

Cyclocephala (Coleoptera: Scarabaeidae: Dynastinae) evolution in Lesser West Indies indicates a Northward colonization by *C. tridentata*

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Abstract

A dual cytogenetic and molecular analysis was performed in four species of *Cyclocephala* (Coleoptera: Scarabaeidae: Dynastinae) from Lesser Antilles (Martinique, Dominica and Guadeloupe). Two species/sub-species, *C. mafaffa grandis* and *C. insulicola*, are endemic to Guadeloupe. They have their own non-polymorphic karyotype and a fairly homogeneous haplotype of the *COI* gene. *C. melanocephala rubiginosa* has a distinct karyotype. Its *COI* haplotype is homogeneous in Guadeloupe and heterogeneous in Martinique. Finally, *C. tridentata* has highly different karyotypes and haplotypes in the three islands. In Martinique, its karyotype, composed of metacentrics, is monomorphic while its haplotype is fairly heterogeneous. Both are close to those of other *Cyclocephala* and Dynastinae species, thus fairly ancestral. In Guadeloupe, its karyotype is highly polymorphic, with many acrocentrics, and its haplotype fairly homogeneous. Both are highly derived. In Dominica, both the karyotype and the haplotype represent intermediate stages between those of Martinique and Guadeloupe. We conclude that several independent colonization episodes have occurred, which excludes that *C. insulicola* is a vicariant form of *C. tridentata* in Guadeloupe. Both chromosome and *COI* gene polymorphisms clearly indicate a recent colonization with a northward direction for *C. tridentata*.

Keywords: *Cyclocephala*, polymorphism, chromosomes, *COI* mtDNA haplotypes, evolution, Lesser West Indies

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Introduction

West Indies, as other islands, are considered as natural laboratories for understanding species formation,

colonization, diversification and extinction (MacArthur & Wilson, 1963; Woods, 1989; Ricklefs & Bermingham, 2008). They are composed of groups of islands with different origins, ages and geography. The Greater Antilles are old, large and have possibly conserved interactions with the surrounding continents, i.e. North and Central America, since their origin. Consequently, the history of their fauna is complex, with possible repeated episodes of colonization. The Lesser Antilles originated more recently (less than 20 Myr ago), partly as a

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consequence of the active volcanic activity yet going on. They were never connected to each other, neither to the continent. Thus, as oceanic islands, it is generally assumed that they have been colonized by over-water dispersal (Darlington, 1957) prior to any possibility of vicariance. The entomo-fauna of these islands is fairly poor, for the number of both species and individuals per species. This may be a consequence of their recent colonization associated with difficulties of adaptation to endemic flora. *Cyclocephala tridentata* Fabricius 1801 (Coleoptera: Scarabaeidae: Dynastinae) is among the rare species which apparently can pullulate. This root-eater insect found an abundant source of food with sugar cane cultures, intensely developed since the 18th century. It belongs to the largest genus of Dynastinae, mainly distributed in Central and South America (Endrödi, 1985). Four other species of *Cyclocephala* occur in Guadeloupe, including a strictly endemic one, *C. insulicola* Arrow 1937, morphologically very close to *C. tridentata*, but limited to highlands of the rain forest of Basse-Terre, while *C. tridentata* occurs all around, in the rest of Guadeloupe and other islands. Because of its limited distribution area, *C. insulicola* was considered as a possible vicariant of *C. tridentata* (Chalumeau, 1983). Two continental species, *C. maffafa* and *C. melanocephala*, occur as a sub-species (*C. maffafa grandis* Burmeister 1847 and *C. melanocephala rubiginosa* Burmeister 1847). The last species, *C. immaculata* Olivier, 1789, is limited to the north of Grande-Terre.

C. tridentata is also known from Dominica and Martinique. Its possible presence in South America needs evaluation (Endrödi, 1985). Populations from Dominica were considered as a sub-species, *C. t. dominicensis*, while those of Guadeloupe and Martinique remained in the same sub-species *C. t. tridentata* (Chalumeau, 1983). A first cytogenetic investigation in Guadeloupe revealed some surprising data (Dutrillaux *et al.*, 2007). The karyotypes of *C. insulicola* and *C. m. grandis*, composed of sub-metacentrics, were very close to each other and fairly comparable to those of other Dynastinae species. At contrast, the karyotype of *C. t. tridentata*, mainly composed of acrocentrics and highly polymorphic, appeared very different from others. It was postulated that it was highly derived from an ancestral karyotype close to that of other species.

DNA-based techniques have proven useful in many areas of entomological research, particularly in the study of taxonomic and phylogenetic relationships (reviewed in Caterino *et al.*, 2000) and population genetics (Behura, 2006). Several gene regions have been investigated but the two regions most often targeted for sequencing in insect systematics are mitochondrial DNA (mtDNA) and nuclear ribosomal DNA (rDNA) (Simon *et al.*, 1994; Caterino *et al.*, 2000; Greenstone, 2006; Stouthamer, 2006). The genes most commonly used include cytochrome oxidase I and II (*COI*, *COII*) for mtDNA, and the 16S and 12S subunits of rDNA. Generally, the *COI* gene is also widely used in DNA barcoding that involves the sequencing of a particular fragment of DNA as a way of identifying an organism. Comparison of the *COI* sequence from an unidentified specimen to DNA sequence databases of identified and characterized species may allow the identification and phylogenetic classification of the specimen. As universal primers are used in the initial PCR assay, barcoding can identify new or previously unknown species, cryptic species or strains. However, as a single character (i.e. one portion of a gene) is used for the identification of a specimen, this technique may have limited phylogenetic resolution unless combined with other genetic, morphological and/or ecological information (Moritz &

Cicero, 2004; Dasmahapatra & Mallet, 2006). Therefore, DNA taxonomy is a current controversy (e.g. Hebert *et al.*, 2002; Lipscomb *et al.*, 2003; Tautz *et al.*, 2003; Will & Rubinoff, 2004). Regardless of differing theoretical or practical views about the larger 'barcoding' programme, a portion of variable DNA sequence can aid diagnosis minimally. Circumstances might include the identification of a limited set of well-known taxa or groups in which traditional diagnostic techniques are exceptionally difficult. Current examples have focused primarily on the identification of species when specimens are especially similar or indistinguishable using traditional types of data.

To understand phylogenetic and taxonomic relationships between *Cyclocephala* species in Lesser West Indies and the origins of island populations, a dual chromosomal and molecular study was developed in specimens from Guadeloupe, Dominica and Martinique, which indicate a northward colonization of these islands.

Material and methods

All animals were captured by light traps in the three islands. Captures were performed at several locations of Basse-Terre of Guadeloupe, near: Petit-Bourg (16°12'N, 61°39'W), Deshayes (16°19'N, 61°45'W), Sofaïa (16°18'N, 61°43'W) and in the west versant of the rain forest (16°11'N, 61°45'W) in March or December 2006, 2007 and 2009. All insects from Dominica were caught at Trafalgar Falls (15°19'N, 61°22'W) in December 2009. Those from Martinique were caught at Le Prêcheur (14°48'N, 61°13'W), Sainte-Anne (14°26'N, 60°53'W) and Ajoupa-Bouillon (14°50'N, 61°08'W) in November 2009.

The insects were kept alive in earth and fed with apple during one to ten days, until they could be dissected in the INRA laboratory from Guadeloupe (Domaine Duclos) or MNHN laboratory in Paris. Dividing cells for cytogenetic studies were obtained from mid gut or testis (Angus, 1982, 1988) and treated according to our usual methods (Dutrillaux *et al.*, 2006, 2010).

Sequencing analysis of the mitochondrial *COI* gene

Mitochondrial DNA was isolated from 152 individuals: 124 specimens *Cyclocephala tridentata* (87 from Guadeloupe, 26 from Dominica and 11 from Martinique), 17 *Cyclocephala melanocephala rubiginosa* (eight from Guadeloupe and nine from Martinique), four *Cyclocephala insulicola* from Guadeloupe and five *Cyclocephala maffafa*. A 525bp segment at the 3' end of the *COI* gene was amplified using the primers C1J 1718 (5'-GGAGGATTTGGAGGTTGATTAGTTC-3') and C1N 2191 (5'-CCCGGTAATAATATAAACTTC-3') (Simon *et al.*, 1994). PCR reactions (50 µl) contained 200–500 ng DNA, 10 × Taq buffer, 2 mM MgCl₂, 0.2 mM of each dNTP, 50 pmoles of each primer and 1 U Taq polymerase (Invitrogen, Carlsbad, CA, USA). The cycling conditions consisted of an initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 40 s and extension at 72°C for 1 min, with a final extension at 72°C for 10 min. All amplified samples were screened for polymorphism using the Single-Strand Conformation Polymorphism (SSCP) method. This method allows the detection of single base polymorphisms in short DNA stretches due to mobility differences of single-stranded DNA fragments during electrophoresis in polyacrylamide gels (Orita *et al.*, 1989). Preliminary SSCP tests were performed with samples known to carry different sequences. Samples that showed the same SSCP

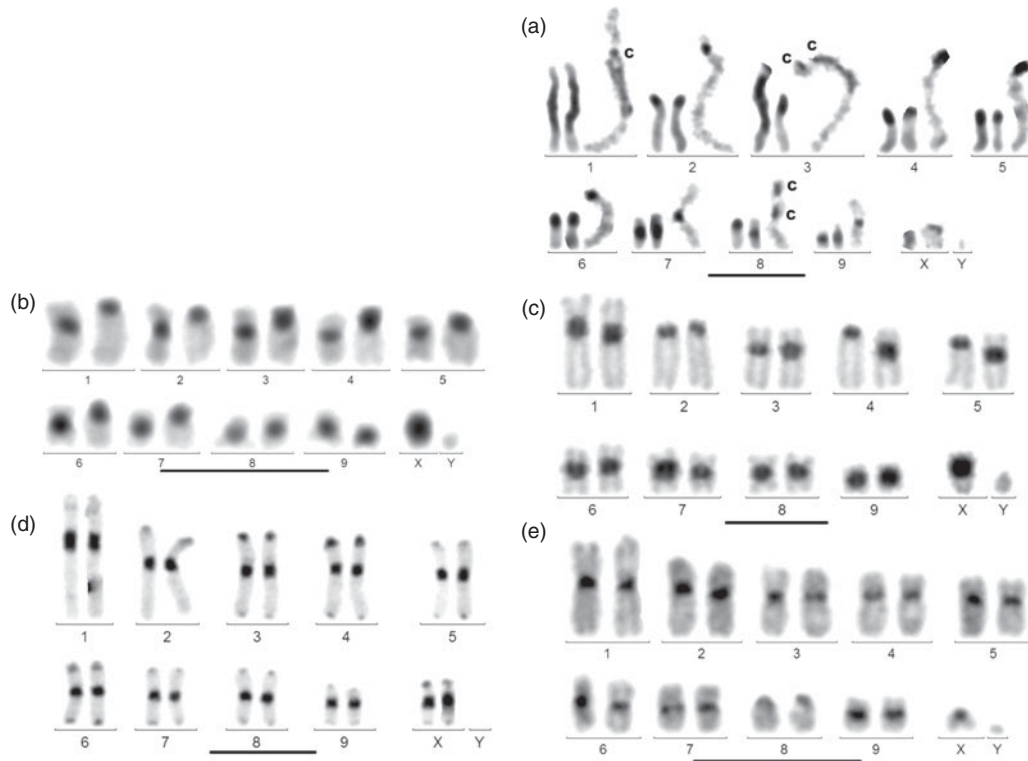


Fig. 1. (a) C-banded karyotype of *C. tridentata* from Guadeloupe with three large sub-metacentrics (N°1, 1 and 3) carrier of amplified heterochromatin (dark). Homozygosity for pair 1 and heterozygosity for pairs 3 and 8 is demonstrated by bivalents staining at pachytene stage of meiosis I (large elements on the right). C, centromere locations on bivalents. (b) C-banded karyotype of a hybrid *C. tridentata* × *C. insulicola*. The large acrocentrics (right) are from *C. tridentata*. The large sub-metacentrics (left) are from *C. insulicola* and do not carry amplified heterochromatin. (c) C-banded karyotype of *C. tridentata dominicensis* carrier of four large acrocentrics. The homo- and heterozygote status has been determined by pachynema analysis, as shown in (a). (d) C-banded karyotype of *C. tridentata* from Martinique. The small telomeric C-bands are variable, but all specimens had only sub-metacentric chromosomes. (e) C-banded karyotype of *C. melanocephala rubiginosa* from Martinique. One pair of small chromosomes (N° 7 or 8) and the X are acrocentric. Chromosome 1 is more metacentric than in other species. Bar = 10 μm.

pattern were grouped, and representative samples from each profile were purified using QIAquick PCR purification kit (QIAGEN Cat. No. 28106, Valencia, CA, USA) and were sequenced directly and bi-directionally by Macrogen Inc. Nucleotide sequences were aligned using ClustalX (Larkin *et al.*, 2007).

For all haplotypes, base composition, nucleotide variation, polymorphic and parsimony informative sites were assessed using MEGA version 4.0 (Tamura *et al.*, 2007). Phylogenetic associations among lineages were assessed with PAUP* 4.0 beta 10 version (Swofford, 1998). To determine the appropriate model of sequence evolution and statistically compare successively nested more parameter-rich models for this data set, the program MODELTEST Version 3.6 (Posada & Crandall, 1998) was used. With a statistical significance of $P=0.01$ the HKY85 model (Hasegawa *et al.*, 1985), with gamma correction, obtained the best likelihood score and was thus selected for the neighbor-joining (NJ) analysis. Parsimony and ML trees were constructed under the heuristic search option with 100 random-taxon-addition replicates and tree bisection–reconnection branch swapping, using PAUP*. Node support was assessed on the basis of 1000 bootstrap replicates.

A Bayesian analysis was also performed with MRBAYES version 3.1 (Huelsenbeck & Ronquist, 2001), under the HKY85 model of sequence evolution. Depending on the data set,

random starting trees run for 2×10^6 to 8×10^6 generations were used, sampled every 100 generations. Burn-in frequency was set to the first 25% of the sampled trees. For all trees, the sequences of *Cyclocephala mafaffa grandis* were used as out-group. The software Network 3.1.0.1 (Fluxus Technology Ltd, downloaded from <http://www.fluxus-technology.com/sharenet.htm>) was used to construct a median joining (MJ) network based on the restriction site data.

Results

Chromosomes

Cyclocephala tridentata (CT)

Guadeloupe (CTT). Following our study of ten males (Dutrillaux *et al.*, 2007), 36 additional specimens were karyotyped. All had $2n=20$ chromosomes, with a number of acrocentrics ranging from 8 to 14 (fig. 1a). All large non-acrocentrics had a very unusual conformation, with long heterochromatic centromeric regions recalling HSRs (homogeneously staining regions) carrier of amplified DNA. When sub-metacentrics were present in a karyotype, their homo- or heterozygote status could be identified by bivalent analyses at pachynema (fig. 1a). We could estimate that up to six chromosome pairs can be involved in this polymorphism.

The karyotype of a sterile specimen, without spermiogenesis, captured in the rain forest at an altitude of about 450 m was obviously hybrid between *C. t. tridentata* and *C. insulicola* (fig. 1b), with seven acrocentric/sub-metacentric heterozygote pairs, none of them carrying HSR-like structures, as in *C. insulicola* karyotype (not shown). Considering their homo- or heterozygote status, 18 different karyotype formulae could be established in addition to that of the hybrid.

Dominica (CTD). The 11 karyotyped specimens had $2n=20$ chromosomes. The number of acrocentrics ranged from 3 to 6. None of the large sub-metacentrics had the HSR-like structures found in specimens from Guadeloupe (fig. 1c). Meiotic analyses indicated that three pairs were involved in this polymorphism, probably by pericentric inversions.

Martinique (CTM). The nine specimens studied also had $2n=20$ chromosomes. All were sub-metacentric. Most of them had a discrete C-banding at telomeric regions, not detected in specimens from Guadeloupe and Dominica (fig. 1d). No variations of euchromatin were observed among the specimens from the three different investigated locations of the island.

Cyclocephala melanocephala rubiginosa (CMR). Fairly similar to that of CTM, its karyotype differed by pair N°1, more metacentric and pairs N°8 and X, acrocentric (fig. 1e). Only specimens from Martinique (CMRM) were karyotyped.

Cyclocephala mafaffa grandis (CMG). Described in Dutrillaux *et al.* (2007), its karyotype differs from that of CTM by the morphology of its chromosome X, acrocentric as in CMR, and the lack of telomeric heterochromatin.

Cyclocephala insulicola (CIN). Also described in Dutrillaux *et al.* (2007), its karyotype, is very similar to that of CTM, but does not exhibit telomeric heterochromatin.

Nucleotide analysis and phylogenetic reconstruction

Sequences obtained, primers excluded, corresponded to a 525bp segment. Haplotypes were evaluated and compared to published sequences in Genbank. All corresponded to new sequences and thus were submitted to GenBank. Their accession numbers are: JF309340, JF309341, JF309342, JF309344, JF309345, JF309355 and JF309356 for *C. t. tridentata* from Guadeloupe, JF309360, JF309361, JF309358 and JF309363 for *C. tridentata* from Martinique, JF309343, JF309354 and JF309349 for *C. insulicola*, JF309350 and JF309359 for *C. mafaffa grandis*, JF309351, JF309352, JF309353 and JF309357 for *C. t. dominicensis* and finally JF309364, JF309362, JF309348, JF309347 and JF309346 for *C. melanocephala rubiginosa*.

Nuclear mitochondrial pseudogenes (numts) seem to commonly occur in most eukaryotic species studied so far (Richly & Leister, 2004; Cameron *et al.*, 2009). However, the absence of insertions, deletions or in-frame stop codons within the sequences studied indicated that they correspond to functional mitochondrial COI gene fragments and are not derived from numts. The high percentage (59.3% on average) observed for the A + T content, is a common feature of animal mitochondrial genes (Brown, 1985). Out of the 525 sites, 141

were variable while 132 of them were informative for parsimony. Among the 141 variable sites, a pattern typically observed in segments under strong functional constraints was revealed given that 78.7% are third codon positions, while first and second codon positions are much more conserved (19.9% and 1.4%, respectively). A number of insect molecular studies using COI are in accordance with these findings (Cognato & Sperling, 2000; Villalba *et al.*, 2002). Similarly, of the 141 variable sites, only ten result in amino acid substitution. Sequence divergence within groups and species ranged from 0.2% for *C. t. dominicensis* (CTD) to 1.2% for *C. melanocephala rubiginosa* (CMR). The lowest sequence divergence (3.7%) was observed between *C. t. tridentata* (CTT) and *C. t. dominicensis* (CTD) and the highest (18.2%) between *C. t. tridentata* (CTT) and *C. melanocephala rubiginosa* (CMR). With some minor differences, the median joining (MJ) network (fig. 2) and the phylogenetic trees based on the Bayesian analysis (fig. 3), as well as on pairwise haplotype divergence (NJ) (tree not shown, available on request), showed similar topologies. The main branches exhibited high bootstrap values (NJ) and high posterior probabilities (Bayesian analysis). In all analyses, specimens of *C. tridentata* from the three islands as well as the three other species were clustered in clearly separated groups without clade overlaps. Using *C. mafaffa grandis* as outgroup, both trees showed that, within *C. tridentata* species, the group of *C. tridentata* from Martinique had a more basal (ancestral) position followed by the groups from Dominica and Guadeloupe (fig. 3).

To estimate the time of separation between groups and species, two different base substitution rates were applied: a divergence rate of 2.3% per million year (Myr), which has been obtained for mitochondrial genes in various organisms (e.g. Brower, 1994; Carisio *et al.*, 2004), and a divergence rate of 3.54% Myr for the COI gene based on 'known' dates (Papadopoulou *et al.*, 2010). Based on the above molecular clocks, separation times that might have occurred between groups and species ranged from 1.0 table 1).

Discussion

Ancestral karyotype and chromosome evolution

Although always composed of 20 chromosomes, the karyotypes of *Cyclocephala* species from Lesser Antilles exhibit a large range of variations. Autosomes may be exclusively sub-metacentric, as in CMG, CIN and CTM, or principally acrocentric, as in CTT. The X chromosome may be also either sub-metacentric, as in CTT, CTD, CTM and CIN or acrocentric as in CMG and CMR. No chromosomal data exist for other Cyclocephalini, but some other Dynastinae were karyotyped. Most often, autosomes are sub-metacentric, and the X chromosome is acrocentric (euchromatin on long arm only) and carries the Nucleolus Organizer Region (NOR) on its short arm (more or less heterochromatin rich) (Dutrillaux *et al.*, 2007; Vidal *et al.*, 1977; Vidal & Giacomozzi, 1978; Vitturi *et al.*, 2003; personal data). The X is also recurrently acrocentric in Melolonthinae, Rutelinae, and Cetoniinae (Dutrillaux *et al.*, 2008 and unpublished data; Moura *et al.*, 2003), suggesting that the acrocentric morphology may be ancestral for Dynastinae. For this chromosome as for autosomes, inversions appear to have frequently occurred during evolution. One of them originated the sub-metacentric X of CTT, CTD, CTM and CIN, transposing the NOR at the extremity of the long arm. It occurred after the divergence of CMR and CMG.

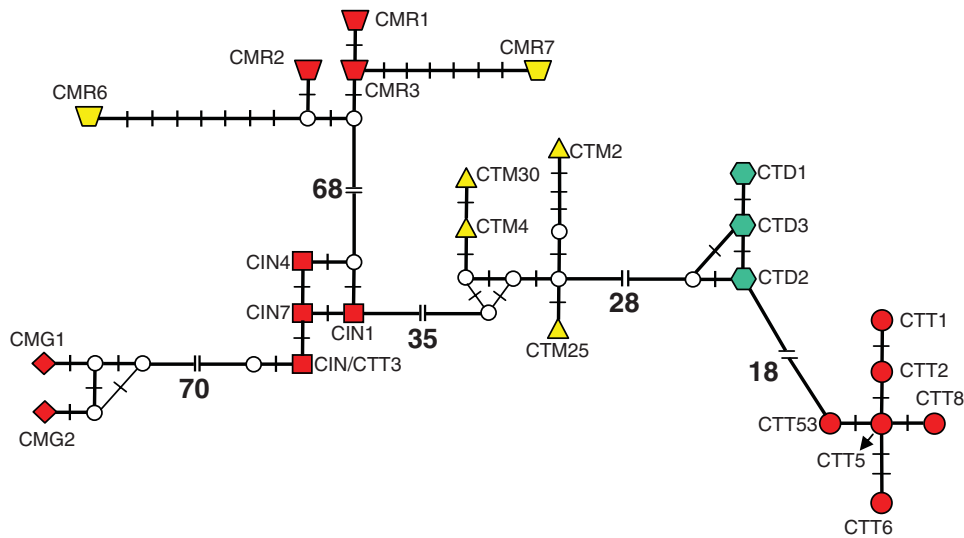


Fig. 2. Median-joining (MJ) network showing the mutational relationships among 25 *Cyclocephala* haplotypes detected in a sample of 152 individuals. Each coloured scheme represents a haplotype, and each group is represented by a different scheme. Each haplotype is identified by its corresponding number, as in the text. Vertical bars on connecting lines between schemes represent a single mutational change, while numbers in bold indicate the total of mutational changes separating the different groups. Red schemes indicate individuals from Guadeloupe, green from Dominica and yellow from Martinique. For the three letter codes, see the Results section. CIN/CTT3 corresponds to the hybrid (fig. 1b). Its haplotype indicates that the mother was CIN.

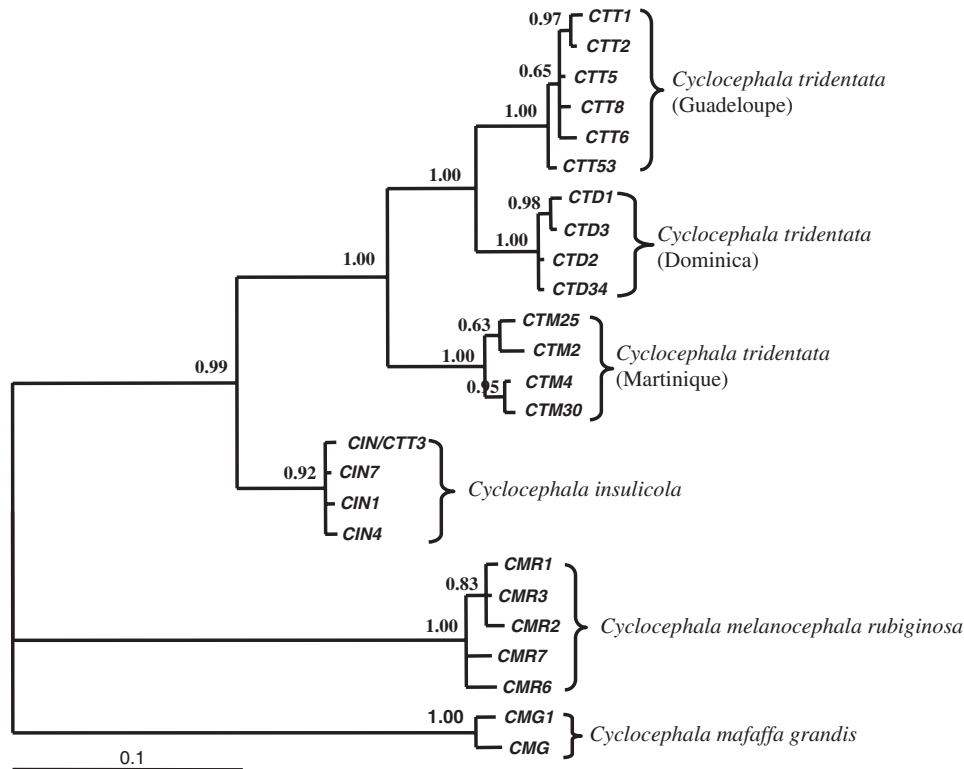


Fig. 3. Phylogenetic tree resulting from the Bayesian analysis, clustering the 25 different haplotypes from *Cyclocephala* groups. The topology of the clusters was similar for the NJ tree. Numbers above branches of the major clusters correspond to posterior probabilities from the Bayesian analysis. Because different specimens showed the same haplotype, haplotypes in the tree have been chosen randomly. For the three letter codes, see the Results section. Each haplotype is identified by its corresponding number, as in text. CIN/CTT3 corresponds to the hybrid (fig. 1b). Its haplotype indicates that the mother was CIN. Trees were rooted with sequences of *Cyclocephala mafaffa grandis*.

Table 1. Pairwise estimates of nucleotide divergence ($\times 10^2$) among *Cyclocephala* species and groups.

	CTT (0.4)	CTD (0.2)	CTM (0.9)	CIN (0.3)	CMG (1.0)	CMR (1.2)
<i>C. tridentata</i> (CTT)	–					
<i>C. tridentata</i> (Dominica) (CTD)	3.7 (1.0–1.6)	–				
<i>C. tridentata</i> (Martinique) (CTM)	5.9 (1.6–2.5)	5.8 (1.6–2.5)	–			
<i>C. insulicola</i> (CIN)	9.8 (2.8–4.3)	9.3 (2.6–4.0)	7.5 (2.1–3.3)	–		
<i>C. mafaffa grandis</i> (CMG)	16.4 (4.6–7.1)	16.8 (4.8–7.3)	17.4 (5.0–7.5)	16.1 (4.6–7.0)	–	
<i>C. melanocephala rubiginosa</i> (CMR)	18.2 (5.1–7.9)	17.3 (4.9–7.4)	17.7 (5.0–7.7)	15.4 (4.4–6.7)	17.6 (5.0–7.6)	–

Numbers in the first line, next to the three letter codes indicate nucleotide divergence within taxa. Numbers in parentheses, next to pairwise nucleotide divergences, indicate estimates of the time of separation between groups and species in million years (Myr).

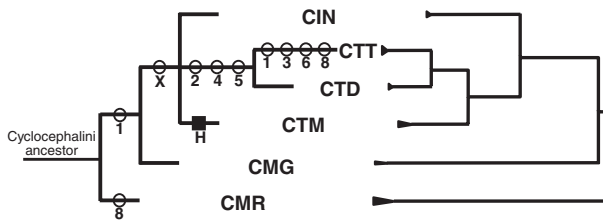


Fig. 4. Phylogenetic tree based on chromosome rearrangements having occurred since the divergence from the presumed ancestral karyotype of Cyclocephalini (left). For the three letter codes, see the Results section. \circ , pericentric inversions; \blacksquare , heterochromatin (H) addition. Numbers under symbols indicate the chromosomes involved. Chromosome polymorphism in CTT and CTD is not indicated. On the right, summary of the tree reconstructed by *COI* gene analysis.

The precise morphology of the ancestral sub-metacentric autosomes remains impossible to reconstruct. However, the fairly distal location of the centromere of chromosome 1, observed in some of the *Cyclocephala* species only, may be of recent origin. Still more recent appear to be the accumulations of acrocentrics, observed in CTD and CTT. These data make possible to propose a scheme of chromosome evolution (fig. 4). *C. melanocephala* ancestors would have separated first, followed by those of *C. mafaffa*. These two species are still present in the American continent. *C. insulicola* and *C. tridentata* from Martinique ancestors would have diverged, after the inversion of the X, without acquisition of any other structural rearrangement. Then, the accumulation of inversions, involving chromosomes 1 to 6, isolated *C. t. dominicensis*, and finally *C. t. tridentata*. This scheme fits very well with the reconstruction obtained by *COI* gene sequence analysis.

Genome evolution and biogeography

Two species, *C. melanocephala* and *C. mafaffa*, occur in the American continent. The first one is also present, as a subspecies, in the three investigated islands, whereas the second one is present in Guadeloupe only. In the chromosomal scheme of fig. 4, they diverged shortly after the origin of the tree, but are clearly separated by inversions. They are also well separated by a high number of gene mutations (fig. 2), with a high genetic distance (17.6%). *C. mafaffa* is genetically homogeneous, as is *C. melanocephala* from Guadeloupe. At contrast, *C. melanocephala* from Martinique is heterogeneous. This may indicate the unique colonization of Guadeloupe vs repeated colonization of Martinique. Both chromosomal and mitochondrial DNA schemes indicate the monophyly of

C. insulicola and *C. tridentata*, with *C. tridentata* from Martinique and *C. insulicola* which remain close to each other and to the origin. *C. t. dominicensis* and *C. t. tridentata* are clearly derived, both genetically and chromosomally, with *C. t. dominicensis* in intermediate position between *C. t. tridentata* and *C. insulicola*/*C. tridentata* from Martinique. Thus, the colonization by *C. tridentata* followed a northward direction, at least from Martinique to Guadeloupe, probably by over-water dispersal. According to Endrödi (1985), the species also occurs in Suriname and Colombia. Thus, it may originate from South America.

The relationships between *C. tridentata* and *C. insulicola*, indicated by both gene and chromosomal evolution, were totally unexpected. The two species are morphologically not much different; and, in Guadeloupe, the distribution of *C. t. tridentata* surrounds that of *C. insulicola*, which is limited to the forest of altitude from Basse-Terre. The latter was thus logically considered as a vicariant form of the former. Obviously, neither mitochondrial DNA nor chromosomes favor this interpretation. The only possible origins of *C. insulicola* are either *C. tridentata* from Martinique or, more likely, a common ancestor from South America. Thus, the colonization of Guadeloupe by *C. insulicola* is independent from that done by *C. tridentata* and came either directly from the continent or via Martinique. Mitochondrial data indicate an ancient origin and thus favor a continental origin. *C. insulicola* appears to be a relict species, only maintained in forest of altitude. We noticed that *C. t. tridentata* was present in 2008–2009 at a place where only *C. insulicola* was found ten years before. One specimen, morphologically considered as a sterile male (gonad atrophy) of *C. insulicola*, was found to be hybrid between *C. insulicola* and *C. t. tridentata* by both mitochondrial DNA and karyotype analyses (fig. 2). Thus, the population of *C. insulicola* may be in regression and that of *C. t. tridentata* in progression towards highlands. In Dominica, where there is no *C. insulicola*, *C. tridentata* occurs in forests at various altitudes. The origin of the Lesser Antilles is double: one ancient (around 50 Myr) corresponds to carbonate sediments, and another, more recent (less than 5 Myr), to volcanic formations. These datings are indeed compatible with the molecular clock giving species or group separations of 1.0 to 7.9 Myr.

Genome polymorphism

Data on mitochondrial DNA indicate that all the species from Guadeloupe and Dominica have low variations, while those from Martinique (*C. melanocephala* and *C. tridentata*) appear more heterogeneous. This may be interpreted as the consequence of the old colonization of Martinique, compared to Dominica and Guadeloupe.

Interestingly, no chromosomal variation was observed in species from Martinique. This well-established karyotype indicates these species are far from periods of active chromosomal speciation processes, which is not in contradiction with the DNA heterogeneity. The situation is very different for *C. tridentata* in Dominica and Guadeloupe. Considering the variations of the acrocentric number and the bivalent synopsis of meiotic cells, we estimated that a minimum of four and 18 different karyotypic formulae existed in Dominica and Guadeloupe, respectively. Such a high chromosomal polymorphism seems to be quite exceptional in Coleoptera. The status of Dominica, with three to six acrocentrics is clearly intermediate between that of Martinique, without acrocentric, and that of Guadeloupe, with 9 to 13 acrocentrics. This fits with DNA data and supports the idea that the chromosome polymorphism has been maintained in Dominica, after migrations to Guadeloupe, and in Guadeloupe, since the beginning of its colonization. Once again, mitochondrial DNA and chromosome data are not contradictory, but quite complementary.

During the last decade, the COI gene was used to accurately place individuals into the correct higher taxonomic levels but also into the correct species. In a survey, aiming to examine the extent of sequence diversity at COI in the major animal phyla, Hebert *et al.* (2002) analyzed more than 13,000 congeneric pairs. They found that in 11% of the 891 species of Coleoptera examined, COI sequence divergence ranged from 3.0 to 8.0%. Therefore, based on the COI sequence divergence of the present study, it is worth wondering on the taxonomic status of *C. tridentata* from the three islands. Sequence divergences between *C. t. tridentata* - *C. t. dominicensis* and *C. tridentata* (Martinique) were 3.7 and 5.9%, respectively, and between *C. t. dominicensis* and *C. tridentata* (Martinique) 5.8%. Thus, their geographical, chromosomal and genetic separation suggests they may constitute three true species. A reassessment of their comparative morphology is in progress to propose their new taxonomic status.

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