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1 **4 years of DNA barcoding : current advances and prospects**

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13

14 **Abstract**

15 L. Frézal and R. Leblois – 4 years of DNA barcoding: current advances and prospects -
16 Infection, Genetics and Evolution

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19 Research using cytochrome *c* oxidase barcoding techniques on zoological specimens was
20 initiated by Hebert et al. (2003). By March 2004, the Consortium for the Barcode of Life started to
21 promote the use of a standardized DNA barcoding approach, consisting of identifying a specimen as
22 belonging to a certain animal species based on a single universal marker: the DNA barcode
23 sequence. Over the last four years, this approach has become increasingly popular and advances as
24 well as limitations have clearly emerged as increasing amounts of organisms have been studied. Our
25 purpose is to briefly expose DNA Barcode of Life principles, pros and cons, relevance and
26 universality. The initially proposed Barcode of life framework has greatly evolved, giving rise to a
27 flexible description of DNA barcoding and a larger range of applications.

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34 ***Index Key words:* DNA barcode, cytochrome *c* oxidase, COI, DNA taxonomy, species**
35 **identification, international species databank, BOLD**

36 1- Introduction

37

38 Species identification and classification have traditionally been the specialist domain of
39 taxonomists, providing a nomenclatural backbone and a key prerequisite for numerous biological
40 studies. Indeed, today's society has to resolve many crucial biological issues, among which are the
41 need to maintain biodiversity, to ensure bio-security, to protect species and to avoid pandemics. The
42 achievement of such goals and the success of subsequent action programs require efficient global
43 networks and rely on our capacity to identify any described species. As Dayrat (2005) clearly
44 expressed, 'delineating species boundaries correctly – and also identifying species – are crucial to
45 the discovery of life's diversity because it determines whether different individual organisms are
46 members of the same entity or not'. The identification of species depends on the knowledge held by
47 taxonomists whose work cannot cover all taxon identification requested by non specialists. To deal
48 with these difficulties, the 'DNA Barcode of Life' project aims to develop a standardized, rapid and
49 inexpensive species identification method accessible to non-specialists (i.e. non-taxonomists).

50 The idea of a standardized molecular identification system emerged progressively during the
51 1990s with the development of PCR-based approaches for species identification. Molecular
52 identification has largely been applied to bacterial studies, microbial biodiversity surveys (e.g.
53 Woese, 1996; Zhou et al., 1997) and routine pathogenic strains diagnoses (e.g. Maiden et al., 1998,
54 Sugita et al., 1998; Wirth et al., 2006) due to a need for culture-independent identification systems.
55 PCR-based methods have also been frequently used in fields related to taxonomy, food and forensic
56 molecular identification (Teletchea et al., 2005) and for identification of eukaryotic pathogens and
57 vectors (e.g. Walton et al., 1999). Several universal systems for molecular-based identification have
58 been used for lower taxa (e.g. nematodes, Floyd et al., 2002) but were not successfully implemented
59 for broader scopes. The Barcode of Life project soon after became that attempt, aiming to create a
60 universal system for a eukaryotic species inventory based on a standard molecular approach. It was
61 initiated in 2003 by researchers at the University of Guelph in Ontario, Canada
62 (<http://www.barcoding.si.edu>) and promoted in 2004 by the international initiative 'Consortium for
63 the Barcode of Life' (CBOL). By then, it had more than 150 member organizations from 45
64 countries including natural history museums, zoos, herbaria, botanical gardens, university
65 departments as well as private companies and governmental organizations. The DNA barcode
66 project does not have the ambition to build the tree of life nor to perform molecular taxonomy
67 (Erbach and Holdrege, 2005; Gregory, 2005), but rather to produce a simple diagnostic tool based on
68 strong taxonomic knowledge that is collated in the DNA barcode reference library (Schindel and
69 Miller, 2005). The DNA Barcode of Life Data System (BOLD, <http://www.boldsystems.org>) has

70 progressively been developed since 2004 and was officially established in 2007 (Ratnasingham and
71 Hebert, 2007). This data system enables the acquisition, storage, analysis and publication of DNA
72 barcode records.

73 In the present paper we briefly review the current state of DNA barcode advances, trends and
74 pitfalls. The main methods of the DNA barcoding approach are given. The feasibility of a universal
75 barcoding approach and interest in the DNA barcoding approach for microbial studies are discussed.

76
77

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79 **2- The DNA Barcoding approach: definitions and objectives**

80

81 *2-1 DNA barcode definition and primary objectives*

82

83 The DNA barcode project was initially conceived as a standard system for fast and accurate
84 identification of animal species. Its scope is now that of all eukaryotic species (Hebert et al., 2003;
85 Miller, 2007). The DNA barcode itself consists of a 648 bp region 58-705 from the 5'-end of the
86 cytochrome *c* oxidase 1 (COI) gene using the mouse mitochondrial genome as a reference. It is
87 based on the postulate that every species will most likely have a unique DNA barcode (indeed there
88 are 4^{650} possible ATGC-combinations compared to an estimated 10 million species remaining to be
89 discovered, Wilson, 2004) and that genetic variation between species exceeds variation within
90 species (Hebert et al., 2003; Hebert, 2004a). The two main ambitions of DNA barcoding are to (i)
91 assign unknown specimens to species and (ii) enhance the discovery of new species and facilitate
92 identification, particularly in cryptic, microscopic and other organisms with complex or inaccessible
93 morphology (Hebert et al., 2003).

94

95 *2-2 When is the DNA barcode useful?*

96

97 Access to a public reference database of taxa allowing identification of a wide range of species
98 will be beneficial whenever accurate taxonomic identifications are required. The DNA barcode can
99 in this way be of great support to numerous scientific domains (e.g. ecology, biomedicine,
100 epidemiology, evolutionary biology, biogeography and conservation biology) and in bio-industry.
101 The cost and time-effectiveness of DNA barcoding enables automated species identification, which
102 is particularly useful in large sampling campaigns (e.g. Craig Venter's Global Ocean sampling team,
103 Rusch et al., 2007). In this way, DNA barcoding could also improve large surveys aiming at
104 unknown species detection and identification of pathogenic species with medical, ecological and

105 agronomical significance (Armstrong and Ball, 2005; Ball and Armstrong, 2006). Besides, it is
106 important to be able to recognize, detect and trace dispersal of patented organisms in agro-
107 biotechnology, either to certify the source organism (e.g. truffles, Rastogi et al., 2007) or secure
108 intellectual property rights for bioresources (Gressel and Ehrlich, 2002; Kress and Erickson, 2007;
109 Taberlet et al., 2007).

110 One obvious advantage of DNA barcoding comes from the rapid acquisition of molecular data.
111 As a contrast, morphological data gathering can be time consuming, in some cases totally confusing
112 and in others, almost impossible (e.g. Dinoflagellate taxonomy, Litaker et al., 2007; *diatomea*, Evans
113 et al., 2007; earthworms, Huang et al., 2007). Furthermore, in three important situations, relevant
114 species identification must necessarily be molecular-based. First, in determining the taxonomic
115 identity of damaged organisms or fragments of (e.g. goods, food and stomach extracts). The DNA
116 barcoding tool is thus potentially useful in the food industry, diet analyses, forensic sciences and in
117 preventing illegal trade and poaching of endangered species (e.g. fisheries, trees, bushmeat). Second,
118 molecular-based identification is necessary when there are no obvious means to match adults with
119 immature specimens (e.g. fish larvae, Pegg et al., 2006; amphibians, Randrianiaina et al., 2007;
120 coleoptera, Caterino and Tishechkin, 2006; Ahrens et al., 2007; fungal sexual stage, Shenoy et al.,
121 2007). The third case is when morphological traits do not clearly discriminate species (e.g. red algal
122 species, Saunders, 2005; fungal species, Jaklitsch et al., 2006; and field-collected mosquito
123 specimens, Kumar et al., 2007), especially when size precludes visual identification (i.e. ‘unseeable
124 animals’, Blaxter et al., 2005; Webb et al., 2006) or if species have polymorphic life cycles and/or
125 exhibit pronounced phenotypic plasticity (e.g. Lamilariales, Lane et al., 2007).

126

127

128 *2-3 DNA Barcoding as a driving force in biological sciences*

129

130 More than being a species identification tool for non specialists, DNA barcoding is also of
131 interest to specialists. To achieve the CBOL objectives, species have to be taxonomically described
132 before their deposit in BOLD, which leads researchers to resolve analytical, technical and
133 fundamental issues beforehand. It also brings together (and complements) taxonomy, molecular
134 phylogenetics and population genetics (Hajibabaei et al., 2007b). According to Rubinoff and
135 Holland (2005), DNA barcoding can be regarded as a ‘tremendous tool’ to accelerate species
136 discovery and initiate new species descriptions (DeSalle et al., 2005; DeSalle, 2006). Moreover, it
137 re-opens the debate on species concepts (Fitzhugh, 2006; Rubinoff et al., 2006b; Balakrishnan, 2007;
138 Miller, 2007; Vogler and Monaghan, 2007). Unlike other well-known sequence libraries (e.g.

139 NCBI), BOLD is an interactive interface where deposited sequences can be revised and
140 taxonomically reassigned. The compiling of sequences, from one or few common loci improves
141 synergic studies at large geographic scales and across numerous genera (Hajibabaei et al., 2007b).
142 Such information on the global distribution of species, their genetic diversity and structure will
143 enhance the speed and effectiveness of local population studies.

144

145

146 **3- Advances in Barcoding**

147

148 *3-1 State of the art*

149

150 By March 2008, the total available DNA Barcode records were at 363,584 sequences (50,039
151 species), of which 136,338 sequences (13,761 species) satisfied DNA barcoding criteria (i.e.
152 minimum sequence length of 500 bp and more than 3 individuals per species). At this date, more
153 than 65% of all barcoded specimens had been collected in the last five years. The majority of the
154 specimens (over 98%) are from the animal kingdom with more than 65% representing Insecta. The
155 International Barcode of Life project (iBOL) is now under development by the new Canadian
156 International Consortium Initiative (ICI). Researchers from 25 countries will be involved in this
157 large-scale and collaborative program, which aims at building a comprehensive DNA barcode
158 registry for eukaryotic life. The program's starting date is tentatively set at January 2009 and within
159 the first 5-year period there are plans to acquire DNA barcode records for 5 million specimens
160 representing 500,000 species (out of more than an estimated 10 million species to be discovered).

161 So far, the COI gene has proved to be suitable for the identification of a large range of animal
162 taxa, including gastropods (Remigio and Hebert, 2003), springtails (Hogg and Hebert, 2004),
163 butterflies (Hebert et al., 2004a; Hajibabaei et al., 2006a), birds (Hebert et al., 2004b; Kerr et al.,
164 2007), mayflies (Ball et al., 2005), spiders (Greenstone et al., 2005), fish (Ward et al., 2005), ants
165 (Smith et al., 2005), Crustacea (Costa et al., 2007) and recently, *diatomea* and Protista (Evans et al.,
166 2007). Hajibabaei et al. (2006a) showed that 97.9% of 521 described species of Lepidoptera possess
167 distinct DNA barcodes and furthermore that the few instances of sequence overlap of different
168 species involve very similar ones.

169 The efficiency of DNA barcoding has been reported in the detection and description of new
170 cryptic species (Hansfield and Hansfield, 2006; Smith et al., 2006b; Anker et al., 2007; Bucklin et
171 al., 2007; Gomez et al., 2007; Pfenninger et al., 2007; Tavares and Baker, 2008) and of sibling
172 species (Hogg and Hebert, 2004; Amaral et al., 2007; Van Velzen et al., 2007). This identification
173 tool can clearly give support to improve classifications and to critically examine the precision of

174 morphological traits commonly used in taxonomy. Indeed, several studies have already illustrated
175 the advances provided by the iterative processes between morphological- and DNA barcode- based
176 studies in taxonomy (Hebert et al., 2004a; Hebert and Gregory, 2005b; Page et al., 2005; Carlini et
177 al., 2006; Smith et al., 2006a; Smith et al., 2007; Van Velzen et al., 2007).

178

179

180 3-2 *New insights into ecology and species biology*

181

182 New insights into ecology and species biology have already emerged from the DNA barcoding
183 project. For example, the identification of organisms contained in stomach extracts allows the
184 elucidation of wild animal diets, especially when behavioural studies are not feasible (e.g. Krill diets,
185 Passmore et al., 2006; affirmation of polyphagy of the moth *Homona mermerodes*, Hulcr et al.,
186 2007; *Xenoturbella bocki* diet, Bourlat et al., 2008). DNA barcoding could also become an efficient
187 tool to clarify host-parasite and symbiotic relationships (Besansky et al., 2003) and in turn give new
188 insights on host spectra, as well as on the geographical distributions of species (host, parasites and/or
189 endangered species). Moreover, the tool is suitable to elucidate the symbiont and parasite
190 transmission pathways from one host generation to the next as illustrated in the interaction of beetles
191 (*Lecythidaceae*) with their endosymbiotic yeasts (*Candida spp.* clades and other undescribed yeast
192 species) (Berkov et al., 2007). Molecular dating of symbiotic relationships can also be deduced using
193 barcoding tools (Anker et al., 2007).

194

195 3-3 *Technical advances in barcoding*

196

197 The purpose of the DNA barcoding project is to rapidly assemble a precise and representative
198 reference library. Thus it is based on conventional and inexpensive protocols for DNA extraction,
199 amplification and sequencing. With time, the reference library will become increasingly useful,
200 enabling the rapid identification of low taxonomic level taxa with specific short-DNA sequences (i.e.
201 mini-barcode 100bp, Hajibabaei et al., 2006c; 300 bp, Min and Hickey, 2007.). It has been shown
202 that species identity can be validated or inferred from a small number of polymorphic positions
203 within the COI-barcode ('microcoding' of 25bp, Summerbell et al., 2005; DNA arrays-based
204 identification, Hajibabaei et al., 2007; SNP-based discrimination, Xiao et al., 2007). Other new
205 molecular technologies used in bioengineering (e.g. silicon-based microarrays, nylon membrane-
206 based macroarrays, etc.) are becoming cheaper and may be integrated into the 'second step of DNA
207 barcoding' (Summerbell et al., 2005). Furthermore, new sequencing techniques such as

208 pyrosequencing (454, Solexa, SOLID) enable rapid and representative analyses of mixed samples
209 (e.g. stomach contents, food, blood or water columns. Largely used in the emerging field of
210 metagenomics, this advance could be promising for future DNA barcoding initiatives.

211 DNA barcoding could also be used as a technical enhancer. Indeed, one condition for data
212 submission to BOLD is the conservation of entire morphological reference for species (voucher).
213 Indeed, new techniques of non-destructive DNA extraction from recently collected specimens have
214 already been developed (Pook and McEwing, 2005; Hunter et al., 2007; Rowley et al., 2007) and
215 additional improvements in specimen conservation may arise. One major drawback of molecular-
216 based studies as for example DNA barcoding is our incapacity to extract DNA from specimens
217 conserved in formalin. Indeed, museum collections of animals represent the major part of voucher
218 specimens from which species have been described and most of these are conserved in formalin. The
219 ultimate challenge is to find the appropriate ways to extract DNA from formalin-conserved
220 specimens and harvest DNA barcodes from them.

221

222

223 **4- What can be learnt from the limitations of DNA barcoding?**

224

225 Despite the promises of the global barcoding initiative, some crucial pitfalls must be
226 mentioned. We believe that these limitations should be clearly identified and resolved in the library
227 construction phase, otherwise the BOLD database will not ever become universally relevant.

228

229

230 *4-1 The under-described part of biodiversity*

231

232 The sampling shortage across taxa can sometimes lead to ‘barcoding gaps’ (Meyer and Paulay,
233 2005), which highlights the care that must be accorded to sampling quality during the database
234 construction phase (Wiemer and Fiedler, 2007). The individuals chosen to represent each taxon in
235 the reference database should cover the major part of the existing diversity. Indeed, in the
236 interrogation of BOLD, identification difficulties arise when the unknown specimens come from a
237 currently under-described part of biodiversity (Rubinoff, 2006; Rubinoff et al., 2006). Meyer and
238 Paulay (2005) estimated the error rates for specimen assignment in well-characterized phylogenies
239 and in partially known groups. They showed that the DNA barcode exclusively promises robust
240 specimen assignment in clades for which the taxonomy is well understood and the representative

241 specimens are thoroughly sampled. Their conclusions are totally concordant with the example of the
242 *Muntjac* described in DeSalle et al. (2005).

243

244 4-2 Inherent risks due to mitochondrial inheritance

245

246 The diversity of mitochondrial DNA (mtDNA) is strongly linked to the female genetic
247 structure due to maternal inheritance. The use of mitochondrial loci can thus lead to overestimate
248 sample divergence and render conclusions on species status unclear. For instance, in *Homona*
249 *mermerodes* (Lepidoptera) mtDNA polymorphism is structured according to the host plants on
250 which females feed, and the two clades produced by phylogenetic analyses are artefacts of female
251 nutritional choice (Hulcr et al., 2007). Heteroplasmy and dual uniparental mitochondrial inheritance
252 (e.g. Mussels, Terranova et al., 2007) are further misleading processes for mitochondrion-based
253 phylogenetic studies.

254 The mitochondrial inheritance within species can also be confounded by symbiont infection.
255 Firstly, indirect selection on mitochondrial DNA arises from linkage disequilibria with
256 endosymbionts, either obligate beneficial micro-organisms, parasitically- or maternally- inherited
257 symbionts (Funk et al., 2000; Whitworth et al., 2007). Such symbionts are very common in
258 arthropods (e.g. *Wolbachia* infects at least 20% of Insecta and 50% of spiders, Hurst and Jiggins,
259 2005; *Cardinium* infects around 7% of arthropods, Weeks et al., 2007) and are probably widespread
260 in many other Metazoa. Secondly, interspecific hybridization and endosymbiont infections can
261 generate transfer of mitochondrial genes outside an individual's evolutionary group (Dasmahapatra
262 and Mallet, 2006). Examples are the cross-generic mitochondrial DNA introgression observed
263 between *Acreae* (Lepidoptera) and *Drosophila* (Diptera) coming from the vertically transmitted
264 *Wolbachia* (Hurst and Jiggins, 2005), or the cross-kingdom horizontal mtgene transfer detected
265 between sponges and their putative fungal symbionts (Rot et al., 2006). Finally, one host species can
266 bear different symbionts (e.g. european populations of *Adalia* bearing three symbionts, *Spiroplasma*,
267 *Rickettsia* and *Wolbachia*, Hurst et al., 1999), leading to intraspecific (i.e. inter-population) variation
268 in mtDNA sequences.

269 In all these cases, nuclear loci are required to resolve phylogenetic relationships and may
270 serve as a validating tool during the database construction stage. Besides, special care must be
271 accorded to the compilation of reference sequences (i.e. DNA barcode), especially for species with
272 already known disturbed mitochondrial inheritance. The presence of such potentially misleading
273 effects should be explicitly indicated in the BOLD. However, unknown endosymbionts or exclusive

274 causes of mtDNA inheritance disturbance could also be revealed during the DNA barcoding
275 database filing.

276

277

278 *4-3 Nuclear copies of COI (NUMTs)*

279

280 Nuclear mitochondrial DNAs (NUMTs) are nuclear copies of mitochondrial DNA sequences
281 that have been translocated into the nuclear genome (Williams and Knowlton, 2001). In eukaryotes,
282 the number and the size of NUMTs are variable, ranging from none or few in *Anopheles*,
283 *Caenorhabditis* and *Plasmodium*, to more than 500 in humans, rice and *Arabidopsis* (Richly and
284 Leister, 2004). As reported by Ann Bucklin (Oral comm., the 3rd international Cons. Gen.
285 symposium, New York, 2007) using DNA barcoding in investigations on marine zooplankton, and
286 by Lorenz et al. (2005) performing primate DNA barcoding, nuclear COI copies can sometimes
287 greatly complicate the straightforward collection of mitochondrial COI sequences. Disturbance due
288 to NUMTs must be seriously considered, in both DNA barcode library construction and further
289 specimen identification. Owing to their particular codon structure, non-synonymous mutations,
290 premature stop codons and insertion-deletions (Strugnell and Lindgren, 2007), NUMTs can be
291 recognized in the sequence and in the amino acid alignments. In the sequence acquisition stage,
292 NUMTs can be detected by the sequence checking process proposed in BOLD (i.e. rejection of
293 inconsistent amino acid alignment), and in such cases, their occurrence should be referenced in
294 BOLD. Only recently integrated NUMTs that are difficult to detect (Thalmann et al., 2004), could be
295 ignored. Although it is more difficult, it is nevertheless possible to get the true mtCOI sequence of
296 voucher specimens with the reverse transcription (Collura et al., 1996). In the diagnostic stage, there
297 may be cases where NUMT occurrence is unknown, which highlights the care that should be taken
298 in DNA barcode alignments.

299

300

301 *4-4 Rate of evolution in COI*

302

303 The rate of genome evolution (mitochondrial or nuclear) is not equal for all living species.
304 Notably, molluscs have a higher evolutionary rate than other bilateral metazoans (Strugnell and
305 Lindgren, 2007). In contrast, diploblast sponges and cnidarians have an evolutionary rate 10-20
306 times slower than in their bilaterian counterparts, a consequence of which is the lack of COI-
307 sequence variation that prevents distinction below the family level (Erpenbeck et al., 2006). The rate

308 of evolution can even differ at the ordinal level, as shown between six dermapteran (Insect) species
309 (Wirth et al., 1999). In the same way, the level of variation in mitochondrial sequences in the plant
310 kingdom excludes species identification based on COI sequence polymorphism (Kress et al., 2005).

311

312 More generally, the lack of resolving power of COI-sequence reported for some taxa has led the
313 CBOL to envisage the transition from the primary single-gene method (i.e. BARCODE) to a multi-
314 region barcoding system, when it is justified (i.e. in cases where COI is not species specific, or for
315 taxa with low mitochondrial evolutionary rates) taxon-specific reference regions (i.e. nuclear plus/or
316 organelle genes), also called non-COI barcode (Bakker, Second International Barcode of Life
317 Conference, TAIPEI, September 2007).

318

319

320 *4-5 The intra-specific geographical structure should be taken into account*

321

322 Geographical structure, if ignored, can blur and distort species delineation. Actually, high rates
323 of intraspecific divergence can derive from geographically isolated populations (Hebert et al., 2003),
324 and thus, must be considered in the setting up of the DNA barcode reference database. This point
325 stresses a key challenge for the DNA barcoding initiative, from both the fundamental and analytical
326 points of view. What is the boundary between a population and a species? Does it exist? To solve
327 this issue, wide-ranging intra-specific sampling should be integrated in the reference database, and
328 one must consider species boundaries not as a definitive but as a revisable concept. The relevance of
329 the reference DNA barcode database depends on the exhaustiveness of intra-taxon sampling.

330

331 To prevent misleading results, the current data format for submission to BOLD should be
332 complemented with new fields related to the limitations mentioned above (i.e. NUMT occurrence,
333 known endosymbiont, available insight on molecular clock, genetic structure and geographical
334 distribution). Besides the biological limitations, DNA barcoding raises analytical and statistical
335 issues.

336

337

338 **5- DNA-Sequence Analysis, a double trend: pure assignment vs delimitation of species**

339

340 *5-1 Query sequence assignment*

341

342 The main and unambiguous objective of DNA barcoding analysis is to assign one query
343 sequence to a set of referenced tagged-specimen sequences extracted from BOLD. The method
344 currently used in BOLD combines similarity methods with distance tree reconstruction in the
345 following way: (i) First, the query sequence is aligned to the global alignment through a Hidden
346 Markov Model (HMM) profile of the COI protein (Eddy, 1998), followed by a linear search of the
347 reference library. The 100 best hits are selected as a pre-set of "closely related tagged-specimens";
348 (ii) Second, a Neighbor-Joining tree is reconstructed on this preset plus the query sequence to assess
349 the relationship between the query sequence and its neighboring referenced sequences (Kelly et al.,
350 2007). The query sequence is then assigned to the species name of its nearest-neighboring referenced
351 sequence, whatever the distance between the two sequences. This method is direct and rapid, but its
352 main shortcomings are high prevalence of sampling-dependent accuracy, high rates of false-positive
353 assignments (Koski and Golding, 2001) and the fact that there is no other way to infer the reliability
354 of the query assignment than computing percentages of similarity or genetic distances, two measures
355 that are known to be irrelevant for taxonomic relationship (Ferguson, 2002). The loss of character
356 information is also inherent in distance methods, as computing distances erase all character-based
357 information (DeSalle, 2006). Moreover, as both similarity and distance methods strongly depend on
358 the disparity between intra- and inter-specific variations, incomplete taxonomic sampling (i.e.
359 barcoding gaps) will artificially increase the accuracy of such methods.

360 Various alternative methods have been proposed to analyse DNA barcode data amongst which
361 we can distinguish four main categories of approaches: (i) similarity approaches, based solely on the
362 similarity between the total DNA barcode sequences or small parts of them (e.g. oligonucleotide
363 motifs, Dasgupta et al., 2005; Little et al., 2007); (ii) classical phylogenetic approaches, using either
364 genetic distances or maximum likelihood / Bayesian algorithms and assuming different mutational
365 models (e.g. Neighbor-Joining, phyML, MrBayes, Elias et al., 2007); (iii) multiple-character based
366 analysis (DeSalle et al., 2005) (vi) pure statistical approaches based on classification algorithms
367 without any biological models or assumptions (CAOS, Sarkar et al., 2002 a, b); and (v) genealogical
368 methods based on the coalescent theory using demo-genetic models and maximum likelihood /
369 Bayesian algorithms (Matz and Nielsen, 2005; Nielsen and Matz, 2006; Abdo and Golding, 2007).
370 The question here is whether it is worthwhile to adopt a biological, populational and/or phylogenetic
371 rationale for DNA barcode sequence analyses or, whether pure statistical approaches are more
372 efficient to assign a query sequence to a species name. Note that character-based methods (either
373 character-based phylogenetics, i.e. not distance-based, or statistical classification) are consistent with
374 the phylogenetic species concept (Goldstein and DeSalle, 2000), whereas distance-based methods
375 are not (Lipscomb et al., 2003). CAOS of Sarkar et al. (2002 a, b) is an example of character-based

376 analysis, in which the nucleotide sequence is considered as a chain of characters. In the same way,
377 DeSalle et al. (2005) proposed the combination morphological and molecular characters, which has
378 the advantage of bridging the gap between the classical taxonomy and ‘molecular-taxonomy’ and the
379 DNA barcoding approach.

380 At present, global comparisons between all these approaches are clearly missing. However,
381 few studies have already compared some of these algorithms (Elias et al., 2007, Ross et al., 2008).
382 For example, Austerlitz et al., (Second International Barcode of Life Conference TAIPEI, September
383 2007) compared phylogenetic tree reconstruction with various supervised classification methods
384 (CART and Random Forest, Support Vector Machines and Kernel methods, Breiman et al., 1984) on
385 both simulated and real data sets. Their main conclusions are: (i) maximum likelihood phylogenetic
386 (PhyML, Guindon and Gascuel, 2003) approaches always seem to be more accurate than distance-
387 based (Neighbor-Joining) phylogenetic inferences; (ii) computation times are much higher for
388 maximum likelihood phylogenetic reconstruction than for statistical classification; and (iii) the
389 accuracy of all the methods strongly depends on sample size and global variability of the taxa.
390 Supervised classification methods outperform phylogenetic analyses only when the reference sample
391 per species is large ($n \geq 10$).

392
393 Rigorous assignment relevancy depends on our capacity to estimate the probability of a false-
394 assignment event. False species assignments can be due to three types of errors (Nielsen and Matz,
395 2006): (i) the true species may not be represented in the database; (ii) the random coalescence of
396 lineages in populations and species may not necessarily lead the query sequence to be the most
397 closely related to the true species sequence; (iii) the random process at which mutations arise on
398 lineages may cause the sequence representing another species to be more similar to the query.
399 Population genetics theory, and more specifically coalescent theory, can help to assess the
400 probability of the occurrence of the last two events. Recently, model-based decision theory
401 framework based on the coalescence theory (Matz and Nielsen, 2005; Nielsen and Matz 2006; Abdo
402 and Golding, 2007) has been established, and should lead to greater accuracy in query sequence
403 assignment with an estimation of the degree of confidence with which this assignment can be made.
404 However, the major drawbacks of such model-based decision tools are high computation times and
405 the requirement of large data sets (e.g. more than 10 sequences per species) for enough genetic
406 information to perform accurate analyses. Moreover, the mitochondrial neutrality has recently being
407 put into question (Bazin et al., 2006), which may invalidate inferences using neutral coalescent
408 processes.

409 To conclude on the query assignment method, it would be advisable to adopt a sequential
410 investigation. Firstly, to search the complete database with similarity methods thus reducing the total
411 data set to the genus or family of the query sequence. Then, to use statistical classification and/or
412 phylogenetic tools to more precisely assign the query sequence to a given species. If still no obvious
413 assignment emerges, it should then be made using population genetic methods based on coalescence.
414 However, even if the assignment with classification or phylogenetic methods seems unambiguous,
415 coalescent-based methods running on the closest neighbours of the query sequence should give an
416 idea of the degree of uncertainty associated with an identification.

417

418

419 *5-2 Delimitation of species*

420

421 The second and more controversial objective of DNA barcode analyses is to define clusters of
422 individuals and consider them as species, in other words, to do molecular taxonomy on unidentified
423 taxa. Unlike the approaches mentioned above, clustering is an unsupervised learning problem that
424 involves identifying homogeneous groups in a data set. Beside all the well-justified discussions
425 between taxonomists about the molecular delimitation of species, such a clustering approach is much
426 more complicated than pure assignment to a pre-identified taxonomic group. Three main approaches
427 have been put forward so far.

428 Hebert et al. (2004b) first proposed the use of a divergence-threshold to delimit species. The
429 underlying idea was that intra-species divergence is lower than inter-species divergence. The
430 standard divergence threshold value advised was of ten times the mean intraspecific variation ('10-
431 fold rule') with the reciprocal monophyly. Despite the efficiency of the threshold approach reported
432 for fishes (Ward et al., 2005), crustaceans (Lefebure et al., 2006), North American birds (Hebert et
433 al., 2004b), tropical lepidopterans (Hajibabaei et al., 2006a) and cave-dwelling spiders (Paquin and
434 Hedin, 2004), the use of thresholds in species delineation has been strongly discouraged. Indeed, the
435 divergence-threshold methods lack strong biological support and undoubtedly could not become a
436 universal criterion suited to animal species delineation (Meyer and Paulay, 2005; Hickerson et al.,
437 2006; Wiemer and Fiedler, 2007). By their literature survey of mitochondrial DNA studies on low
438 taxonomic-level animal phylogeny, Funk and Omland (2003) detected species-level paraphyly or
439 polyphyly in 23% of 2,319 assayed species, demonstrating that NJ-tree analysis will fail to assign
440 query sequences in a significant proportion of cases (Ross et al., 2008). The question is thus to
441 clearly characterize the proportion of non-monophyletic species and the relationship between intra-

442 and inter-specific variability in various taxa to globally assess the relevance of such threshold
443 approaches.

444 The second approach to delimitate species has been developed by Pons et al. (2006) using a
445 mixed model combining a coalescent population model with a Yule model of speciation. Their
446 approach is based on the differences in branching rates at the level of species and populations. Such
447 a model allows them to infer a time of branching regime change, from the coalescent rate to the
448 speciation rate, and to define species as being the clusters for which all individuals are branched
449 inside the coalescent time frame. Even if their approach is currently oversimplified because they
450 consider a unique rate of coalescence (i.e. all population sizes are the same) and a unique shift from
451 population to species processes, it is a promising step that combines the principles of population
452 genetics and those of speciation processes.

453 The third methodology, which also uses principles of population genetics, is the extension of
454 the coalescent-based models of Matz and Nielsen (2005), Nielsen and Matz (2006) and, Abdo and
455 Golding (2007). This approach has not yet been fully developed but is suggested in the three above-
456 mentioned papers. The underlying idea is that maximum likelihood or Bayesian inference, using
457 coalescent models, should help to assess divergence times and/or presence or absence of gene flow
458 between the clusters considered. Estimates of divergence times and gene flow can then be used to
459 infer species status of clusters, based on the biological definition of species. As for the assignment
460 methods, the main drawback of such coalescent-based methods are computation times and large
461 intra-specific sampling requirements.

462 We emphasize that, whatever approach is used, every taxonomic decision using DNA Barcode
463 data should be validated by other independent lines of evidence.

464

465

466 **6- What level of universality can the DNA barcode reach?**

467

468 *6-1 The choice of the genome region(s)*

469

470 The main difficulty of DNA barcoding is to find the ideal gene that discriminates any species
471 in the animal kingdom. Hebert et al. (2003, 2004a,b) argue in favor of the mitochondrial 5' COI
472 region (Folmer et al., 1994), a choice justified by its great resolving-power for birds, lepidopteran
473 and dipteran species discrimination. Ideally, a single pair of universal primers (e.g. Folmer primers,
474 Folmer et al., 1994) would amplify the DNA barcode locus in any animal species. The development
475 of taxon-specific primers and their combinations are however sometimes necessary to obtain greater

476 intra-generic accuracy (e.g. coral reef, Neigel et al., 2007), as illustrated by the primer combinations
477 and cocktails required to obtain DNA barcodes from fish species (Ward et al., 2005; Ivanova et al.,
478 2007), or the primer sets needed to distinguish between primate genera (Lorenz, 2005). The COI
479 amplification does not always ensure the success of the specimen identification. Indeed, the COI-
480 based identification sometimes fails to distinguish closely related animal species, underlining the
481 requirement of nuclear regions (e.g. *Cyrb* and *Rhod* to identify all teleost fish species, Sevilla et al.,
482 2007): the idea of a multi-locus DNA barcoding approach is progressively emerging.

483 The extension of DNA barcoding to other kingdoms is also progressing. The efficiency of
484 COI-based barcoding has been documented for few groups of fungi (e.g. *Penicillium sp.*, Seifert et
485 al., 2007), macroalgae (Rhodophyta, Saunders, 2005) and two ciliophoran protists genera
486 (*Paramecium* and *Tetrahymenas*, Barth et al., 2006; Lynn and Strüder-Kypke, 2006; Chantangsi et
487 al., 2007), suggesting that the DNA barcode standardization may be harder to reach than expected. It
488 is now commonly accepted that the universality of the initial COI-based CBOL project is unlikely.
489 Indeed, considering mitochondria solely would not solve problems of differential evolutionary rates
490 among close genera, of inheritance discrepancy, of mtDNA introgression processes and of the
491 intron-size variations that prevent COI-sequence alignment (e.g. fungi, plants). Besides, methods of
492 sequence assignment based on a single-locus will often lack accuracy (Elias et al., 2007). The
493 ineluctable future trend for species identification through DNA barcoding is to develop a multi-locus
494 system, including COI-region or/and independent markers (Rubinoff and Holland, 2005;
495 Dasmahapatra and Mallet 2006; Kress and Erickson, 2007; Smith et al., 2007; Sevilla et al., 2007).
496 Additional molecular markers have already been proposed, among which the nuclear subunit
497 ribosomal RNA genes are promising candidates because of their great abundance in the genome and
498 their relatively conserved flanking regions. Moreover, the use of rRNA allows efficient species
499 distinction (e.g. for amphibians, Vences et al., 2004, 2005; for truffles, El Karkouri et al., 2007), and
500 can sometimes provide classifications into molecular taxonomic units, MOTU (e.g. for nematodes,
501 Floyd et al., 2002; Blaxter et al., 2005).

502 In higher plants, the mitochondrial genome evolves much more slowly than in animals. The
503 COI-region is thus inappropriate for plant species distinction (Rubinoff et al., 2006). The CBOL
504 plant working group (PWG) agrees that plant barcoding will be multi-locus, with one ‘anchor’ (i.e.
505 universal across the plant kingdom) and ‘identifiers’ to distinguish closely related species (Bakker,
506 Second International Barcode of Life Conference TAIPEI, September 2007). Several combinations
507 of DNA regions have been recently proposed (Kress et al., 2005; Chase et al., 2006; Kress and
508 Erickson, 2007; Pennisi, 2007; Lee et al., Second International Barcode of Life Conference TAIPEI,
509 September 2007). At present, there is still no consensus on which candidate markers are the best

510 plant DNA barcoding region (Pennisi, 2007). The future combination will certainly contain non-
511 coding intergenic spacers (e.g. *trnH-psbA*, Kress et al., 2005; Chase et al., 2006; Kress and Erickson,
512 2007) and plastidial coding sequences (e.g. *matK*, Chase et al., 2007). Recently, Lahaye et al. (2008)
513 working on a large representative sample (>1600 plants specimens) strongly converged with Chase
514 et al.'s (2007) conclusion, and advocates the *matK* locus as the best universal 'anchor' for DNA
515 barcoding of plant taxa. However, they also agree with the need for extra loci (i.e. 'identifiers') to
516 resolve lower taxon identification. In addition, Taberlet et al. (2007) focused on the feasibility of
517 barcoding plants from highly degraded DNA that is of interest for ancient DNA studies (e.g.
518 permafrost samples) and other applied fields (e.g. processed food, customs, medicinal plants). They
519 promoted the chloroplast *trnL* (UAA) intron or a shorter fragment of this intron (the P6 loop, 10-143
520 bp), which, despite the relatively low resolution, can be amplified with highly conserved primers.

521

522 If the prior universality of a single locus and a single primer set remains utopian, the use of a
523 few common loci is still a great advance for future diversity assessments within higher taxa. Steady
524 common features of the DNA barcoding approach will remain, but will certainly evolve in kingdom-
525 or even lower taxon- specific technical approaches.

526

527 6-2 The challenge: barcoding microscopic biodiversity

528

529 One of the greatest challenges for the Barcode of Life project is to account for the diversity of
530 unicellular life (i.e. archaea, bacteria, protists, and unicellular fungi). As a matter of fact, with an
531 evolutionary history dating back to 3.5 billion years, microscopic life (<1mm) represents the largest
532 part of biodiversity. Besides, microscopic species are the causal agents of numerous diseases and are
533 keys to the functioning of trophic networks (Chantangsi et al., 2007). In oceans, microbial life is
534 responsible for 98% of the primary production and the mediation all the biogeochemical cycles
535 (Sogin et al., 2006). One of the striking characteristics of the microbiosphere is the unstable
536 population size over short periods of time, one population can be dominant at a specific time or
537 location, but rare, and thus difficult to survey at another time or location. The low-abundance
538 populations (e.g. rare biosphere) that account for most of the phylogenetic diversity are masked by
539 the dominant populations, leading to an underestimation of the diversity of microbial life (Sogin et
540 al., 2006).

541 The microbial diversity DNA barcoding has yet been poorly studied. The emerging community
542 genomics (DeLong et al., 2006) and the metagenomics approaches promise great insights on
543 prokaryote biodiversity and molecular evolution (DeLong, 2004; Tyson et al., 2004; Venter et al.,

544 2004; Tringe et al., 2005; Leclerc et al., 2007). But, for microscopic eukaryotes, the routine use of
545 the metagenomics approach is not technically conceivable and too expensive today, highlighting the
546 interest of DNA barcoding on pooled samples (i.e. where multiple species are present or
547 communities) to assess eukaryotic microbial diversity (Johnson and Slatkin, 2008).

548 Exploring the microscopic eukaryotic life diversity can be achieved by the COI-based barcode
549 (Blaxter et al., 2005, Chantangsi et al., 2007; Evans et al., 2007). But as for the macroscopic species,
550 a specific and multi-locus barcoding approach may be required. The 5'-end region (600 bp) of the
551 small ribosomal subunit has already been reported to assign isolates to specific subtypes of the
552 human parasite *Blastocystis hominis* (Sciicluna et al., 2006) and successfully reached types or
553 species in the deep seas (Sogin et al., 2006).

554

555 6-3 Universality of the Barcode Of Life Data system (BOLD)

556 The universality of the Barcode of Life mainly resides in the synergic and standard approach
557 for data acquisition and their compilation into BOLD, which is the central connection of the CBOL
558 initiative. The current format for data submission to BOLD is composed of five fields for voucher
559 specimen characterization: (i) the specimen identifier (the catalogue and collection codes, the
560 institution responsible for providing the specimen samples); (ii) the taxonomic status; (iii) the
561 specimen characteristics (sex, life stage, reproduction); (iv) the collection data (collector, collection
562 date and location with GPS coordinates); (v) the DNA barcode sequence (gene name and location,
563 trace file, alignment details, primers used to generate the amplicon). Next to this, pictures of
564 vouchers and the trace of their DNA Barcode must be submitted. All the guidelines are specified on
565 the BOLD website (<http://www.barcodinglife.com/docs/boldtutorial.html>).

566 Recently the international sequences depository (NCBI) have accepted to put in place a
567 BARCODE keyword into their search facilities for entries that conform to the minimal CBOL
568 requirements (i.e. traces file, collection location). The BOLD barcode Submission tool associates the
569 'Barcode submissions' with a further submission to NCBI using the 'My NCBI' user name. Note
570 that for data that satisfies the BARCODE-keyword conditions, the NCBI taxonomy browser gives
571 the direct link to the BOLD taxonomy browser. A keyword related to the concept of 'non-COI
572 barcode' may be soon envisaged.

573 BOLD will provide an increasing amount of DNA barcode records (either COI- or non-COI
574 barcodes) to clearly identify unknown specimens, which will enable accurate query assignments and
575 will facilitate comparison between data obtained in geographically dispersed institutions. Recently,
576 an increasing amount of initiatives for global data recording have been proposed to manage clinical
577 and molecular information about infectious diseases (epiPATH, Amadoz and Gonzales-candelas,

578 2007), but also to focus on either human pathogenic bacteria (e.g. pathoMIPer, Thiyagarajan et al.,
579 2006; pyloriBASE, Ahmed et al. 2007; VectorBase, Lawson et al., 2007; DengueInfo, Schreiber et
580 al., 2007) or on plant pathogens (e.g. PhiBASE, Winnenbourg et al., 2006). BOLD could serve as the
581 universal starting point for species identification, which would convey users to refer to specialized
582 databases (e.g. pathogenic strains, disease vector species and endangered species). The CBOL has
583 already initiated the new International Network for the Barcoding of Invasive and Pest Species
584 (INBIPS; www.barcoding.si.edu/INBIPS.htm) that will help to coordinate the collection of barcode
585 data on pest species around the world (Ball and Armstrong, 2006).

586

587

588

589 **7-Conclusion**

590

591 After four years, the animal DNA barcoding approach has become less controversial, its
592 relevancy is now supported by numerous successes and by the increasing amount of DNA barcoding
593 projects, among which the barcoding of 500,000 species planned by the iBOL. However, clear
594 limitations arise from the incomplete coverage of the existing diversity, the inherent characteristics
595 of the mitochondrial DNA (evolutionary rate, inheritance, introns, neutrality) and the single-locus
596 initial strategy. With its enlargement to all eukaryote taxa, the Barcode of life project has also
597 evolved to a more flexible framework. The approach reveals to be more complex than the system
598 projected by the CBOL initially. The multi-locus barcoding approach is now commonly accepted,
599 particularly to discriminate between low level taxa and to increase the power of the sequence
600 assignments.

601 The BOLD data system is central to the DNA barcoding approach. The specificities of BOLD
602 are (i) to assemble standard information on voucher specimens using common description fields
603 (DNA tag, specimen taxonomy, specimens details, collection information, voucher pictures), and,
604 (ii) its dynamic status that allows taxonomic revisions and reassignment of the deposited sequences.

605 The final point concerning the Barcode of Life project is that, beyond the construction of a
606 standard approach based on the existing taxonomic knowledge, it has enhanced communication
607 between different scientific communities, including taxonomists, phylogeneticists and population
608 geneticists.

609

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614

615

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