (92 OTUs) in comparison with the local  
390 diversity. The full tree includes one representative of all Lepidoptera, Diptera, Hemiptera and  
391 Hymenoptera OTUs from a previously reported extensive survey of the Arthropods from  
392 Tahiti and surrounding islands (Ramage et al., 2017). Finer scale taxonomic details can be  
393 found in table S1. Vertical bars on the surface indicate the species included in the present  
394 analysis.

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398 **Figure 3.** Distribution of median RAD distances within the 92 OTUs under study.