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Phylogenetics, species boundaries and timing of resource tracking in a highly specialized group of seed beetles (Coleoptera: Chrysomelidae: Bruchinae)

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ABSTRACT

Though for a long time it was hypothesized that the extraordinary diversity of phytophagous insects was better explained by a synchronous pattern of co-diversification with plants, the results of recent studies have led to question this theory, suggesting that the diversification of insects occurred well after that of their hosts. In this study we address this issue by investigating the timing of diversification of a highly specialized group of seed beetles, which mostly feeds on legume plants from the tribe Indigofereae. To that purpose, a total of 130 specimens were sequenced for six genes and analyzed under a Bayesian phylogenetic framework. Based on the resulting trees we performed several analyses that allowed a better definition of the group boundaries and to investigate the status of several taxa through the use of molecular species delimitation analyses in combination with morphological evidences. In addition the evolution of host plant use was reconstructed and different molecular-dating approaches were carried out in order to assess the ages of several clades of interest. The resulting framework suggests a more ancient than previously thought origin for seed beetles, and a pattern of rapid host plant colonization. These findings call for further similar studies in other highly specialized groups of phytophagous insects.

1. Introduction

The rapid diversification of angiosperms in the late Cretaceous and early Tertiary has provided countless ecological opportunities that likely explain the extreme diversity of insect lineages specialized on angiosperm tissues (Labandeira et al., 1994; Farrell, 1998; Farrell and Sequeira, 2004; Lopez-Vaamonde et al., 2006; Gómez-Zurita et al., 2007). A vibrant testimony of these past plant-insect interactions is provided by fossil traces of insect damages, which can be found in abundance in the plant fossil record (Wilf et al., 2001, 2005; Labandeira et al., 2002; Wing et al., 2009). On the contrary, in numerous insect groups the fossil record is sparse (see Grimaldi and Engel (2005) for a review), and for a long time it was difficult to provide accurate estimates for the age of insect lineages (e.g., Labandeira et al., 1994; Labandeira and Philips, 1996). It was not until the development of molecular dating analyses that it was feasible to investigate more precisely the timing of insect-plant associations in various insect groups, starting with the works of Farrell (1998) or Pellmyr and Leebens-Mack (1999). In beetles (Coleoptera), recent studies on different families have unravelled more recent than previously thought ages for the insect lineages in comparison with those of their host plants (Sequeira et al., 2000; Gómez-Zurita et al., 2007; Hunt et al., 2007; McKenna et al., 2009). These results sharply contrast with former postulates on insect-plant evolution, especially with reference to the coevolutionary theory introduced by Ehrlich and Raven (1964), in which the insects and their host plants experience reciprocal selective responses and are thus expected to have similar ages. With reference to the latter only a few studies have recovered relatively synchronous ages, like the study on *Tetraopes* longhorn beetles made by Farrell (2001) or the study on *Blepharida* leaf-beetles made by Becerra (2003). These two genera have in common a high level of specialization, as *Tetraopes* mostly feed on *Asclepias* milkweeds (Asclepiadaceae) whereas most *Blepharida* feed on *Bursera* torchwood trees (Burseraceae). One can thus make the hypothesis that the more intimate the relation between an insect group and its host plant group is (i.e. the level of specialization), the closer their ages will be. To investigate this hypothesis, we have chosen to focus on representatives of one of the most (if not the most) specialized beetle groups (Southgate, 1979; Johnson, 1981; Farrell and Sequeira, 2004), the seed beetles (Chrysomelidae, Bruchinae).

Bruchine beetles are well known for their obligate seed-feeding habit (hence their trivial name) and because several species of economic importance are found among their ranks (Southgate, 1979; Delobel and Tran, 1993), such as the bean bruchid (*Acanthoscelides*

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obtectus) or the pea weevil (*Bruchus pisorum*). Comprehensive host plant records for the subfamily (e.g., Johnson, 1981) reveal that more than 70% of seed beetle species are associated with legume plants (Leguminosae). The level of specialization of seed beetles is very high: unequivocal host records (i.e. based on the rearing of seeds collected in the field) indicate that a given seed beetle species usually develops in a few species of plants, which generally belong to the same genus or botanical tribe (Janzen, 1980; Johnson, 1981; Jermy and Szentesi, 2003; Delobel and Delobel, 2003, 2005, 2006). The high level of specialization of bruchines has even led several authors to postulate a possible coevolution pattern between the seed beetles and their host plants (Janzen, 1969; Center and Johnson, 1974). Yet, this theory was later discarded by its proponents (Janzen, 1980; Johnson, 1990) in favour of less constrained schemes of evolution such as sequential evolution (Jermy, 1984) in which there are no reciprocal evolutionary changes between the insects and their hosts. In bruchines, the pattern of host plant associations is also conserved over time, as molecular studies reveal that phylogenetically related species are generally associated with host plants that are also closely related (Silvain and Delobel, 1998; Kergoat et al., 2004, 2005a, 2005b, 2007b, 2007c, 2008; Tuda et al., 2006; but see also Morse and Farrell (2005) and Kato et al. (2010) for discussions on complementary patterns). A late Cretaceous origin for seed beetles is supported by molecular clock calibrations (Kergoat et al., 2005a) and by the recent discovery of *Mesopachymerus antiqua* (Bruchinae: Pachymerini) recovered from Cretaceous Canadian amber (approximately 79 Myr; Poinar Jr., 2005). More recent specimens also include other members of the tribe Pachymerini found in British Columbia shale (approximately 52–54.5 Myr; Archibald and Mathewes, 2000), Dominican amber (approximately 15–45 Myr; Poinar Jr., 1999) and Florissant shale (approximately 35 Myr; Kingsolver, 1965). Despite these information on bruchine age, no studies have investigated in a detailed manner the timing of diversification and resource tracking in any group of seed beetles.

In this study, we focus on a paleotropical group that encompasses the majority of seed beetle species associated with legume plants from the tribe Indigofereae (Delobel, 2010). Despite including species from three distinct genera (*Bruchidius, Bruchus* and *Conicobruchus*; see Table 1), this group is morphologically homogeneous: all corresponding species possess distinctive male genitalia and a trapezoid or compressed pronotum with more or less concave sides (Delobel and Le Ru, 2009, 2010a, 2010b). A high level of specialization characterizes these species as they are only

Table 1
List of species belonging or related to the genus *Conicobruchus*. When known, areas of distributions and host plant genera are reported. Taxa sampled in this study are indicated with an asterisk.

Conicobruchus alticola Decelle, 1958	DR Congo, Rwanda and Somalia	
*Conicobruchus atrosuturalis** (Pic, 1939)	DR Congo, Ethiopia, Kenya and Rwanda	*Crotalaria*
Conicobruchus bedfordi (Pic, 1941)	Sudan and Zaire	
Conicobruchus flabellicornis (Boheman, 1829)	Angola, Burundi, DR Congo, Mozambique, Sierra Leone, Tanzania and Zimbabwe	*Indigofera*
Conicobruchus kashmiricus (Pic, 1929)	India	
Conicobruchus impubens (Pic, 1927)	India	
Conicobruchus indicus (Pic, 1909)	India	*Crotalaria*
*Conicobruchus strangulatus** (Fahraeus, 1839)	DR Congo, Ethiopia, Mali, Nigeria and Senegal	*Crotalaria*
Conicobruchus veddarum (Decelle, 1975)	Sri Lanka	
*Bruchidius adouanus** (Pic, 1929)	Central, East and West Africa	*Indigofera*
*Bruchidius albopubens** (Pic, 1931)	Burkina Faso, India, Pakistan, Senegal and Sudan	*Cyamopsis, Indigofera*
*Bruchidius astragalinae** Delobel and Le Ru, 2010b	Senegal	*Indigofera*
Bruchidius bilineatithorax Pic, 1952	South Africa	
Bruchidius decoratus (Fahraeus, 1871)	Angola, DR Congo, Lesotho, Mozambique, South Africa, Tanzania and Zambia	
*Bruchidius eriosemae** nomen nudum	Ivory Coast and Kenya	*Eriosema*
*Bruchidius fuligineus** Delobel and Le Ru, 2010b	Kenya	*Indigofera*
*Bruchidius kidevuensis** Delobel and Le Ru, 2010b	Tanzania	*Indigofera*
Bruchidius hargreavesi (Pic, 1933)	Uganda	
Bruchidius incaeruleus var. *impressicollis* (Pic, 1924)	DR Congo, Ethiopia and Rwanda	
Bruchidius innocuus (Fahraeus, 1871)	South Africa	
*Bruchidius lineatopygus** (Pic, 1924)	Cameroon, DR Congo, Ivory Coast, Kenya, Mali, Niger, Senegal and Togo	*Eriosema, Indigofera*
*Bruchidius lubaicus** Delobel and Le Ru, 2010b	Ivory Coast	*Indigofera*
*Bruchidius mahangoensis** nomen nudum	Namibia	*Indigofera*
*Bruchidius malindiensis** Delobel and Le Ru, 2010b	Kenya	*Indigofera*
*Bruchidius massaicus** Decelle, 1973	Tanzania	
*Bruchidius medaniensis** Decelle, 1982	Kenya, United Arab Emirates and Sudan	
*Bruchidius nigricornis** (Fabricius, 1801)	DR Congo, Kenya, Mauritius, Rwanda, South Africa, Tanzania and Uganda	*Indigofera*
*Bruchidius nodieri** (Pic, 1943)	Eastern, western and central Africa	*Eriosema, Indigofera*
*Bruchidius pilosus** (Boheman, 1829)	Angola, DR Congo, Ethiopia, Ghana, Guinea, Ivory Coast, Rwanda, Sierra Leone and Tanzania	*Indigofera*
*Bruchidius skaifei** (Pic, 1928)	Kenya, Tanzania, South Africa, United Arab Emirates and Zimbabwe	*Indigofera*
*Bruchidius sokokensis** Delobel and Le Ru, 2010b	Kenya	*Indigofera*
*Bruchidius subdolus** Delobel and Le Ru, 2010b	Burkina Faso, Gambia, Kenya, Namibia and Senegal	*Indigofera*
*Bruchidius umbratus** Delobel and Le Ru, 2010a	Comoro islands (Anjouan)	
*Bruchidius watamuensis** Delobel and Le Ru, 2010a	Kenya	*Indigofera*
*Bruchus cicatricosus** Fahraeus, 1839	South Africa and Zimbabwe	*Crotalaria*
Bruchus cicatricosus var. *pallidioripennis* Pic, 1941	South Africa	
Bruchus diegosensis Pic, 1913	Madagascar	
Bruchus obscurus var. *densepubens** Pic, 1929	Cameroon, Congo, Kenya, South Africa and Rwanda	*Indigofera*
Bruchus obscurus var. *longithorax* Pic, 1934	Cameroon, DR Congo, Kenya, Mozambique and Uganda	
*Bruchus rubricollis** Pic, 1903	Kenya, South Africa and Zimbabwe	
*Bruchus sakeensis** (Pic, 1953)	DR Congo, Kenya, Rwanda and the Congo	*Indigofera*
Bruchus (*Acanthoscelides*) *sublineatus* Pic, 1943	DR Congo	
Bruchus turneri Pic, 1929	South Africa	

known to develop on *Cyamopsis* spp., *Eriosema* spp. and *Indigofera* spp. from tribe Indigofereae (Leguminosae) or on *Crotalaria* spp. from tribe Crotalarieae (Leguminosae) (see Table 1).

To better understand the relevance of this grouping of species that belong to different genera, it is important to consider the history of seed beetle taxonomy and systematics. From the beginning, almost all species of seed beetles were described in the genus *Bruchus* Linnaeus, 1758 (Borowiec, 1987). Subsequently several new genera were defined, but some authors (most notably M. Pic) kept on describing species in the genus *Bruchus* instead of placing them in the newly erected genera. Pending an extensive revision of all corresponding type material, numerous species (e.g. see the catalogue of Udayagiri and Wadhi, 1989) are thus still assigned to genus *Bruchus* despite being completely unrelated to the now well-circumscribed *Bruchus* genus (Borowiec, 1987; Kergoat et al., 2007c; Kergoat and Álvarez, 2008). Another classical issue in seed beetle taxonomy is the fact that species that failed to be affiliated to better-circumscribed genera are usually dispatched in the poorly defined genera *Acanthoscelides* (for the New World species) or *Bruchidius* (for the Old World species) (Borowiec, 1987; Kergoat, 2004; Kergoat et al., 2005a, 2007a). Unsurprisingly these two genera clearly appear paraphyletic in recent phylogenetic studies (Morse, 2003; Kergoat and Silvain 2004; Kergoat et al., 2005a, 2005b, 2008, Álvarez et al., 2006). In a similar way, the genus *Conicobruchus*, as currently circumscribed, is likely paraphyletic because of its current equivocal definition. Historically, the genus *Conicobruchus* was defined by Decelle (1951) for the species *Bruchus strangulatus* Fahraeus, 1839 and three other African *Bruchus* species (*B. atrosuturalis* Pic, 1939, *B. bedfordi* Pic, 1941 and *B. flabellicornis* Boheman, 1829). This new genus was mostly defined on the basis of a characteristic shape of the pronotum, which is conical with sides slightly concave in *C. strangulatus* and related species (Decelle, 1951; Borowiec, 1987). Later on, six other species were either described (Decelle, 1958) or transferred from *Bruchus* to *Conicobruchus* by several authors (Arora, 1977; Singal and Pajni, 1986). Another species (*C. veddarum* Decelle, 1975) was also transferred from *Cornutobruchus* to *Conicobruchus* by Borowiec (1987), putting up a total of 11 valid species for the genus *Conicobruchus*. Two species were further withdrawn from *Conicobruchus* (Varaigne-Labeyrie and Labeyrie, 1981; Delobel and Le Ru, 2010a) because they do not exhibit a distinctive compressed pronotum (Varaigne-Labeyrie and Labeyrie, 1981; Kingsolver 1982). The fact that other seed beetle species (listed in Table 1) share many similarities with *Conicobruchus* species (pronotum shape, structure of male genitalia) while being assigned to other genera stresses the need for a general clarification. It is especially the case for the nine taxa (including Pic's varieties) that are still assigned to the genus *Bruchus*. Additionally, no less than 23 species are currently assigned to the large paraphyletic genus *Bruchidius*: this large number can be partially explained by the fact that numerous species were recently described or assigned to this genus in a transitory way (Delobel and Le Ru, 2009, 2010a, 2010b), pending a global reassessment of Old World bruchine systematics. Changes of status, new combinations or even proper descriptions are required for all these taxa, not to mention the species considered as nomina nuda (e.g. *Bruchidius eriosemae* or *Bruchidius mahangoensis*) because they have been named but further left undescribed. This complex situation underlines the need of a wide phylogenetic appraisal based on biological, morphological and molecular data, in order to better redefine the limits of a larger and better-circumscribed genus *Conicobruchus*.

In the present work, first we aim to clarify the status of the genus *Conicobruchus* and of its presumably related species, by providing a comprehensive and well-supported phylogenetic framework. To do so we will rely on extensive analyses of a six genes data set under Bayesian inference (BI). The resulting pattern will allow us to investigate the monophyly of taxa and groups of species, and to better define the boundaries of the genus *Conicobruchus*. We will also assess the usefulness of a molecular species delimitation approach (Pons et al., 2006) in disentangling puzzling species complexes within *Conicobruchus* and closely related species. All these information will be compared to morphological evidences in order to propose taxonomical modifications for the corresponding taxa. The second objective of this study is to investigate the evolution of host plant association and the timing of resource tracking of the species associated with Indigofereae. To do so we will: (1) map the evolution of host plant associations using maximum likelihood; (2) provide divergence time estimates for the sampled seed beetle species, through the use of geological and fossil constraints. The resulting timelines will then be compared with results of studies that have examined the timing of legume diversification (e.g., Lavin et al., 2005), with a focus on plants from tribe Indigofereae (Schrire et al., 2009).

2. Materials and methods

2.1. Taxon sampling and species identification

Most of the specimens used in this study were reared from seeds or legume pods collected on the field between 1994 and 2010 (see supplementary Table 1). The sampling effort was particularly important in Africa, with dozen of thousand legume pods (encompassing about 50 species of Crotalariae and Indigofereae) sampled in more than 300 localities. Additional specimens were also obtained from various collaborators and museums. Overall individuals from 25 countries and 86 localities were included for the purpose of this study. The identity of almost all seed beetle specimens was determined or confirmed by A. Delobel, who has also studied most of the available type material for *Conicobruchus* and related species: during this process, possible morphological variations within putative species were carefully investigated through the examination of hundreds of specimens (see also Delobel and Le Ru, 2009, 2010a, 2010b). All possible members of genus *Conicobruchus* and related species were tentatively included, using whenever possible several individuals from different localities or countries of origin (these specimens are listed as "*Conicobruchus* sensu lato" in supplementary Table 1).

For some taxa, the sampling process was complicated by the existence of potential species complexes that are suggested by noticeable morphological variations among individuals: these distinctive specimens were listed using the cf. abbreviation in supplementary Table 1. It was especially the case for the taxa formerly assigned to *Bruchus obscurus* s.l. (see Udayagiri and Wadhi (1989) for a list of varieties). Despite the fact that *Bruchus obscurus* was put in synonymy with *Bruchidius nigricornis* by Decelle (1969a), this change of status is highly questionable because of the existence of noticeable morphological differences among individuals. For instance, dissections of series of specimens from diverse origins (including the type specimen conserved in the Museum National d'Histoire Naturelle of Paris) have revealed that the taxa listed as *Bruchus obscurus* var. *densepubens* can be separated from *Bruchidius nigricornis* specimens because they possess slightly different male genitalia. In a similar way, specimens formerly considered as *Bruchus obscurus* var. *obscurus* are distinguishable from other *Bruchidius nigricornis* specimens by the presence of two pygidial specula in females. However, several specimens (often listed with the nomen nudum *Bruchidius salamensis* in collections) present intermediate characters: more or less well-defined pygidial mirrors in female, male genitalia that are intermediate of those of *Bruchidius nigricornis* and *Bruchus obscurus* var. *obscurus*. For this study we were able to sample morphologically diverse individuals of *Bruchus obscurus*

s.l. that present almost all possible combinations of characters: these specimens were either listed as *Bruchidius* cf. *nigricornis* or *Bruchus obscurus* var. *densepubens* in supplementary Table 1. Other taxa that exhibit noticeable variations are Western individuals of *Bruchidius subdolus* (see also Delobel and Le Ru, 2010b), Southern specimens of *Bruchidius albopubens* (also known as *Bruchidius mahangoensis* in. litt.), and Eastern individuals of *Conicobruchus strangulatus*.

Although we manage to obtain sequences for 90 specimens of *Conicobruchus* and related species, our sampling is far from exhaustive (out of 39 potential species 25 were sampled; see Table 1 for details). The latter is mostly accountable to the fact that numerous species are only known from unstable countries or from countries for which collecting permits are hard to obtain at best. In addition, for two species (*Bruchidius lubaicus* and *Bruchidius medaniensis*) we were unable to obtain suitable DNA material from dried museum specimens. To assess the monophyletic status of *Conicobruchus* and related species, potential outgroups were picked among almost all known *Bruchidius* species group (species groups *astragali, bimaculatus, centromaculatus, cinerascens, foveolatus, kiliwaensis, niger, pauper, serraticornis, submaculatus, tibialis, tuberculatus, unicolor, varius* and *villosus*). For all corresponding species group but one (*serraticornis* group) we were able to include the name bearer of the species group (for *serraticornis* group we used *Bruchidius quinqueguttatus* instead). In addition, an extensive sampling of species from *unicolor* group was performed because species from this group have often been recovered in a sister group position with *Conicobruchus* (and their related species) in previous phylogenetic analyses (Kergoat et al., 2005a, 2008). We also deliberately included two species endemic to the Canary Islands (*Bruchidius antennatus* and *Bruchidius guanchorum*) in anticipation of divergence time analyses. To complement this sampling, we used results of previous molecular analyses (Kergoat et al., 2005a, 2008, unpublished) to select specimens from closely related genera (*Callosobruchus, Decellebruchus, Megabruchidius, Pygobruchidius*), as well as individuals from other genera (*Bruchus, Paleoacanthoscelides*) of the same tribe (Bruchini). Finally, the most distant outgroups were chosen among representatives of two distinct seed beetle tribes (Kytorhinini and Pachymerini) and of the sister group subfamily Sagrinae (Reid, 1995; Farrell, 1998; Duckett et al., 2003; Gómez-Zurita et al., 2007; Hunt et al., 2007). On average more than two individuals per species were sequenced (nearly four if only considering the *Conicobruchus* s.l.). Collected plants were identified in the laboratory by A. Delobel and B. Le Ru, who have followed the latest available version of ILDIS World database of Legumes for the botanical names (ILDIS, 2010). For the plant material from East Africa further confirmation was made by S. Mathenge (Botany department, University of Nairobi).

2.2. DNA extraction and polymerase chain reaction

Total genomic DNA was extracted by grinding up whole specimens or hind legs using QIAGEN's DNeasy Tissue kit. Four mitochondrial gene fragments were obtained using the following polymerase chain reaction primers: primers CB-J-10933, CB-N-11367 (Simon et al., 1994) and CP1 (Harry et al., 1998) were used to amplify 783 base pairs (bp) of the cytochrome b (*cob*) gene; primers C1-J-1751, C1-N-2191 (Simon et al., 1994), TONYA and HOBBES (Monteiro and Pierce, 2001) were used to amplify 1016 of the cytochrome oxidase subunit 1 (*cox1*) gene; primers SR-J-14233 and SR-N-14588 (Simon et al., 1994) were used to amplify 414 bp (including gaps) of the 12S ribosomal RNA (*rrnS*) gene; primers LR-J-12887 and LR-N-13398 (Simon et al., 1994) were used to amplify 560 bp (including gaps) of the 16S ribosomal RNA (*rrnL*) gene. Two nuclear gene fragments were also amplified using the following primers: primers 28S-F01 and 28S-R01 (Kim et al., 2000) were used to amplify 788 bp (including gaps) of the domains D2–D3 of 28S nuclear rRNA (*28S D2-D3*) gene, while primers 28S.F.D4-5 and 28S.R.D4-5 (Belshaw and Quicke, 2002) were used to amplify 713 bp (including gaps) of the domains D4–D5 of 28S nuclear rRNA (*28S D4-D5*) gene. All these genes were chosen because they are commonly used to assess interspecific relationships in various groups of Coleoptera, including seed beetles (e.g. Álvarez et al., 2006; Tuda et al., 2006; Kergoat et al., 2007c). Polymerase chain reaction amplifications were conducted as described in previous studies (see Belshaw and Quicke (2002) and Kergoat et al. (2004, 2005b) for cycling conditions). Polymerase chain reaction products were sequenced in both directions using the ABI (Applied Biosystems) technology. None of the coding genes had insertion or deletion making alignment unambiguous. The alignment of non-coding genes (*rrnS*, *rrnL*, *28S D2-D3* and *28S D4-D5*) was performed with ClustalX using default option (Thompson et al., 1997) and then reviewed by eye under Mesquite v2.74 (Maddison and Maddison, 2010). When concatenated, the sequenced gene fragments represent a total of 4274 bp. The new sequences reported in this study have been deposited in GenBank (see supplementary Table 1 for the corresponding accession numbers).

2.3. Phylogenetic analyses and hypothesis testing

For each gene best-fit models of evolution were determined by using the Akaike's information criterion (AIC; Akaike, 1974), as implemented in Modeltest v3.06 (Posada and Crandall, 1998). The General time reversible (GTR) + I + G model (Yang, 1994; Gu et al., 1995) was indicated as the best-fit model for all genes, and was further used in all BI analyses. Phylogenetic analyses were carried out under Bayesian inference (BI) using MrBayes v3.12 (Ronquist and Huelsenbeck, 2003). To increase the fit of evolutionary models with data, we used partitioned analyses, which allow subsets of the data to evolve under distinct models and parameters (Nylander et al., 2004; Brandley et al., 2005). Four partitioning strategies were defined a priori: strategy P1, which corresponds to an unpartitioned analysis; strategy P2, which implements one partition for the mitochondrial genes and one partition for the nuclear genes; strategy P3, which implements a partition for each gene (with the two non-contiguous regions of 28S nuclear rRNA being treated as different genes); and strategy P4, which uses one partition for each non-coding gene, and three partitions for the two mitochondrial coding genes (one partition per codon position was used). For each partitioning strategy, two independent BI runs were carried out, each one with four chains (with incremental heating) of 10,000,000 generations, with random starting trees, default priors and trees sampled every 100 generations. A conservative burn-in of 2,500,000 generations was adopted for all partitioning strategies: 25,000 of the saved trees were discarded and the remaining 75,000 trees were used to construct the BI trees. For all resulting consensus trees, the robustness of clades was assessed by clade posterior probabilities (CPP) estimates. The best-fit partitioning strategy was then determined through the estimation of Bayes factors (B_F), using twice the difference of harmonic means and a standard threshold of 10 (see Brandley et al., 2005). The harmonic means were estimated through the *sump* command in MrBayes (with a burn-in of 2,500,000 generations).

The monophyly of the sampled *Conicobruchus* and related species was assessed through additional BI analyses in which these taxa (listed as *Conicobruchus* s.l. in supplementary Table 1) were constrained to be monophyletic. The corresponding estimates of harmonic means were subsequently compared with those of the unconstrained analyses (using B_F) to determine whether they were statistically significantly less supported.

2.4. Species delimitation analyses

Identification of potential species-level branches was conducted using the generalized mixed Yule-coalescent (GMYC) approach of Pons et al. (2006), which investigates branch lengths to divide an ultrametric tree into inter- and intraspecific portions. The method is implemented in the R package GMYC (available at http://r-forge.r-project.org/projects/splits/) and generally relies on a single threshold to delimit nodes defining the most recent common ancestors of species (see also Monaghan et al. (2009) for multiple threshold approaches). Nodes younger than this threshold are assumed to be intraspecific portions. To implement the GMYC approach, the BI tree resulting from the best-fit partitioning strategy (as determined by the B_F) was used as reference tree. The use of a tree based on a concatenated dataset follows the view of several authors that advocate the use of multilocus data to carry out species delimitation analyses (e.g., Fontaneto et al., 2007; Jousselin et al., 2009) because it generally increases the accuracy of species delimitation (Knowles and Carstens, 2007). Due to some known artefacts resulting from incomplete sampling in species delimitation analyses (Papadopoulou et al., 2008), we only analyzed the subset of the tree that encompasses all sampled *Conicobruchus* and related species: all other taxa were excluded from the analysis. The program PATHd8 (Britton et al., 2007) was then used to transform the pruned tree (with branch lengths scaled as evolutionary rate) into an ultrametric tree (with branch lengths proportional to time) with the mean path length (MPL) method (Britton et al., 2002). This approach was preferred because it does not require any specific calibration: it is thus less sensitive to potential overestimations of divergence times in terminal branches that may occur when most available time constraints are outside the clade of interest (Ho et al., 2008).

2.5. Evolution of host plant association

Host plant associations were determined based on extant literature (e.g. Udayagiri and Wadhi, 1989; Jermy and Szentesi, 2003; Delobel and Delobel, 2003, 2005, 2006; Delobel and Le Ru, 2009, 2010a, 2010b) or directly compiled from data obtained during field missions. Because one of the main purposes of this study was to investigate the evolution of host plant association in *Conicobruchus* s.l. we chose to use a simple optimization scheme with only four possible character states: (1) associated with Indigofereae; (2) associated with Crotalariae; (3) associated with other host plant groups; (4) host plant unknown. This setting is expected to maximize the probabilities of having ancestral associations with "other host plant groups", thus permitting to obtain more conservative estimates when looking for the most ancient common ancestor associated with Indigofereae. To limit the possible influence of the overrepresentation of *Conicobruchus* s.l. individuals on ancestral character states estimation, optimizations were conducted on a "species-level" tree (Schluter et al., 1997). The results of GMYC species delimitation analyses were directly used to define the number of terminal taxa for *Conicobruchus* (and their related species) in the "species level" tree. One individual (randomly chosen among specimens with fewest missing data) per putative species was included in the corresponding tree. This pruned tree was constructed with Mesquite, using as guide-tree either the best constrained or unconstrained tree. Ancestral character state estimations were further carried out under maximum likelihood using a one-parameter Markov *k*-state model with symmetrical rates (Lewis, 2001), as implemented in Mesquite. The support of one state over another (at a given node) was considered as significant if the difference between their log-likelihoods was greater than or equal to 2.0 (Schluter et al., 1997).

2.6. Estimation of divergence times

To prevent the systematic overestimation of recent divergence times (Ho et al., 2005; Ho and Larson, 2006), molecular calibrations were conducted on the "species-level" tree that was used to estimate the evolution of host plant association. In a preliminary way, the applicability of a molecular clock was investigated for this tree using PATHd8. Since the hypothesis of a molecular clock was not statistically supported for our dataset ($P < 0.05$), methods of dating that account for rate variation across lineages were used. For comparison purpose we used both Bayesian relaxed clock (BRC) and penalized likelihood (PL) methods.

In BRC approaches, Markov Chain Monte Carlo (MCMC) procedures are used to approximate the posterior distribution of rates and divergence times and simultaneously infer their credibility intervals. In this study BRC analyses were carried out using the BEAST v1.5.4 package (Drummond and Rambaut, 2007), which assumes that substitution rates are uncorrelated across the tree (there is thus no a priori correlation between a lineage's rate and that of its ancestor). Two distinct runs were carried out, each one with four independent chains of 20,000,000 generations, a constant-rate Yule speciation process, default priors and trees sampled every 100 generations. After applying a conservative burn-in of 5,000,000 generations both the mean parameter estimates and the 95% higher posterior densities (95% HPD) were directly estimated using TreeAnnotator v1.5.4 (Drummond and Rambaut, 2007).

In the PL approach, divergence times are approximated with a nonparametric likelihood-based method that relaxes the stringency of the clock assumption using smoothing algorithms (Sanderson, 2002). The optimality criterion is the log likelihood of a given branch minus a nonparametric penalty function, which is used to penalize rates that change too quickly from branch to neighbouring branch (Sanderson, 2002). The weight of the penalty function is determined by the smoothing value: the higher it is, the higher the penalty cost will be. In contrast with BRC approaches, PL only requires a given phylogenetic tree with its estimated branch lengths (hence no molecular matrix is needed for PL analyses). Penalized likelihood analyses were carried out with r8s v1.71 (Sanderson, 2003): in all analyses we used the default truncated Newton algorithm in order to better handle age constraints (Sanderson, 2004). To estimate the optimal smoothing value we used a two-step strategy. First, a cross-validation procedure was conducted on a gradient of smoothing value comprises between 0.1 and 10,000. Second, we used the *checkgradient* command to conduct additional checks on the correctness of solutions for each smoothing value that has successfully passed the cross-validation procedure (Sanderson, 2004). The aim of the latter analysis was to determine the smoothing value that minimizes the number of "active constraints", which correspond to constraints for which age estimates seem to run right up against minimum or maximum age constraints (Sanderson, 2004). Confidence intervals on parameters were estimated through the inference of additional phylograms with the same topology but different sets of branch lengths. To do so we conducted additional MrBayes analyses (same settings, best-fit partitioning strategy) on a species-level dataset. After randomly picking 100 of the resulting trees, additional r8s analyses were carried out (one analysis per supplementary tree, same settings) to provide a confidence interval for each node (see Sanderson (2004) and Lopez-Vaamonde et al. (2009) for details).

For all BRC and PL analyses, five age constraints were enforced to provide a more precise estimation of divergence times. First, a conservative estimate of 183 Myr for the angiosperm age (Bell et al., 2010) was used to set an upper limit for the root age. This particular estimate was chosen because the study of Bell et al.

(2010) likely constitutes the most rigorous and comprehensive calibration study ever done on angiosperm in term of taxonomic coverage, methodology and number of fossil constraints. Second, we have used the age of the oldest known bruchine fossil (79 Myr: Poinar Jr., 2005) to set a minimum age for the seed beetle crown group. Finally three geological constraints based on the age of volcanic islands were enforced: a maximum age of 24 Myr (see Carracedo (2008) for details on the geology) was set for the nodes leading to the species endemic of Canary islands (*Bruchidius antennatus* and *B. guanchorum*); a maximum age of 11.5 Myr (see Emerick and Duncan (1982) and Nougier et al. (1986) for details on the geology) was set for the node leading to the species endemic to the Anjouan island in the Comoros archipelago (*Bruchidius umbratus*).

Under BRC geological constraints were either treated as hard bounds (like in the PL analyses) or as soft bounds (see Yang and Rannala, 2006; Sanders and Lee, 2007). Constraints with soft bounds allow nonzero probabilities for ages that lie outside specified bounds, and as such they are useful to deal with possible calibration errors or uncertainties (Yang and Rannala, 2006; Sanders and Lee, 2007; Ho and Phillips, 2009). Here we used soft bounds to account for possible missing lineages (either gone extinct or not sampled) between constrained nodes and the clades that include the taxa endemic to volcanic Islands (Ho and Phillips, 2009). Soft bounds were not used for the remaining constraints because: (i) the upper limit on the root age is already very conservative; (ii) both the age and the assignation of the bruchine fossil are unequivocal. Soft bounds were implemented in BEAST analyses through BEAUti v1.5.4 (Drummond and Rambaut, 2007): for each geological constraint we used a gamma distribution (default settings were used for the shape and scale of the distribution) and the *mean* value was chosen so that only 5% of the distribution lied above the upper age limit (see Ho and Phillips (2009) for more information on the rational of these settings).

3. Results

3.1. Phylogenetic analyses and hypothesis testing

The four distinct partitioning strategies recover the following harmonic means estimates: −48735.40 for the unpartitioned strategy P1, −48116.48 for strategy P2, −47445.69 for strategy P3 and −46321.29 for strategy P4. All corresponding B_F comparisons (obtained by calculating twice the difference of harmonic scores) significantly favour the strategy P4, which is thus considered as the best-fit strategy in our analyses. Overall, the resulting topology (Fig. 1) is well supported by the CPP as most nodes are supported by CPP > 90%. A congruent topology was recovered in all but one analysis, which corresponds to the BI run that uses the strategy P2 (see below for more details). The seed beetle clade is strongly supported by a CPP of 100%; at the base of this clade, *Pachymerus cardo*, the member of the tribe Pachymerini (Borowiec, 1987) is recovered in a sister group position with the clade encompassing the member of the tribe Kytorhinini, *Kytorhinus thermopsis* and the remaining sampled seed beetle species from the tribe Bruchini.

All sampled representatives of the tribe Bruchini form a well-supported monophyletic group (CPP of 100%). Within this clade, the genera *Bruchus* and *Bruchidius* appear clearly paraphyletic, as their members are completely scattered in different parts of the tree. Almost all *Conicobruchus* s.l. individuals are recovered together in a well-supported clade (CPP of 100%). The sole exception is made by the two specimens of *Bruchidius sokokensis*, which are found outside the *Conicobruchus* s.l. clade, in a sister position (CPP of 95%) of a clade constituted *by Megabruchidius tonkineus* and the members of the species groups *niger*, *pauper* and *unicolor*. Interestingly this placement was not recovered in one of the competing partitioning strategy analysis (P2), which gathers all sampled *Conicobruchus* s.l. individuals in the same clade (yet with a low CPP support <50%). The paraphyletic status of *Conicobruchus* s.l. is also not statistically supported by the B_F: a non-significant value ($B_F = 2\ (46323.54 - 46321.29) = 4.50$) was found for the B_F that results from the comparison of the harmonic score of the unconstrained analysis with those of the analysis in which all *Conicobruchus* s.l. are constrained to be monophyletic.

Within *Conicobruchus* s.l. almost all taxa are recovered monophyletic with high CPP. One noticeable exception is found in a large clade that groups together the individuals belonging to the potential *Bruchidius nigricornis* species complex. In this clade, the individuals that are either assigned to *Bruchidius nigricornis, B.* cf. *nigricornis* or *Bruchus obscurus* var. *densepubens* do not constitute monophyletic groups. Yet, some level of genetic structuring can be perceived especially in reference to members of *Bruchus obscurus* var. *densepubens*, which appear more closely related than other *Bruchidius nigricornis* or *B.* cf. *nigricornis* individuals. It is also interesting to note that the resulting phylogenetic pattern definitely shows some congruence in relation to the structures of male genitalia (as underlined by the illustration of various *Conicobruchus* s.l. male genitalia in Fig. 1).

3.2. Species delimitation analyses

The species delimitation analyses (see Fig. 2) recover a pattern of clustering that can be associated with 27 species entities for the *Conicobruchus* s.l. clade. At least five new taxa are suggested by the latter scheme as either two (for *Bruchidius albopubens, B. lineatopygus* and *Conicobruchus strangulatus*) or three (for *Bruchidius subdolus*) species clusters were recovered for each putative species. On the contrary, only one species cluster is found for the members of the large clade that encompasses the individuals assigned to *Bruchidius nigricornis, B.* cf. *nigricornis* or *Bruchus obscurus* var. *densepubens*. For *B. lineatopygus* and *C. strangulatus*, distinct geographic clusters are recovered with western individuals (from Cameroon, Mali and/or Senegal) on the one hand, and eastern individuals (from Kenya) on the other hand. With reference to *B. albopubens*, the two species clusters either correspond to western individuals (from Senegal) or southern specimens (from Namibia). A more complex pattern is inferred for *B. subdolus*: similarly to the pattern found in *B. lineatopygus* and *C. strangulatus*, *B. subdolus* specimens from West Africa (Burkina-Faso) and East Africa (Kenya and Tanzania) are associated with distinct species clusters. However, the species delimitation analyses also suggest that the specimens from Kenya and Tanzania constitute distinct species entities. Interestingly, this result is also in agreement with their branching order, as they are recovered paraphyletic in the phylogenetic tree. It is also worthy to note that in the two largest clades (*Bruchidius sakeensis* and *Bruchidius nigricornis*, respectively), specimens with distinct geographic origins (Cameroon, Kenya, Mali or Tanzania) are not clustered together.

In addition, the species delimitation analyses provide additional evidence to support the species status of *B. eriosemae* in litt., as a distinct species cluster was recovered for the sampled specimens.

3.3. Evolution of host plant association

Using the unconstrained tree as a guide-tree, the mapping of the evolution of host plant association (Fig. 3) suggests two independent colonizations of Indigofereae (one for *B. sokokensis* and one for the common ancestor of the other *Conicobruchus* s.l., the latter with a probability of 81.13%). Alternatively only one colonization event is suggested for the mapping analysis that uses the constrained tree as guide-tree (probability of 95.55% for the com-

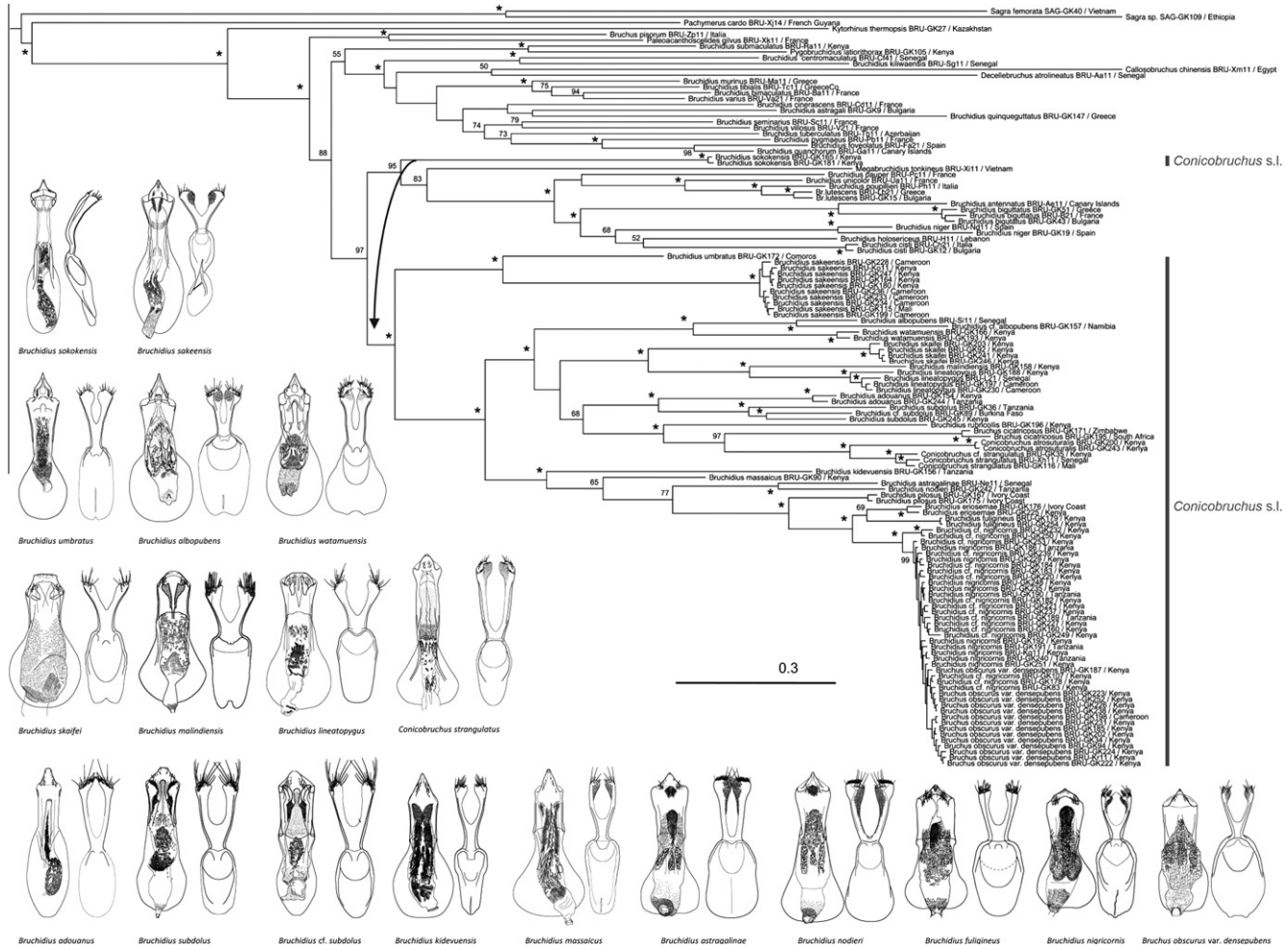

Fig. 1. Bayesian tree resulting from the phylogenetic analysis of the complete dataset under MrBayes (10,000,000 generations with four MCMC chains, best-fit partitioning strategy). Clade posterior probabilities are indicated for most nodes (asterisks were used for nodes with CPP of 100%). The alternative placement of the two members of *Bruchidius sokokensis* is figured using a descending arrow. Drawings of male genitalia for 19 taxa that either belong or are related to the genus *Conicobruchus* are also included for discussion purpose.

mon ancestor of the sampled *Conicobruchus* s.l. to be associated with Indigofereae). Both analyses recover a similar pattern in reference with the four taxa that feed exclusively on Crotalariae, with only one colonization event for the common ancestor of *Bruchus cicatricosus*, *Conicobruchus atrosuturalis*, *C. strangulatus* and *C.* cf. *strangulatus* (ancestral state supported by probabilities of 96.66% and 97.32%, depending on the guide-tree). All these shifts are statistically supported by the likelihood comparisons (log-likelihood difference of one state over another >2.0).

3.4. Estimation of divergence time

Overall, the age estimates do not differ widely between the BRC analyses that have either implemented soft or hard bounds (see Table 2). By contrast PL analyses consistently yield younger estimates, especially when considering ages of the sampled representatives of tribe Bruchini (Table 2). For PL, both the cross-validation and *checkgradient* procedures recover an optimal smoothing value of 0.2 (out of a gradient of values comprises between 0.1 and 10,000). This small optimal smoothing value indicates that much rate variation is allowed in the PL analyses, which is consistent with a dataset that is far from being clocklike (Sanderson, 2004). Using this smoothing value there is still one active constraint (instead of two or three for the other smoothing values), which corresponds to the constraint (maximum age of 11.5 Myr) assigned to the node between *Bruchidius sakeensis* and the species endemic to Anjouan, *Bruchidius umbratus* (node 36 in Table 2). The latter indicates that the PL analysis was constrained to use a non-optimal age (which corresponds exactly to the upper limit assigned to this node: 11.5 Myr) for the corresponding node. In comparison, the BRC analysis with hard bounds found a slightly younger age (10.9 Myr) for the same node whereas the BRC analysis with soft bounds (Fig. 3) clearly supports a much older age and has pushed backward this upper limit to 21.3 Myr. Finally, it should be added that the other geological constraints (upper limit of 24 Myr for common ancestors of taxa endemics to the Canary Islands) were neither considered as active constraints in the PL analysis nor violated by the BRC analysis with soft bounds.

With respect to the age of Bruchinae, very comparable estimates were recovered by all analyses, with ages ranging from 82.6 Myr to 85 Myr (node 1 in Table 2). These estimates significantly predate the ages recovered in previous molecular studies (70 Myr in Kergoat et al., 2005a and 49 Myr in Gómez-Zurita et al., 2007). An old origin is suggested for the clade that gathers most *Conicobruchus* s.l. (39.3 Myr, 46.8 Myr or 49.7 Myr for the analyses using either PL, BRC with soft bounds or BRC with hard bounds). Interestingly, these estimates are not too distant from the estimate (41 Myr) obtained for the age of the common ancestor of the two *Conicobruchus* s.l. specimens sampled in the study of Kergoat et al. (2005a). Within *Conicobruchus* s.l., an age comprised

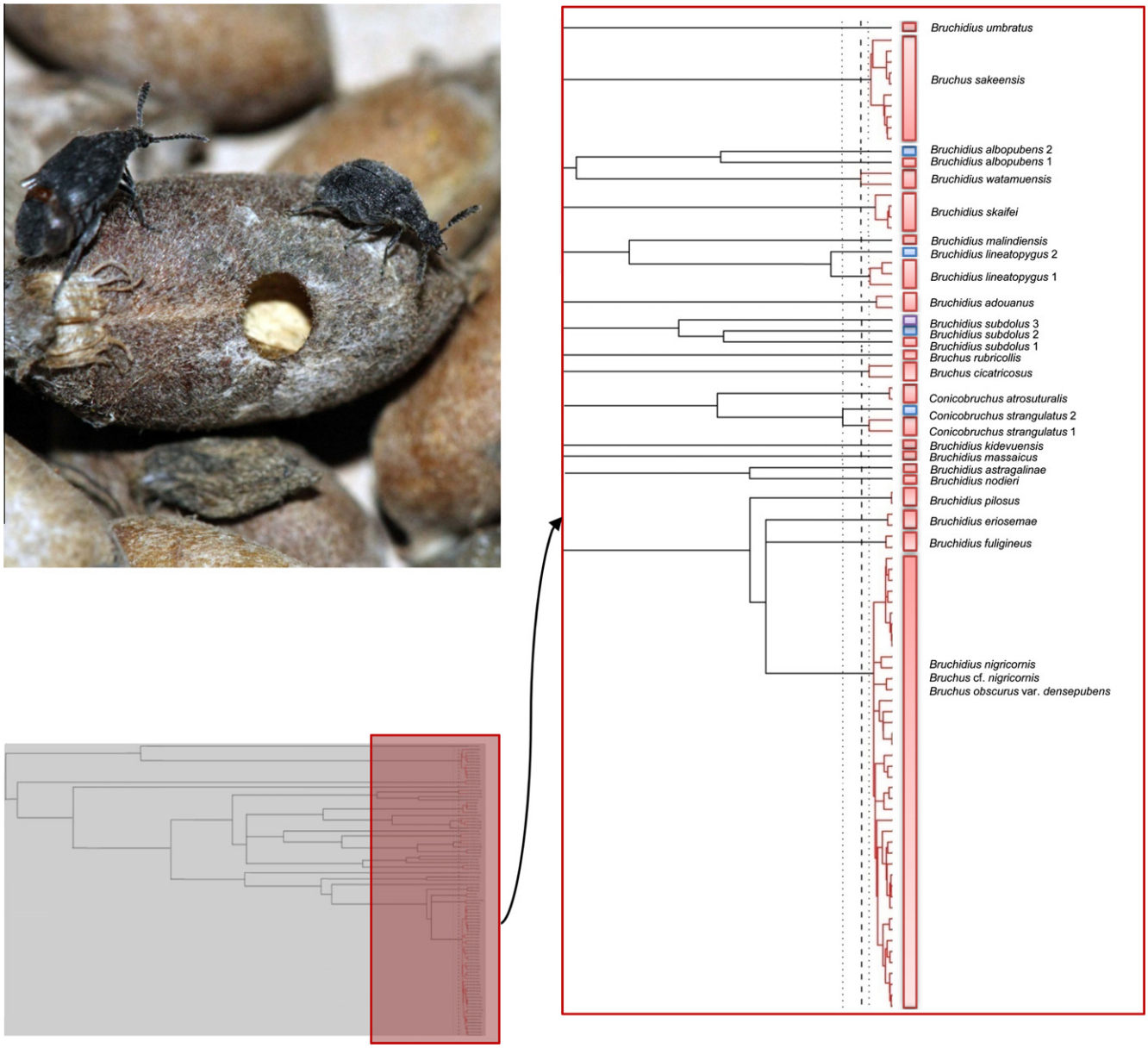

Fig. 2. Result of the molecular species delimitation analysis (for more clarity an enlargement of the excerpt of the corresponding tree is figured on the right). The inter- and intraspecific portions of the tree are divided with a dotted line (95% confidence intervals are figured using thinner dotted lines). Inferred species clusters are highlighted on the right side, using distinct lateral bars (one per species cluster). Distinct colours and tones combinations were used to distinguish potential new taxa. For illustration purpose, a picture of freshly emerged specimens of *Conicobruchus strangulatus* is provided on the upper left (picture by G. Kergoat). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between 15.4 Myr (PL) and 24.1 Myr (BRC with soft bounds) is suggested for the clade of four species that feed exclusively on Crotalariae.

4. Discussion

4.1. Toward a monophyletic genus Conicobruchus?

The results of molecular analyses support for the most part the monophyletic status of the sampled *Conicobruchus* and related species since all species but *Bruchidius sokokensis* are consistently recovered in a well-supported clade. Their monophyly is also supported by a preliminary cladistic analysis of a morphological data set (Delobel, unpublished data), which recovers a monophyletic *Conicobruchus* s.l. group. The fact that these taxa clearly belong to the same clade thus strongly argues for a revision of their generic status. As a first step, we propose to assign all these taxa to the genus *Conicobruchus* Decelle, 1951 (see Table 3 for the proposed nomenclatural changes).

With reference to *B. sokokensis*, it appears that its phylogenetic placement is debatable owing to the results of several molecular analyses that do not unequivocally support its position outside the *Conicobruchus* s.l. clade. On one hand the result of the partitioned BI analysis that recovers the monophyly of *Conicobruchus* s.l. does not constitute a well-supported evidence because the corresponding partitioning strategy is not optimal (not to mention the low CPP value supporting the corresponding clade). On the other hand, the fact that the analysis in which all *Conicobruchus* s.l. are constrained to be monophyletic constitutes a statistically well-supported alternative (as indicated by the B_F) suggests that the issue of the phylogenetic placement of *B. sokokensis* is far from re-

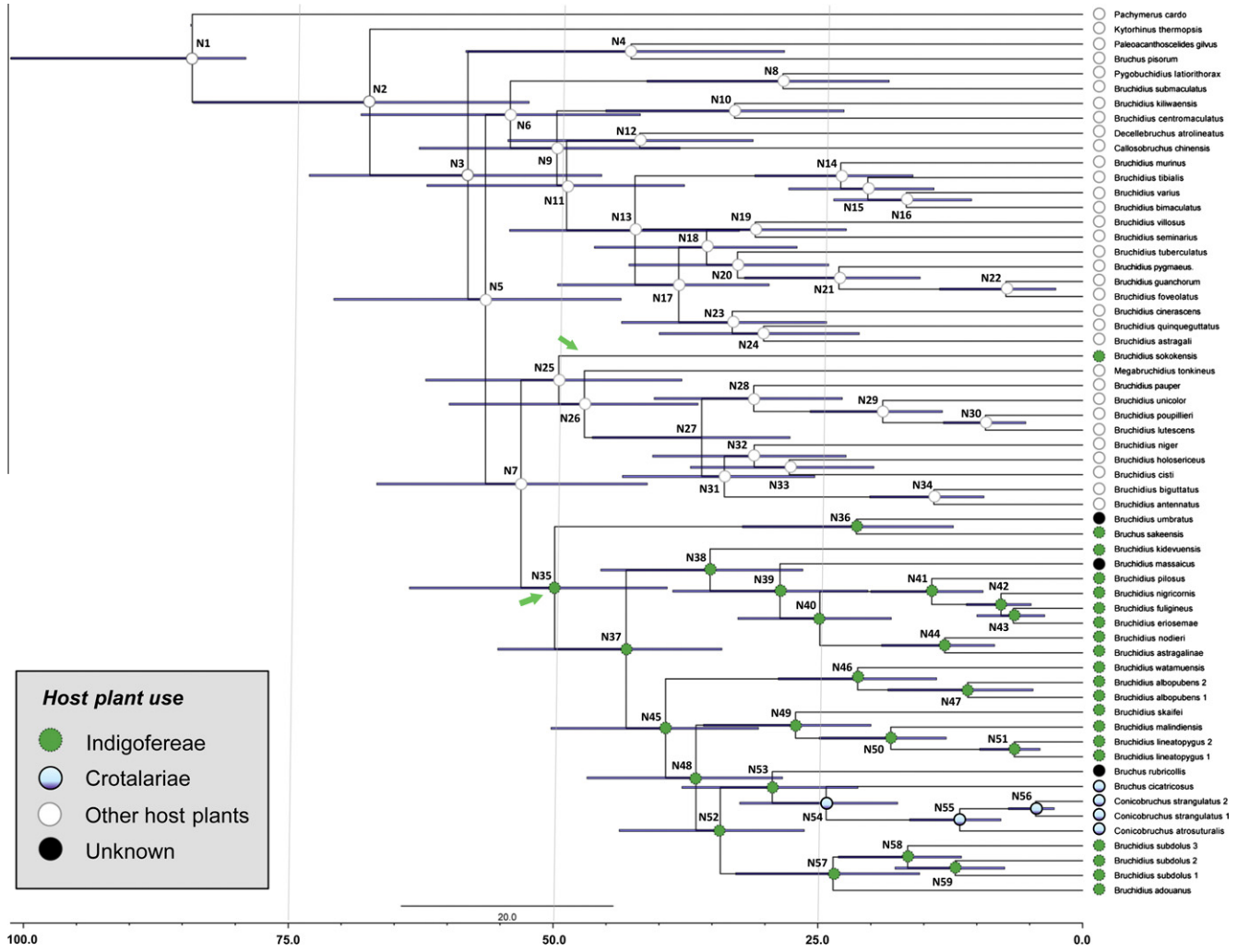

Fig. 3. Mapping of host plant associations on the chronogram that results from the BRC analysis with soft bounds. Significantly supported ancestral character states are figured on nodes using filled circles. Labels on each node correspond to those used in Table 3. Ninety-five percent higher posterior densities confidence intervals are indicated with blue bars. Green arrows also highlight the two independent colonization of Indigofereae. The two most basal outgroups (*Sagra* spp.) have been pruned for more clarity. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

solved. From a morphological point of view, *B. sokokensis* is also extremely difficult to categorize. Though several external characters (e.g. presence of a large triangular femoral spine, lack of tibial brush in males) suggest a close affinity with three other *Conicobruchus* s.l. species (*Bruchidius bilineatithorax*, *B. innocuus* and *B. sakeensis*; Delobel and Le Ru, 2010b), the structure of the male genitalia of *B. sokokensis* is quite different from those of *B. innocuus* or *B. sakeensis*, not to mention other *Conicobruchus* s.l. Strikingly, the male genitalia of *Bruchidius sokokensis* definitely shares similarities with those of *Bruchidius* species groups *pauper* and *unicolor* (e.g. the presence of a tegminal strut). To conclude it seems that the absence of clear morphological evidences combined with the fact that this species is consistently recovered in an unstable position in molecular analyses stresses the need for further analyses (with more specimens and/or genes). Therefore here we prefer to adopt a conservative approach by provisionally keeping this species in the genus *Bruchidius*.

A second step was to reassess the status of all remaining *Conicobruchus* s.l. species, which are listed in Table 1. As underlined in the introduction, all corresponding taxa are morphologically homogeneous as they generally exhibit the following diagnostic characters: trapezoid or compressed pronotum, with more or less concave sides; male median lobe with densely packed tubercles, spines or strong teethes and a triangular ventral valve. It is even possible to determine the degree of relatedness of several species on the basis of the common sharing of unique internal structures of male genitalia (see Fig. 1). For instance *Bruchus obscurus* var. *longithorax* and *Bruchidius lubaicus* possess an elongated sclerotized plate that is homologous to the structure found in *Bruchidius adouanus*. In a similar way, *Bruchus incaeruleus impressicollis* has a central column of densely packed tubercles, which is only found in the clade that includes *B. eriosemae* in litt., *B. fuligineus, B. nigricornis* and *B. pilosus*. From an ecological point of view, these taxa are also quite distinguishable because of their high level of specialization on Crotalariae or Indigofereae. Though the latter pattern is not unique in seed beetles, when associated with their specific morphological features it nonetheless constitutes a unique combination that is well suited to characterize the genus *Conicobruchus*. All these elements strongly argue for a revision of the unsatisfactory status of the taxa that are not listed as *Conicobruchus* s.s. in Table 1 and for which we have no molecular data. In order to make progresses in the necessary reassessment of bruchine systematics and taxonomy we propose to assign the corresponding taxa to the genus *Conicobruchus* (see Table 3). It should be added that

Table 2
Age estimates (Myr) using two methods: PL as implemented in r8s and BRC as implemented in BEAST. The latter is divided into two analyses using either hard bounds or soft bounds. For each node the median age and the 95% higher posterior densities (95% HPD) are reported.

Node	Ancestor of	PL		BRC (hard bounds)		BRC (soft bounds)	
		Median	95% HPD	Median	95% HPD	Median	95% HPD
1	*P. cardo* – *B. adouanus*	85.0	79.0–92.3	82.6	79.0–94.4	84.0	79.0–101.3
2	*K. thermopsis* – *B. adouanus*	62.1	54.2–78.1	63.9	50.6–76.8	67.2	52.2–83.9
3	*B. pisorum* – *B. adouanus*	51.1	40.2–61.8	54.8	43.5–65.6	57.9	45.1–72.1
4	*B. pisorum* – *P. gilvus*	38.7	22.3–51.4	40.3	26.5–53.8	42.5	28.3–58.0
5	*P. latiorithorax* – *B. adouanus*	48.6	41.5–66.7	53.0	42.1–63.7	56.2	43.9–70.9
6	*P. latiorithorax* – *B. astragali*	47.4	38.7–61.3	50.6	41.0–61.7	53.9	41.8–68.1
7	*B. sokokensis* – *B. adouanus*	43.4	34.0–57.7	49.9	40.0–60.3	52.8	40.9–66.3
8	*P. latiorithorax* – *B. submaculatus*	25.3	11.6–37.0	27.0	17.7–38.2	28.3	17.7–40.5
9	*B. kiliwaensis* – *B. astragali*	44.0	32.2–49.9	46.0	36.1–56.0	49.4	38.3–68.0
10	*B. kiliwaensis* – *B. centromaculatus*	29.5	18.2–39.0	30.4	21.0–40.7	32.7	22.0–44.9
11	*D. atrolineatus* – *B. astragali*	36.6	27.3–48.3	45.1	35.9–55.1	48.5	37.6–52.1
12	*D. atrolineatus* – *C. maculatus*	44.0	37.2–56.5	38.4	29.0–48.8	41.6	31.2–54.5
13	*B. murinus* – *B. astragali*	38.4	31.7–43.5	38.9	31.0–48.1	42.0	32.0–53.9
14	*B. murinus* – *B. varius*	24.0	14.3–37.7	21.2	15.0–28.5	22.8	15.9–30.9
15	*B. tibialis* – *B. varius*	21.1	9.0–26.9	18.7	12.7–25.2	20.2	13.9–27.8
16	*B. bimaculatus* – *B. varius*	16.5	8.4–28.1	15.3	9.3–21.0	16.6	10.4–23.5
17	*B. villosus* – *B. astragali*	35.4	26.5–44.4	35.2	27.6–43.5	38.0	28.4–48.6
18	*B. villosus* – *B. foveolatus*	32.8	25.1–49.9	32.8	25.6–40.8	35.4	26.7–46.0
19	*B. villosus* – *B. seminarius*	27.8	21.3–43.1	28.7	21.2–36.9	30.8	22.2–41.5
20	*B. tuberculatus* – *B. foveolatus*	29.8	19.0–38.5	30.0	23.0–38.5	32.4	23.9–42.8
21	*B. pygmaeus* – *B. foveolatus*	19.6	9.7–26.9	21.6	15.4–28.9	22.9	15.3–31.9
22	*B. guanchorum* – *B. foveolatus*	9.7	1.2–15.3	7.7	2.5–14.4	7.2	2.6–13.5
23	*B. cinerascens* – *B. astragali*	30.9	22.0–39.9	30.2	22.8–38.4	32.9	24.1–43.5
24	*B. quinqueguttatus* – *B. astragali*	28.6	18.6–36.5	27.4	20.4–35.8	30.0	21.0–39.9
25	*B. sokokensis* – *B. biguttatus*	40.4	27.0–51.5	46.2	36.9–56.1	49.3	37.8–62.0
26	*M. tonkineus* – *B. biguttatus*	37.8	25.8–50.6	43.9	34.8–53.1	46.9	36.2–59.8
27	*B. pauper* – *B. biguttatus*	26.9	15.9–40.6	33.4	27.3–41.9	35.9	27.4–46.3
28	*B. pauper* – *B. lutescens*	24.2	17.3–32.5	28.7	21.7–36.1	31.0	22.7–40.6
29	*B. unicolor* – *B. lutescens*	13.1	6.7–18.6	17.3	11.8–23.2	18.8	13.0–25.5
30	*B. poupillieri* – *B. lutescens*	5.6	2.0–8.5	8.5	5.3–12.5	9.1	5.4–13.2
31	*B. niger* – *B. biguttatus*	25.1	15.1–34.8	31.2	24.7–38.8	33.7	24.7–43.1
32	*B. niger* – *B. cisti*	21.8	15.7–33.6	28.4	22.1–36.5	30.9	22.5–40.9
33	*B. holosericeus* – *B. cisti*	19.1	13.0–30.9	25.6	18.4–33.0	27.6	19.7–37.0
34	*B. antennatus* – *B. biguttatus*	8.0	4.0–13.7	13.3	9.2–18.8	14.0	9.1–19.9
35	*B. umbratus* – *B. adouanus*	39.3	27.7–53.6	46.8	37.3–57.3	49.7	39.1–63.3
36	*B. umbratus* – *B. sakeensis*	11.5	11.5–11.5	10.9	9.3–11.5	21.3	12.2–32.4
37	*B. kidevuensis* – *B. adouanus*	32.1	21.6–46.2	40.5	32.3–49.4	43.0	33.9–55.0
38	*B. kidevuensis* – *B. nodieri*	26.6	18.9–40.3	32.7	24.5–41.2	35.1	26.4–45.4
39	*B. massaicus* – *B. nodieri*	21.2	7.4–35.7	26.4	19.5–35.7	28.5	20.2–38.6
40	*B. pilosus* – *B. nodieri*	16.4	8.0–27.8	22.8	17.6–29.5	24.7	18.2–32.7
41	*B. pilosus* – *B. fuligineus*	8.5	4.0–19.9	13.2	8.8–18.3	14.2	9.4–20.0
42	*B. nigricornis* – *B. fuligineus*	4.9	1.5–7.7	7.1	4.5–10.5	7.6	4.8–10.9
43	*B. eriosemae* – *B. fuligineus*	4.0	1.4–7.7	6.0	3.2–9.1	6.5	3.6–9.0
44	*B. astragalinae* – *B. nodieri*	8.1	3.5–15.3	12.1	7.8–16.9	12.9	7.9–18.5
45	*B. watamuensis* – *B. adouanus*	28.3	19.2–40.1	36.7	29.1–44.8	39.2	30.6–50.0
46	*B. watamuensis* – *B. albopubens* 1	15.5	7.2–25.0	19.4	13.3–27.4	21.1	13.7–28.7
47	*B. albopubens* 2 – *B. albopubens* 1	7.9	3.4–15.3	10.0	4.3–16.7	10.8	4.6–18.3
48	*B. skaifei* – *B. adouanus*	26.4	16.4–37.9	34.0	27.7–42.8	36.4	28.2–46.5
49	*B. skaifei* – *B. lineatopygus* 1	19.4	11.3–28.4	25.1	18.0–32.4	27.0	19.9–35.6
50	*B. malindiensis* – *B. lineatopygus* 1	11.4	4.6–18.6	16.5	11.0–22.7	18.0	12.5–24.4
51	*B. lineatopygus* 2 – *B. lineatopygus* 1	2.9	0.7–6.6	5.6	3.4–8.4	6.4	3.9–9.7
52	*B. rubricollis* – *B. adouanus*	24.6	13.8–35.6	31.7	24.7–39.1	34.1	26.3–43.7
53	*B. rubricollis* – *C. atrosuturalis*	19.2	8.1–29.9	27.2	20.1–34.3	29.2	21.3–37.8
54	*B. cicatricosus* – *C. atrosuturalis*	15.4	8.7–24.3	22.3	16.4–29.6	24.1	17.4–32.3
55	*C. strangulatus* 1 – *C. atrosuturalis*	6.6	1.4–17.0	10.7	7.2–14.9	11.6	7.4–16.3
56	*C. strangulatus* 1 – *C. strangulatus* 2	1.9	0.9–4.7	4.1	2.3–6.7	4.4	2.6–7.0
57	*B. subdolus* 3 – *B. adouanus*	17.6	2.3–32.0	21.9	14.8–29.4	23.5	15.3–32.7
58	*B. subdolus* 3 – *B. subdolus* 2	9.9	4.4–19.0	15.3	11.1–20.7	16.4	11.0–22.5
59	*B. subdolus* 1 – *B. subdolus* 2	8.0	2.4–12.9	11.1	6.9–15.8	12.0	7.2–17.6

we decided that the status of the taxa considered as subspecies or varieties (see also Table 1) should be left unchanged pending the examination of more material.

Although the phylogenetic relationships and the status of other bruchine genera were beyond the scope of this study, our approach could easily be generalized to other bruchine genera, given a proper sampling. In tribe Bruchini it is especially the case for large paraphyletic genera (such as *Acanthoscelides* and *Bruchidius*), which definitely need to be split into smaller monophyletic genera. Extant species groups could constitute a relevant basis for this extensive task, as most of them are defined using internal characters (structure of male genitalia) that are a lot more informative at this taxonomic level than external characters (Borowiec, 1987; Delobel and Delobel, 2006; Kergoat and Álvarez, 2008).

4.2. Molecular species delimitation approaches faced with biogeography and morphology

The molecular species delimitation approach has come out with five (six if considering the confirmation of the status of *Bruchidius*

Table 3
Proposed nomenclatural changes.

Conicobruchus adouanus (Pic, 1929) comb. nov.
Bruchus adouanus (Pic, 1929: 27)
Bruchidius adouanus: (Decelle, 1969b: 292)
Bruchidius adouanas: (Udayagiri and Wadhi, 1989: 115) (misspelling)

Conicobruchus albopubens (Pic, 1931) stat. rev.
Bruchus albopubens (Pic, 1931: 26)
Conicobruchus albopubens: (Arora, 1977: 34)
Bruchidius albopubens: (Varaigne-Labeyrie and Labeyrie, 1981: 94)

Conicobruchus astragalinae (Delobel and Le Ru, 2010a,b) comb. nov.
Bruchidius astragalinae (Delobel and Le Ru, 2010b: 62)

Conicobruchus bilineatithorax (Pic, 1952) comb. nov.
Bruchidius bilineatithorax (Pic, 1952: 8)

Conicobruchus cicatricosus (Fahraeus, 1839) comb. nov.
Bruchus cicatricosus (Fahraeus, 1839: 39)

Conicobruchus decoratus (Fahraeus, 1871) comb. nov.
Bruchus decoratus (Fahraeus, 1871: 448)
Bruchidius decoratus: (Decelle, 1975: 21)

Conicobruchus diegosensis (Pic, 1913) comb. nov.
Bruchus diegosensis (Pic, 1913: 116)
Bruchus diagosensis (Udayagiri and Wadhi, 1989: 187) (misspelling)

Conicobruchus fuligineus (Delobel and Le Ru, 2010) comb. nov.
Bruchidius fuligineus (Delobel and Le Ru, 2010b: 65)

Conicobruchus hargreavesi (Pic, 1933) comb. nov.
Bruchus hargreavesi (Pic, 1933: 19)
Bruchidius hargreavesi: (Luca, 1965: 58)

Conicobruchus innocuus (Fahraeus, 1871) comb. nov.
Bruchus innocuus (Fahraeus, 1871: 446)
Bruchidius innocuus: (Delobel and Le Ru, 2009: 4)

Conicobruchus kidevuensis (Delobel and Le Ru, 2010) comb. nov.
Bruchidius kidevuensis (Delobel and Le Ru, 2010b: 68)

Conicobruchus lineatopygus (Pic, 1924) comb. nov.
Bruchus lineatopygus (Pic, 1924: 458)
Acanthoscelides lineatopygus: (Luca, 1965: 55)
Bruchidius lineatopygus: (Gillon et al., 1992: 428)

Conicobruchus lubaicus (Delobel and Le Ru, 2010) comb. nov.
Bruchidius lubaicus (Delobel and Le Ru, 2010b: 70)

Conicobruchus malindiensis (Delobel and Le Ru, 2010) comb. nov.
Bruchidius malindiensis (Delobel and Le Ru, 2010b: 72)

Conicobruchus massaicus (Decelle, 1973) comb. nov.
Bruchidius massaicus (Decelle, 1973: 131)

Conicobruchus medianensis (Decelle, 1982) stat. rev.
Conicobruchus medaniensis (Decelle, 1982: 282)
Bruchidius medaniensis (Delobel and Le Ru, 2010a: 25)

Conicobruchus nigricornis (Fabricius, 1801) comb. nov.
Bruchus nigricornis (Fabricius, 1801: 400)
Bruchus obscurus (Fahraeus, 1839: 67; Decelle, 1969a: 252) (syn.)
Bruchidius nigricornis: (Decelle, 1969a: 251)

Conicobruchus nodieri (Pic, 1943) comb. nov.
Bruchus (*Acanthoscelides*) *nodieri* (Pic, 1943: 6)
Bruchidius nodieri: (Decelle, 1969b: 291)

Conicobruchus pilosus (Boheman, 1829) comb. nov.
Bruchus pilosus (Boheman, 1829: 108)
Bruchidius pilosus (Decelle, 1975: 19)

Conicobruchus rubricollis (Pic, 1903) comb. nov.
Bruchus rubrithorax (Pic, 1903: 169)
Bruchus rubricollis (Pic, 1913: 45) (name preoccupied)

Conicobruchus sakeensis (Pic, 1953) comb. nov.
Acanthoscelides lineatopygus var. *sakeensis* (Pic, 1953: 4)
Bruchus sakeensis: (Decelle, 1956: 424)

Conicobruchus skaifei (Pic, 1928) comb. nov.
Bruchus obscurus var. *skaifei* (Pic, 1928: 20)
Conicobruchus subdolus (Delobel and Le Ru, 2010) comb. nov.
Bruchidius subdolus (Delobel and Le Ru, 2010b: 77)

Conicobruchus sublineatus (Pic, 1943) comb. nov.
Bruchus (*Acanthoscelides*) *sublineatus* (Pic, 1943: 5)
Acanthoscelides sublineatus (Udayagiri and Wadhi, 1989: 65)

Conicobruchus turneri (Pic, 1929) comb. nov.
Bruchus turneri (Pic, 1929: 26)

Conicobruchus umbratus (Delobel and Le Ru, 2010) comb. nov.
Bruchidius umbratus (Delobel and Le Ru, 2010a: 26)

Conicobruchus watamuensis (Delobel and Le Ru, 2010) comb. nov.
Bruchidius watamuensis (Delobel and Le Ru, 2010a: 27)

eriosemae in litt.) potential new species clusters that are partially congruent with our assumptions. Overall the suggested speciation pattern is congruent with a classical allopatric speciation scheme in which potential new species clusters generally present a clear disjunct distribution (West Africa vs. East Africa) associated with more or less important differences in internal (e.g. see the male genitalia *of Bruchidius subdolus* and *B.* cf. *subdolus* in Fig. 1) or external morphology. Though in some cases we do recover distinct species clusters for specimens with noticeable morphological variations (*Bruchidius albopubens*, *B. subdolus* and *Conicobruchus strangulatus*), by contrast we also recover additional species clusters for taxa that did not exhibit noticeable morphological variations such as western or eastern individuals of *Bruchidius lineatopygus*. An even more complex pattern is suggested for two members of the *B. subdolus* species cluster, which are not found in a sister-species position in the molecular analyses. The corresponding specimens come from neighbouring countries (Kenya and Tanzania) and they exhibit no clear morphological differences. Elucidating the relationships in this species complex will probably require a more intensive sampling of the geographical populations of *B. subdolus*, especially in reference to the inclusion of specimens from Namibia, which are known to differ from West African and Kenyan material (Delobel and Le Ru, 2010b). An interesting outcome of this analysis is also related to the previous work of Decelle (1969a) who put in synonymy *Bruchus obscurus* with *Bruchidius nigricornis*. From a morphological point of view it was clear to us that the numerous morphological variations found among the sampled *B. nigricornis* specimens (including the specimens previously considered as distinct species and varieties) should have generated a clear pattern after being processed by molecular analyses. On the contrary, a very low level of molecular differentiation was found among our specimens, with no apparent genetic structure except for the taxa previously listed as *Bruchus obscurus* var. *densepubens*. Interestingly, these specimens can be clearly differentiated by their poorly defined and loose column of tubercles (Delobel and Le Ru, 2010b), as illustrated in Fig. 1. Nonetheless, the species delimitation analysis places them in the species cluster that includes the typical form of *Bruchidius nigricornis*, in agreement with the revisional work of Decelle (1969a). Because male genitalia structures have been thought to be one of the most informative ways of distinguishing closely related beetle species, our results for the *B. nigricornis* complex thus advocate for more thorough analyses such as finer-scale population genetics studies. Yet, pending the results of such analyses, the status of *Bruchidius nigricornis* remains undisputed.

Our findings illustrate both the potential usefulness and limitations of molecular species delimitation analyses. Like any other methods of species delineation, the GMYC approach is sensitive to undersampling (Lohse, 2009; Papadopoulou et al., 2008). Though this potential issue warns against drawing hasty conclusions, a wide range of empirical data nonetheless support the effectiveness of the GMYC method in inferring species clusters that correspond to species defined by independent criteria (Papadopoulou et al., 2008). As underlined by several authors (e.g. see Wheeler, 2004; Will and Rubinoff, 2004), it seems also extremely hazardous to justify lumping or splitting on the sole basis of molecular evi-

dence, especially when considering morphologically difficult groups such as seed beetles (Borowiec, 1987). For instance, in our study it is difficult to assess whether the potential species clusters recovered in taxa for which no morphological variation was found correspond to relevant cryptic species complexes. On the contrary, the interest of these approaches is obvious when the species clusters correspond to well-characterized groups of specimens that can be distinguished on the basis of morphological characters.

4.3. New insights from time divergence estimates

Interestingly, the old age that was recovered for the origin of Bruchinae (mean age comprised between 82.6 and 85 Myr, depending on the methods used) is well in line with the hypothesis of an ancestral association of bruchines with palms (Arecaceae) made by Poinar (2005). Palms have a rich fossil record with the oldest unequivocal fossil (genus *Sabalites*) being dated around 84 Myr (Harley, 2006). When the bruchines started their diversification the Leguminosae were not present (Lavin et al., 2005; Bell et al., 2010) whereas the palm family was already well diversified since palms appeared about 110 Myr ago (Janssen and Bremer, 2004). In a striking way the oldest known seed beetle fossil (*Mesopachymerus antiqua*) is a member of the subtribe Pachymerina, whose extant members are almost exclusively associated with palms (Arecaceae). All these evidences suggest that palm feeding was likely the ancestral feeding condition for seed beetles. With respect to the almost exclusively legume-feeding tribe Bruchini (Borowiec, 1987), mean age estimates ranging from 51 to 57.9 Myr were recovered. This time frame lags closely behind those of legume plants, which have supposedly started their diversification early in the Tertiary period between 60 (Lavin et al., 2005) to 63 Myr (Bell et al., 2010). This finding suggests that seed beetles may have colonized legume plants shortly after their emergence, a scenario that requires more in-depth investigations using a large sampling of seed beetles associated with a vast array of host plants.

Our time calibration analyses also provided a relevant example of the usefulness of soft constraints in molecular calibration procedures. Despite the existence of an upper limit of 11.5 Myr for the node leading to the species endemic to Anjouan, the BRC analysis with soft bounds inferred an older age of 21.3 Myr. In accordance with this result, in the PL analysis this constraint was active and a non-optimal age of 11.5 Myr was recovered. The results of both analyses indicate that our constraint is probably positioned on a node that is too deep in the tree. The latter can be explained by the existence of missing lineages that either correspond to taxa that have gone extinct or to unsampled taxa (Yang and Rannala, 2006; Ho and Phillips, 2009). This also reveals that *B. sakeensis* is probably not the closest relative of *B. umbratus* and that the upper limit constraint of 11.5 Myr cannot be used properly in analyses that only implement hard constraints.

4.4. Evidence for a tracking of host plant resources in Conicobruchus seed beetles?

According to the most recent estimates, the stem group of tribe Indigofereae can be dated around 50 Myr (Lavin et al., 2005; Schrire et al., 2009). However, significantly younger estimates (between 30 and 35 Myr) were recovered for the age of the crown group of Indigofereae (Lavin et al., 2005; Schrire et al., 2009). In comparison, our estimates for the age of *Conicobruchus* s.l. are older since we recovered ages comprised between 39 and 49 Myr. It is only by considering the confidence intervals of divergence time estimates that our dated *Conicobruchus* s.l. ages (node 37 of Table 2) can be accommodated with the age of the crown group of Indigofereae. This unexpected finding either suggests that the common ancestor of *Conicobruchus* s.l. was not feeding on Indigofereae or that there are potential biases in divergence time estimations. Since the pattern of host plant associations in seed beetles is known to be extremely conserved over time (e.g. Kergoat et al., 2005a, 2007c, 2008) the hypothesis of having multiple independent colonizations of Indigofereae by several lineages of *Conicobruchus* s.l. seems unlikely. Therefore the resulting discrepancies are probably better explained by biases in the molecular calibration analyses. As underlined by Lopez-Vaamonde et al. (2009), age estimates may range widely depending on which molecular-dating approach is used. With regard to the latter, Lavin et al. (2005) and Schrire et al. (2009) have used PL for their analyses, a method that has consistently yielded younger ages estimates in our study. In addition, the age of the crown group of Indigofereae in the study the Lavin et al. (2005) is likely underestimated because they only included three representatives for the tribe Indigofereae. It is also important to bear in mind the fact that Schrire et al. (2009) used fixed root ages based on secondary calibrations, which are known to often generate artefacts in divergence time estimations (Graur and Martin, 2004; Ho, 2007; Ho and Phillips, 2009). Similarly we cannot exclude the fact that our analytic pipeline may have led to an overestimation of divergence times, because of possible biases linked to the sampling design of our study or to the choice of dating methods and calibrations points.

Having said that, all these analyses point toward a similar time frame for the origin of the two groups. Though it is not possible to assess the precise timing of colonization of Indigofereae by *Conicobruchus* s.l., the hypothesis of a rapid colonization (less than 5 Myr after the origin of the plant lineage) appears highly probable. A similar scenario is also expected for the group of species that feed exclusively on Crotalariae, which started its diversification between 15.4 Myr (PL) and 24.1 Myr (BRC with soft bounds). For the latter, a clear pattern of a posterior colonization can be inferred because the stem group of tribe Crotalariae can be dated around 41 Myr, whereas their crown group age can be approximated around 25–30 Myr (Lavin et al., 2005).

5. Conclusions

This study has permitted a better circumscription of the limits of the puzzling genus *Conicobruchus* and as such it constitutes a new step toward a global reassessment of the systematic and phylogenetics of Old World seed beetles. For our study model, the use of molecular species delimitation approaches has provided meaningful insights in relation with past taxonomic studies and interesting directions for future studies. For example, it could be interesting to sequences additional specimens of distinct geographical origins (e.g. specimens of *B. albopubens* from Sudan; Delobel and Le Ru, 2010a or unidentified material from DR Congo or Mozambique) to assess whether the species richness of *Conicobruchus* is underestimated. Therefore we feel that the potential benefits of these approaches likely exceed their costs, and that the species delimitation approaches constitute a welcome addition to the ever-growing field of integrative taxonomy (Dayrat, 2005; Will et al., 2005; Schlick-Steiner et al., 2010). With reference to the evolution of host plant association, our analyses have suggested that the host plants have been colonized shortly after their diversification. This result is interesting because it partially counterbalances the conclusions of other studies, which have generally recovered old ages for the host plants in comparison with those of their insect predators (e.g. Gómez-Zurita et al., 2007; Hunt et al., 2007; McKenna et al., 2009). Of course, it remains to be seen whether this interesting finding could be generalized to other seed beetle groups or to other highly specialized group of phytophagous insects.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ympev.2011.03.014.

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