Discussion

The role of CO1 barcoding in taxonomic revision

In traditional morphology-based taxonomy, morphologically discrete forms are tentatively recognized and hypothesized to be species. Taxonomists search for consistent phenotypic discontinuities that may indicate the occurrence of reproductive isolation. Many ant species, however, show considerable geographical variation in morphological characters. An additional complication for morphology-based taxonomy is the difference between castes within the same species, e.g. males, major and minor workers, and queens. Sequence data provide an alternative set of characters to assist in inferring species boundaries. In addition, like morphological data, hypotheses can be evaluated in light of additional data on specimen distribution, biology, and behavior. In the example of *Anochetus* of Madagascar, sequence data impacted the taxonomic process at the following steps:

Caste association. Caste association, including male/female association, is a powerful contribution to taxonomic studies, especially for ants, which vary tremendously in morphology between sexes and castes. In this study, CO1 divergence was the principal source of data for revealing that small and large workers and queens are the same species. Though no morphological distinction in addition to size between the forms was noted, it remained unclear whether they belonged to the same species since no colony collection contained both size classes, even though they are often collected at the same site. One explanation is that small workers are produced by small queens. Small queens may represent an alternative reproductive strategy and may be only rarely produced by large queens. Further research will explore the reproductive biology of this species. The sequence data also confirmed the association of males collected in Malaise traps with the worker caste.

Type designation. The identities of many valid names are in question in Madagascar because insufficient geographic and morphological information was provided in their original descriptions, or type specimens are of uninformative minor worker castes or are damaged. For *Anochetus*, description of new species included the DNA barcode of a specimen from the paratype colony series to provide an additional tool for associating the name with type specimens. This facilitates linking the name to the type specimen if the identity of the type is called into question.

Evolutionary questions and biogeographic patterns. Sequencing revealed patterns of geographic coherence and divergence that were not revealed in morphological analysis. A good example of this is the deep divergence in isolated populations of *A. goodmani*, and *O. coquereli* (see *Species as hypotheses* below). These results will direct future morphological and evolutionary studies on these divergent populations.

Identification. In-depth morphological study, a more time-consuming process than the DNA analysis undertaken in this study, was applied to outliers identified by the DNA analysis. For example, in the inventory described in part I, 22 collections of *Anochetus* from three species were included. All were correctly identified using sequence data. Specimens within the same species that showed high sequence divergence, however, were culled for morphological scrutiny (e.g. *A. madagascarensis* from Amato and Binara).

Biogeography. This combination of traditional taxonomy and DNA barcoding has produced a wealth of biogeographic hypotheses to be tested. Do more basal lineages have more restricted or wider distributions, compared to younger taxa? Are evident patterns of genetic isolation by distance within the ergatoid ponerines examined here shared by all those with wingless queens? Are the mechanisms of isolation the same? Do the phylogeographic groupings correspond with the Wilme *et al.* [29] biogeographic regions hypothesized largely as related to primates? Taxonomy has always had this style of iterative hypothesis testing, but adding an explicit molecular component as with DNA barcoding – allows these hypotheses to be more transparent.

Species as hypotheses

The existence of any species is a hypothesis to be tested, and the transparency of species delimitation is one of the major additions that DNA barcoding brings to systematics. In our analysis, the deep sequence divergences within *A. goodmani* suggests that populations from the north and south of western Madagascar have a long history of isolation, and could in fact be separate species (Fig. 6). However, there are alternate hypotheses. This species has wingless queens. Species of ants that lack winged queens, reproduce by fission and have reduced dispersal ability, particularly when measured using a maternally inherited genetic marker. Thus, we might expect that those populations now restricted to isolated relict pockets of moist habitat in the dry west would show deep divergence [for example – 44–46], and represent distinct, evolutionarily significant units [47], if not distinct species. By contrast in *A. madagascarensis* and *A. grandidieri*, where only winged queens have been observed, within-species sequence divergences are much lower. We are currently testing the hypothesis that female-limited dispersal has caused the extremely site-specific phylogeographic signal by assaying nuclear genes. It is possible that these populations, separated at such a large spatial scale, will show strong genetic differentiation for both nuclear and mtDNA markers between localities [44]. The CO1 analysis does not unequivocally indicate that *A. goodmani* is more than one species, but it does suggest future hypotheses of species membership to be tested.

Molecular approaches to species identification have been criticized for potentially overestimating [48,49], and/or underestimating biodiversity. Species diversity will be underestimated when collections include quickly evolving species-pairs [9] where interspecific divergences are less than or equal to intraspecific variation. Our data set contains one potential example of this phenomenon: individuals of *Anochetus goodmani* collected from Binara on the north east coast and Parc National de Kirindy Mite on the south west coast. Individuals from these populations are separated by, on average, 6.0% sequence divergence. Are these populations operating as separate species? Are these populations members of the same species but highly divergent? Our data alone cannot answer this question. But, of critical import, our data have identified a surprising level of within-species divergence and lays bare these differences to further study. A standard arthropod molecular clock for CO1 is 1.2–1.5% per million years [50–52]. In hymenopterans the rate for this gene is accelerated [53], and therefore average estimates should be interpreted with caution. However, the higher rates suggest that populations have been isolated for several hundred thousand years. The opportunity now exists to employ a suite of approaches (behavioral observations, tests of interbreeding, and phylogeographic resolution of more quickly evolving genetic markers) to test species membership.

CO1 and complementary genetic analyses

Of all the molecular data used here, the CO1 data was by far the easiest to generate and interpret. While an inter-gene/genomic comparison of utility was not the intent of this research, we feel it important to comment on these differences here, while presenting a full multigene phylogeographic analysis of the covariance of genetic diversity and geographic separation in another manuscript.