

Is mimicry a diversification-driver in ants? Biogeography, ecology, ethology, genetics and morphology define a second West-Palaeartic *Colobopsis* species (Hymenoptera: Formicidae)

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The West-Palaeartic *Colobopsis* ant populations have long been considered a single species (*Colobopsis truncata*). We studied the diversity of this species by employing a multidisciplinary approach and combining data from our surveys, museum and private collections, and citizen science platforms. As a result, we have revealed the existence of a second species, which we describe as *Colobopsis imitans* sp. nov., distributed allopatrically from *Co. truncata* and living in the Maghreb, Sicily and southern Iberia. While the pigmentation of *Co. truncata* is reminiscent of *Dolichoderus quadripunctatus*, that of *Co. imitans* is similar to *Crematogaster scutellaris*, with which *Co. imitans* lives in close spatial association, and whose foraging trails it habitually follows, similar to *Camponotus lateralis* and other ant-mimicking ants. The isolation between *Co. imitans* and *Co. truncata* seems to have occurred relatively recently because of significant, yet not extreme, morphometric differentiation, and to mtDNA polyphyly. Both *Co. imitans* and *Co. truncata* appear to employ mimicry of an unpalatable or aggressive ant species as an important defensive strategy; this ‘choice’ of a different model species is motivated by biogeographic reasons and appears to act as a critical evolutionary driver of their diversification.

ADDITIONAL KEYWORDS: adaptation – Batesian mimicry – citizen science – COI mtDNA – discriminant-function analysis – mediterranean – multivariate statistics – North Africa – sibling species – speciation.

INTRODUCTION

Ants (Insecta: Formicidae) are a hyperdiverse group of organisms that counts about 13 860 species (Bolton, 2021) and is extraordinarily successful in

most terrestrial ecosystems (Hölldobler & Wilson, 1990; Gibb *et al.*, 2017; Seifert, 2017). Such high diversification stems from several evolutionary strategies and lifestyles, and enables even hundreds of different ant species to coinhabit the same habitat (Hölldobler & Wilson, 1990, 2008). However, only one or few dominant species generally characterize even the most species-rich ant communities: these species form populous colonies, with large, often permanent, foraging trails, and they defend territories that may extend over hectares (Hölldobler & Wilson, 1990; Andersen, 1995, 1997; Grasso *et al.*, 1998, 1999,

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2005; Arnan *et al.*, 2018). These ants are exposed to higher predation risk compared to the ones whose workers forage solitarily or in small groups, and are accordingly equipped with effective defensive mechanisms (Buschinger & Maschwitz, 1984; Hölldobler & Wilson, 1990; Dornhaus & Powell, 2010; Seifert, 2018). Most ant species live in small colonies and forage solitarily or in small groups, only occasionally form trails and develop evasive anti-predatory strategies (e.g. Hölldobler & Wilson, 1990; Tautz *et al.*, 1994; Andersen, 1995; Dornhaus & Powell, 2010; Helms *et al.*, 2014; Larabee & Suarez, 2015; Seifert, 2018; Grasso *et al.*, 2020). Of the species that live in small colonies, the only ones armed with dangerous defences are some predatory ants that retain the primitive feature of a powerful functional stinger to hunt (Buschinger & Maschwitz, 1984; Hölldobler & Wilson, 1990; Dornhaus & Powell, 2010).

Well-armed ant species, in particular the ones that build large colonies, are a good model for several mimicking organisms, mostly arthropods. Some of these mimics are myrmecophilous organisms: commonly ant predators or parasites, they have adapted to live within, or close to, ant colonies by relying on chemical or acoustic mimicry (e.g. Geiselhardt *et al.*, 2007; Barbero *et al.*, 2009; Cushing, 2012; Parker & Grimaldi, 2014; Parker, 2016; Scarparo *et al.*, 2019). On the other hand, myrmecomorph species resemble their ant model thanks to morphological and/or behavioural adaptations (e.g. Komatsu, 1961; Jackson & Drummond, 1974; Oliveira & Sazima, 1984; Cobben, 1986; McIver, 1987; Oliveira, 1988; McIver & Stonedahl, 1993; Trjapitzin & Trjapitzin, 1995; Cassis & Wall, 2010; Chandler, 2010; Durkee *et al.*, 2011; Huang *et al.*, 2011; Cushing, 2012; Pekár, 2014; Corcobado *et al.*, 2016; Pekár *et al.*, 2017; Harvey *et al.*, 2018; De L. Nascimento & Perger, 2018; Gnezdilov, 2019). The main aim of myrmecomorphism is predation avoidance: compared to the mimics, models usually possess superior defensive mechanisms and are also more numerous. As such, they are usually interpreted as Batesian mimics (e.g. Jackson & Drummond, 1974; McIver, 1987; Durkee *et al.*, 2011; Huang *et al.*, 2011; Cushing, 2012; Harvey *et al.*, 2018), whose evolution can be favoured by model abundance (Kikuchi & Pfenning, 2010).

Many ant mimics are ants themselves. Some are parasites (e.g. inquilines) that act similarly to myrmecophilous organisms (Buschinger, 2009), relying on chemical adaptations to interact with the host species. However, there are some free-living ant species that act as mimics of more aggressive or dominant ant species and are, therefore, interpreted as Batesian mimics. Nonetheless, solid empirical evidence to reject the alternative hypothesis of Müllerian mimicry (see: Müller, 1879;

Pasteur, 1982; Ritland, 1991) is rarely available (Ito *et al.*, 2004; Wagner, 2014). In these species, chromatic mimicry is the prevalent mechanism, while behavioural or morphological adaptations are more rarely documented (Emery, 1886; Forel, 1886; Santschi, 1919; Gobin *et al.*, 1998; Merrill & Elgar, 2000; Ito *et al.*, 2004; Ward, 2009; Gallego-Ropero & Feitosa, 2014; Powell *et al.*, 2014; Pekár *et al.*, 2017; Rasoamanana *et al.*, 2017; Seifert, 2019a). In addition, recurrent behaviour among mimicking ant species is interspecific trail-following, which consists in the mimics regularly infiltrating into the foraging trails of the model and may lead to parasitic behaviour with regard to food resources (Emery, 1886; Santschi, 1919; Gobin *et al.*, 1998; Ito *et al.*, 2004; Menzel *et al.*, 2010; Powell *et al.*, 2014). Unlike specialized parasites (e.g. Visicchio *et al.*, 2001; Buschinger, 2009; de la Mora *et al.*, 2020), no advanced mechanisms of chemical deception exist in most of these cases, so in the eventuality of direct encounters, the model species recognizes and attacks the mimic, which is well-equipped to escape (Goetsch, 1942, 1951; Kaudewitz, 1955; Gobin *et al.*, 1998; Ito *et al.*, 2004; Menzel *et al.*, 2010). The vast majority of the hitherto well-documented cases come from the tropics: mimics mainly belong to diverse lineages from the Formicinae tribe Camponotini (mostly *Camponotus* Mayr, 1861), while their models are phylogenetically scattered, including Ectatomminae, Myrmeciinae, Myrmicinae (Crematogastrini and Stenammini) and Pseudomyrmecinae. Only one case of ant-mimicking ant species is well-documented in the West-Palaearctic zone, i.e. *Camponotus lateralis* (Olivier, 1792). It chromatically mimics the similar *Crematogaster* Lund, 1831 species, *Cr. ionia* Forel, 1911, *Cr. scutellaris* (Olivier, 1792) and *Cr. schmidti* (Mayr, 1853), and follows their trails (Emery, 1886; Baroni Urbani, 1969; Menzel *et al.*, 2010; Wagner, 2014; Seifert, 2018, 2019a). The three species are closely related with neighbouring geographic ranges and differ slightly chromatically (Blaimer, 2012). Interestingly, there seems to be a geographic trend in the chromatic pattern of *Ca. lateralis*, allowing it to better resemble these three *Crematogaster* species in the regions of sympatric occurrence (Wagner, 2014; Seifert, 2019a).

The ant genus *Colobopsis* Mayr, 1861 (Formicinae: Camponotini), recently separated from *Camponotus* (Ward *et al.*, 2016), currently counts 95 valid species and 21 subspecies (Bolton, 2021). It is distributed across the Holarctic, Indomalayan and Australasian regions, and is most diversified in the latter two regions (Janicki *et al.*, 2016; Guénard *et al.*, 2017). *Colobopsis* species are usually arboreal ants that nest in dead wood, form small-sized colonies and behave timidly towards other ants (Wheeler, 1904; Ward *et al.*, 2016).

In the Western Palaearctic region, *Colobopsis truncata* (Spinola, 1808) is the only recognized species of its genus. The queen caste was described by Spinola (1808) from north-western Italy (Liguria region), while the other castes were described later (Dufour & Perris, 1840; Forel, 1874; Emery, 1916). Another species, *Co. fuscipes* (Mayr, 1853), was described from Austria by Mayr (1853), but was later reclassified as a junior synonym of *Co. truncata* (Emery & Forel, 1879). Therefore, *Colobopsis truncata* is considered to have a wide geographic distribution, from the Caucasus to Iberia and from Central Europe to the Maghreb (Janicki *et al.*, 2016; Guénard *et al.*, 2017; Seifert, 2018). It is an arboreal-nesting species, as is typical of the genus, and it preferably nests on broadleaved trees, where it lives in monogynous and often polydomous colonies, rarely exceeding 500 workers (Seifert, 2018). Queens and soldiers are specialized for phragmosis, and soldiers may also function as repletes (living containers of liquid food), seldom leaving the safety of the nests they guard (Brun, 1924; Goetsch, 1950, 1953; Seifert, 2018). Minor workers are usually active outside the nest during both day and night, forage solitarily, do not recruit nest mates to food sources and perform quick evasive movements when encountering other ants (Seifert, 2018).

During field observations across Italy we encountered marked divergences between *Colobopsis* colonies: workers of some colonies resembled *Cr. scutellaris* and followed its trails, while others resembled *Dolichoderus quadripunctatus* (Linnaeus, 1771), two species having remarkably different appearance. This is reflected by several contradictory reports, but which contain no comment on these incongruences. Forel (1874) first referred to Swiss ants by considering *Co. truncata* a Batesian mimic of *D. quadripunctatus*. He later suggested that the two species show pre-adaptations to parabiotic nest-sharing (Forel, 1903). Zimmermann (1934) instead studied ants in Croatia and considered the occasional relationship between *Co. truncata* and *Cr. scutellaris* similar to the one between *Ca. lateralis* and *Cr. scutellaris*. Goetsch (1942) stated that in Spain *Co. truncata* behaves similarly to *Ca. lateralis*, following the trails of *Cr. scutellaris*, yet is also chromatically different and shows no adaptation to mimicry. Baroni Urbani (1971) reported on a case of trail-following between a *Co. truncata* queen and a *Cr. scutellaris* trail from central Italy. More recently, Tinaut (1991) claimed that in southern Iberia *Co. truncata* can be easily confused with *D. quadripunctatus* during field surveys due to their similarity. However, working in the same area, Carpintero *et al.* (2001, 2005) instead affirmed that *Co. truncata* is a visual mimic of *Cr. scutellaris* and follows its trails, and even speculated that after nuptial flights *Co. truncata* queens specifically choose trees occupied

by *Cr. scutellaris* to found their colony. In reviewing the distribution of *Colobopsis* in Iberia, García (2020) mentioned possible chromatic similarity of *C. truncata* with *Cr. scutellaris* and its mimic *Ca. lateralis*, but also *D. quadripunctatus*. More recently, there have been reports on trail-following by *Co. truncata* on *Cr. scutellaris* ants in Italy [mentioned by Seifert (2018), no locality specified; Lake Garda according to H. C. Wagner, personal communication]. Wagner (2019) described a close association between *Co. truncata* and *D. quadripunctatus* in Austria (Vienna), highlighted morphological and chromatic similarity between the two, and reported trail-following of *D. quadripunctatus* trails by *Co. truncata*.

We aimed to test whether the above-mentioned diversity of traits represented intraspecific variation or indicated the existence of multiple *Colobopsis* species in the Western Palaearctic. In order to address the different biological dimensions of this problem, we used a multidisciplinary approach that involves the description of the patterns of diversity within the traditional notion of *Co. truncata*. We relied on the principles of integrative taxonomy (Schlick-Steiner *et al.*, 2010), involving morphological, genetic, ecological, ethological and biogeographical data.

MATERIAL AND METHODS

We combined qualitative morphology through chromatic pattern evaluation, quantitative morphology through the multivariate analysis of morphometric data and genetics in the form of mtDNA (*COI*) sequencing, ecological surveys to study the association between *Colobopsis* and its putative model species, and recorded ethological data to document cross-species trail following. Finally, we compared our results with the known biogeographic patterns of other ant species. We chose this quantitative morphological approach as it is widely regarded as the most practical and reliable single source of evidence for cryptic ant species delimitation, and as a cornerstone in integrative approaches on cryptic species complexes of ants (Seifert, 2009, 2018; Steiner *et al.*, 2011, 2018; Seifert *et al.*, 2014; Wagner *et al.*, 2017; Csősz *et al.*, 2020). Moreover, mtDNA sequencing represents a widespread and cost-effective method to gain preliminary information on species identification, biogeography and cryptic speciation (Hebert *et al.*, 2003, 2016; Ratnasingham & Hebert, 2007), which has developed into an aid to myrmecological faunistic, biogeographic and taxonomic studies (Steiner *et al.*, 2005, 2018; Csősz *et al.*, 2015; Seifert *et al.*, 2017; Schär *et al.*, 2018, 2020; Blatrix *et al.*, 2020). Ecological data on species associations and

ethological data are rarely used in ant taxonomy but appear to be highly relevant to the specific case we are investigating, while biogeography is important to understand species diversity.

For our morphological and molecular analyses, we gathered type material of *Co. truncata* and *Co. fuscipes*, as well as additional non-type material of *Colobopsis* from the Mediterranean region, and relied on our own efforts and the generous contribution of colleagues to achieve a satisfactory geographic coverage. In particular, the type series of *Co. truncata* consists of a single queen with the label ‘*Polyergus* (?) | *F.ca truncata* | Spin. in Ligur. | Genova || 6571’. This queen could be safely identified as the type since it is the sole *Colobopsis* queen in the Spinola collection at the Museo di Scienze Naturali in Turin (Italy), and matched the description given by the author (Spinola, 1808). Concerning *Co. fuscipes*, at least two syntypes are stored in the Museum für Naturkunde, University of Berlin (Germany), and their pictures are available on AntWeb (<https://www.antweb.org>, codes FOCOL2496 and FOCOL2497): these are labelled ‘Oesterreich | Coll. Rhd || *Colobopsis fuscipes* Mayr || Type || 29812 || GBIF-D/FoCol | 2496 | specimen + label | data documented’ and ‘Oesterreich | Coll. Rhd || *Colobopsis fuscipes* Mayr || Type || GBIF-D/FoCol | 2497 | specimen + label | data documented’. Although the label is unlikely to be an original by Gustav Mayr (B. Seifert, pers. comm.), we deem their status as types credible. We also retrieved a worker labelled *Co. fuscipes* in Mayr’s collection at the Natural History Museum of Vienna, but with no explicit indication ensuring its type status. In order to gather information on chromatic variation of Euro-Mediterranean *Colobopsis*, we relied on AntWeb pictures, images from scientific papers or monographs (Glaser, 2009; Wagner, 2014, 2019; Lebas *et al.*, 2016; Seifert, 2018; Scupola, 2018; García, 2020; Salata *et al.*, 2020; Täuşan *et al.*, 2020), and on georeferenced photographs uploaded on citizen science platforms (<https://www.inaturalist.org>, <https://www.biodiversidadvirtual.org>) and on biodiversity-related Facebook groups. A complete list of the material examined, their depositories and collecting data is available in the Supporting Information, File S1. Ecological and behavioural data were obtained through field surveys across Italy.

PIGMENTATION: CHROMATIC MIMICRY

Preliminary observations highlighted that chromatic pattern provides the most evident difference between allopatric *Colobopsis* populations, which resemble two different model ant species in Italy. We pre-emptively

described the two chromatic forms and investigated whether these were consistently able to represent *Colobopsis* diversity across the Mediterranean basin, and whether they occurred intracolony or sympatrically, and also checked for the possible existence of third forms. The two *Colobopsis* model patterns were pre-emptively established by observing ten workers per each form (ten from Sicily and ten from mainland Italy), and all subsequent investigations were carried out by inspecting specimens of well-preserved pigmentation primarily belonging to the worker caste. Queens and soldiers were also examined and identified, only if closely resembling one worker model pattern, while males were not considered due to their different pigmentation. Furthermore, to better describe the differences between the two models, a ratio was calculated between head colour and mesosoma colour by taking dorsal pictures of specimens and calculating the average value of red (RGB colours) between five randomly selected pixels of the head and five of the mesosoma via software ImageJ (Schneider *et al.*, 2012). Calculating a ratio, rather than considering the absolute values, greatly reduces the variation produced by different light conditions and camera settings among different pictures, thereby allowing comparison of pictures from various sources. The same ratio was also calculated for the two putative model species *Cr. scutellaris* and *D. quadripunctatus*. Chromatic ratios were calculated on 20 workers per chromatic pattern or species from across their respective geographic range, using both directly inspected specimens and images from citizen science platforms. Any differences were statistically tested by using the software R 4.0.3 and R Studio 1.3.1056 (R Core Team, 2021), and employing an ANOVA test and subsequent Tukey post hoc test for pairwise comparisons.

The visual examination to verify correspondence to either of the two chromatic models was conducted on 79 directly observed *Colobopsis* colonies (76 of which containing workers), plus images of 136 further specimens (including 76 isolated queens), for a total of 310 workers and 79 queens covering a total of 16 countries from across the Western Palaearctic *Colobopsis* distribution (see Supporting Information, File S1).

The two model patterns are defined as follows:

1. *Crematogaster scutellaris*-like pattern (CSL pattern): head, or head and anterior part of the mesosoma (rarely most of it), uniformly red; rest of the body evidently darker and mostly black. White stripes or dots on the second gastral tergite often absent or weak (present in 10% of the examined workers). See Figure 1.

2. *Dolichoderus quadripunctatus*-like pattern (DQL pattern): head, mesosoma and appendages from reddish to blackish (therefore, chromatically more variable than the *Cr. scutellaris*-like model), head at least slightly darker than the mesosoma or less frequently concolorous, gaster black. White stripes or dots on the second gastral tergite often present (80% of examined workers) and more obvious. Phragmotic heads of soldiers or queens are always reddish in their anterior, heavily sculptured part (approximately one half of the head), while the rest follows the same scheme of workers. See [Figure 1](#).

NUMERIC MORPHOLOGY: MULTIVARIATE ANALYSES OF MORPHOMETRIC DATA

A total of 12 continuous morphometric traits were defined following [Seifert \(2018\) \(Table 1\)](#) and measured on 115 *Colobopsis* workers from 44 nest samples (considering minor workers only, and not the soldiers). All measurements were made in μm by using a pin-holding stage, which allowed rotations around the X-, Y- and Z-axes. An Olympus SZX9 stereomicroscope was used at 150 \times magnification for each character, but with characters larger than the

field of view, 75 \times magnification was applied. Due to the low number of the much rarer queens, males and soldiers in our possession, we recorded only a reduced set of seven morphometric traits aimed at providing a brief description of these castes, without using them in the following statistical analyses. Morphometric data are provided in μm throughout the whole paper.

Repeatability of the recorded size parameters were evaluated via intraclass correlation coefficients (ICC) by using the package ICC ([Wolak et al., 2012](#)), see [Table 1](#). Variables were tested via matrix scatterplots and Pearson product-moment correlation coefficients for error variance and outliers. Each character resulted highly repeatable, except for NOL, which was considered moderately repeatable.

Exploratory analyses through NC-PART clustering

The prior species hypothesis was generated based on workers through combined application of NC clustering ([Seifert et al., 2014](#)) and partitioning based on recursive thresholding (PART) ([Nilsen & Lingjaerde, 2013](#)). The script for NC-clustering combined with PART was written in R and can be found in [appendix S1](#) in [Cs6sz & Fisher \(2016\)](#).

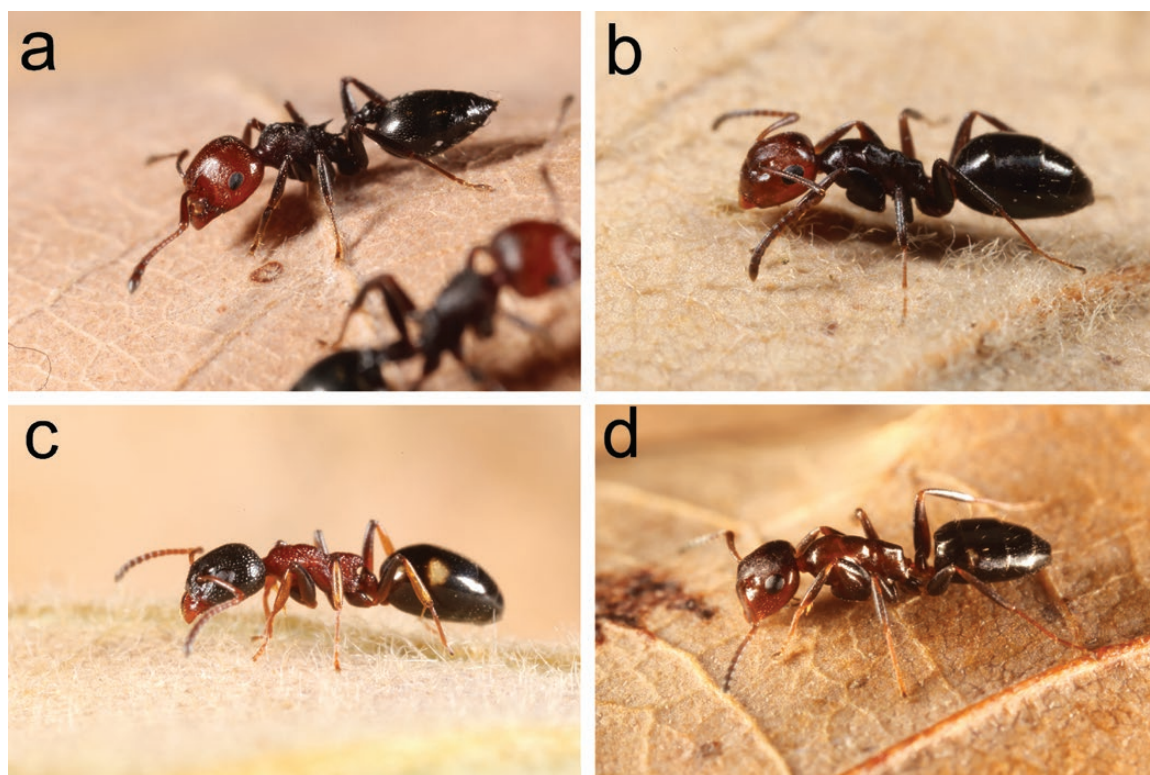


Figure 1. The model species and the two detected chromatic model patterns of *Colobopsis*: A, *Crematogaster scutellaris*; B, CSL *Colobopsis* from Sicily; C, *Dolichoderus quadripunctatus* from Tuscany; D, DQL *Colobopsis* from Tuscany.

Table 1. Abbreviation (Abbr.) of morphometric characters, definition of measurements and ICC (R), a metric for repeatability parameter, are given. Definitions of morphometric characters follow [Seifert \(2018\)](#)

Abbr.	Description of the trait	ICC (R)
CL	Maximum median length of head capsule. The head must be carefully tilted so the maximum length is positioned in the measuring plane.	0.982
CW	Maximum head with including compound eyes. The largest distance between profiles of the two compound eyes in full-face view.	0.951
EL	Eye length. Maximum diameter of the compound eye.	0.967
dAN	Minimum distance of the inner margins of antennal socket rings.	0.985
ML	Diagonal length of the alitrunk in profile. Measured in lateral view from the anteriormost point of anterior pronotal slope to the caudalmost point of the lateral metapleural lobe.	0.969
MW	Maximum width of pronotum.	0.989
NOL	Petiole node length; measured in lateral view, from the center of the petiolar spiracle to the posterior profile.	0.890
PeW	Petiole width. The maximum width of petiole in dorsal view.	0.994
PreOC	Preocular distance. Use a cross-scaled ocular micrometer and adjust the head to the measuring position of CL. Frontal measuring point: median clypeal margin; caudal measuring point: reference line between the frontalmost border of the two compound eyes.	0.951
SL	Scape length. The maximum straight-line scape length excluding the articular condyle.	0.971
HTL	Hind tibia length. Measured from the distalmost point of the tibia to the proximal end where the tibia is narrowest in profile.	0.968
PeSH	Petiole scale height measured from the center of petiolar spiracle to top of the crest.	0.959

Our exploratory data analysis approach follows the protocol described by [Csősz & Fisher \(2016\)](#) with the following specific settings: bootstrap iterations in PART were set to ‘b = 1000’ and the minimum size of clusters was set to ‘minSize = 5’ for both ‘hclust’ and ‘kmeans’. The optimal number of clusters and the partitioning of samples were accepted as the preliminary species hypothesis in any case in which the two clustering methods, ‘hclust’ and ‘kmeans’ through PART, have yielded the same conclusion.

Exploratory analyses via principal component analysis (PCA) with allometrically corrected data

An alternative prior species hypothesis was generated via the principal component analysis (PCA) ordination tool, which searches for discontinuities in continuous morphometric data and display plots in a graph. The use of raw data in PCA (without removal of allometric variance) may lead to weaker performance in ordination because the first vector of the PCA often describes the size component, which is useless information when separating cryptic species of similar size. Residuals were, therefore, used in PCA. Residuals, in which the head length (CL) was used as covariate, were calculated via a linear regression model according to the following steps: (1) scaling properties, intercept and steepness were calculated for each nest sample separately (note: nest samples constituted by a singleton were not

involved in this phase); (2) scaling properties of each nest sample were used to calculate a grand average for steepness and intercept; (3) residuals were calculated for each nest sample (including singletons) based on the grand average. [Table 1](#) shows the residuals of every trait calculated against head length (CL). In contrast to NC-PART clustering, PCA provides no estimate of the number of clusters, and ‘classification’ of objects was based on subjective decision. The coefficients (x any intercept) for removal allometric variance for each trait are given in the [Supporting Information, File S1](#).

Hypothesis testing by confirmatory analysis

The validity of the prior species hypothesis was tested via linear discriminant analysis (LDA). Classification hypotheses were imposed for all samples that were congruently classified by partitioning methods, while wild-card settings (i.e. no prior hypothesis imposed on classification) were given to samples that were incongruently classified by the two partitioning methods. Statistical analyses were conducted through the software R 3.6.3 (R Core Team, 2021).

GENETICS: MITOCHONDRIAL *COI* SEQUENCES

Total genomic DNA was extracted from leg tissues using the NucleoSpin DNA Insect kit (Macherey-Nagel, Düren, Germany), in compliance with

the manufacturer's protocol. A 700-bp region of mitochondrial gene cytochrome *c* oxidase subunit I (*COI*) was amplified by using the primer couple LCO1490/HCO2198 (Folmer *et al.*, 1994). Polymerase chain reaction was carried out in 25- μ L reactions according to the following profile: initial denaturation step at 95 °C for 5 min, 35 amplification cycles (denaturation at 95 °C for 30 s, annealing at 52 °C for 30 s, elongation at 72 °C for 45 s), final elongation at 72 °C for 7 min. Polymerase chain reaction products were sent to Macrogen Europe (Amsterdam, Netherlands) for Sanger sequencing. Chromatograms were checked and edited using SeqTrace (Stucky, 2012). Sequences were aligned via the MUSCLE (Edgar, 2004) algorithm as implemented in AliView (Larsson, 2014). Model selection and maximum likelihood phylogenetic analysis were performed on the IQ-TREE web-server (Trifinopoulos *et al.*, 2016) by using the Eastern Palaearctic *Co. nipponica* (Wheeler, W.M., 1928) and *Co. shohki* (Terayama, 1999), and the Indomalayan *Colobopsis* nr. *saundersi* (Emery, 1889) (GenBank accession numbers AB019417, AB019418 and KU975365, respectively) as outgroups. Ten separate runs were launched, each with 1000 replicates of ultrafast bootstrap. The tree with the best likelihood value out of the ten was then chosen. Twenty-three colony samples were sequenced, consisting in 41 workers from six countries and 18 localities. The sequences obtained have been submitted to GenBank, under accession numbers MW462045–MW462085 (see Supporting Information, File S1).

ECOLOGY: COEXISTENCE WITH MODEL SPECIES

Field surveys were conducted in mainland Italy (Emilia-Romagna, Tuscany; N sites = 5, DQL pattern) and in Sicily (N sites = 8, CSL pattern) in order to test whether the local *Colobopsis* populations, showing a DQL and a CSL phenotype, respectively, actually lived in close proximity with either of the two species indicated as probable mimicry models (see Supporting Information, File S1). We searched for the presence of *Colobopsis* workers on trees in each site to detect those occupied by a *Colobopsis* colony. We then performed a 10-min long continuous sampling within a 1.5 m radius of the point of the tree trunk where *Colobopsis* workers were first observed, recording any presence of *Cr. scutellaris* or *D. quadripunctatus* workers.

Occurrence of *Cr. scutellaris* or *D. quadripunctatus* on trees also occupied by *Colobopsis*, according to the different DQL and CSL models, were statistically tested by using the software IBM SPSS statistics, Italian v.24 and the chi-squared test. All data are presented in the Supporting Information, File S1.

ETHOLOGY: INTERSPECIFIC TRAIL-FOLLOWING BEHAVIOUR

Field surveys were conducted in mainland Italy (Emilia Romagna, Tuscany) and in Sicily (sites as in the section before; also see Supporting Information, File S1) with the aim of quantifying the occurrence of trail-following behaviour by *Colobopsis* ants in relation to *Cr. scutellaris* or *D. quadripunctatus* trails. We selected trees where *Colobopsis* colonies coexisted with *Cr. scutellaris*, *D. quadripunctatus* or both. In accordance with the relevant literature (Gobin *et al.*, 1998; Ito *et al.*, 2004; Menzel *et al.*, 2010; Powell *et al.*, 2014), trail-following was defined as the event of *Colobopsis* workers moving along an established pheromone trail of *Cr. scutellaris* or *D. quadripunctatus* within 1 cm from the trail itself. A 10-min continuous sampling was used to record the presence or absence of this behaviour on each of the trees examined.

We selected 59 trees inhabited by *Cr. scutellaris* to study trail-following on their trails: 29 trees hosted *Colobopsis* colonies exhibiting the CSL pattern (Sicily, four sites) and 30 hosted *Colobopsis* with the DQL pattern (Emilia-Romagna and Tuscany, five sites). Observations on *D. quadripunctatus* trails could be performed only in 23 *Colobopsis* colonies exhibiting the DQL pattern (Emilia-Romagna and Tuscany): since no *D. quadripunctatus* colonies were found in the sites studied in Sicily (where the species is known to be rare; see: Schifani & Alicata, 2018), no *Colobopsis* colonies exhibiting the CSL pattern could be tested in this regard. All data are summarized in the Supporting Information, File S1.

SPECIES CONCEPT

Integration of the evidence provided by different complementary disciplines into an evolutionarily credible species hypothesis is performed according to the principles formulated by Schlick-Steiner *et al.* (2010), i.e. by resolving disagreements through solid evolutionary explanations. Biogeography is here treated as an additional source of information, and plays an important advisory role in the formation of the final species-hypothesis. We follow the universal gene and gene expression (GAGE) species concept proposed by Seifert (2020), which, despite being recently formulated, convincingly summarizes the main theoretical and practical formulae most commonly adopted during the last few decades as a rigorous approach on alpha taxonomy of cryptic ants, especially in Europe.

RESULTS

PIGMENTATION: CHROMATIC MIMICRY

All the colonies are safely assigned to one of the two models and no transitional or third forms are detected

(see [Supporting Information, File S1](#)). No intracolony coexistence of the two models is detected either. The type series of both *Co. truncata* and *Co. fuscipes* show the DQL pattern ([Fig. 2](#)). According to the material examined, the two models occur strictly allopatrically. Samples from the south-western Mediterranean basin, namely Algeria, Sicily (Italy), Morocco, southern Portugal and Andalusia (southern Spain), are assigned to the CSL pattern. The rest is assigned to the DQL pattern, i.e. samples from Austria, Bulgaria, Croatia, Czech Republic, France, Germany, Greece, Hungary, Israel, mainland Italy (Apulia, Campania, Emilia-Romagna, Liguria, Tuscany), Romania, Serbia, Slovenia, Switzerland, the rest of Spain (Aragon, Catalonia, Balearic Islands, Castilla-La Mancha) and Turkey. In addition, the DQL pattern is shown in all photographs of *Co. truncata* specimens present in the European ant fauna guides by [Lebas et al. \(2016\)](#) and [Seifert \(2018\)](#), regional faunistic guides by [Glaser](#)

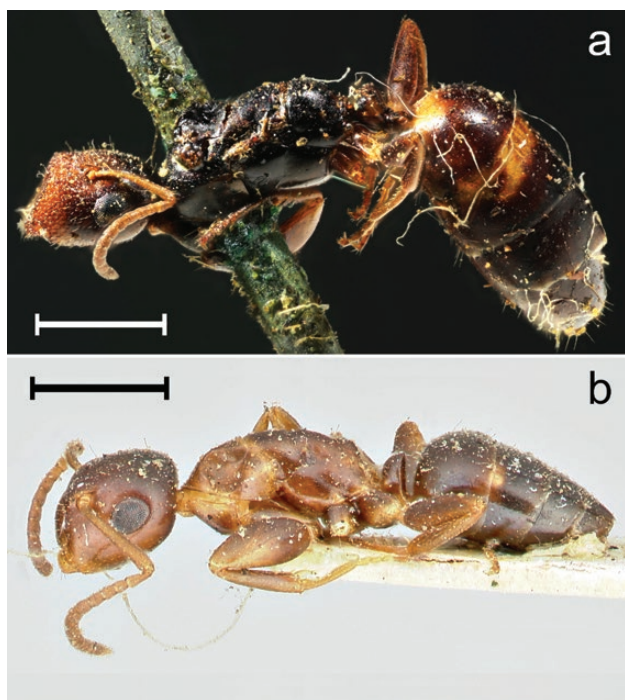


Figure 2. Type material of the described West-Palaeartic *Colobopsis*, all showing to the ‘*D. quadripunctatus*-like’ pattern. **A**, holotype queen of *Colobopsis truncata* from Liguria, Italy, preserved at the Turin Natural History Museum (Italy). **B**, syntype worker of *Colobopsis fuscipes* from Austria (picture from AntWeb.org, FOCOL2496; photographer: Christiana Klingenberg), preserved at the Museum für Naturkunde der Humboldt-Universität Berlin (Berlin, Germany). Note that the queen’s red colour in the anterior heavily sculptured part of the phragmotic head is not relevant to evaluating its chromatic pattern. Scale bars: 0.5 mm.

(2009) (Liechtenstein), [Wagner \(2014\)](#) (Austria’s Carinthia) and [Scupola \(2018\)](#) (Italy’s Veneto), as well as in the Cretan ant fauna monograph by [Salata et al. \(2020\)](#) and in the papers by [Wagner \(2019\)](#) (Austria), [García \(2020\)](#) (Spain) and [Tăușan et al. \(2020\)](#) (Romania).

The head red/mesosoma red ratio is statistically different among the two *Colobopsis* patterns and their models ($F_{3,76} = 152.4$, $P < 0.001$) (also see [Supporting Information, File S1](#)). Pairwise comparisons show no statistically significant difference between CSL *Colobopsis* and *Cr. scutellaris* ($P = 0.817$; mean \pm SD = 1.96 ± 0.36 for CSL *Colobopsis*; mean \pm SD = 2.05 ± 0.38 for *Cr. scutellaris*) and between DQL *Colobopsis* and *D. quadripunctatus* ($P = 0.299$; mean \pm SD = 0.60 ± 0.27 for DQL *Colobopsis*; mean \pm SD = 0.43 ± 0.20 for *D. quadripunctatus*), while all other comparisons are significantly different ($P < 0.001$) ([Fig. 3](#)).

NUMERIC MORPHOLOGY: MULTIVARIATE ANALYSES OF MORPHOMETRIC DATA

Two morphological clusters are identified via NC-clustering combined with ‘kmeans’ and ‘hclust’ ([Fig. 4](#)) that is also supported by PCA calculated on residuals of morphometric traits ([Fig. 5](#)). These two clusters correspond to the CSL pattern and DQL pattern specimens, respectively. All but two samples are congruently classified via both partitioning methods. The two incongruently placed samples (ITA:Mondello-VillaMercadante_col-12, ITA:Mondello-VillaMercadante_col-16; both CSL pattern from Sicily) are classified as belonging to the CSL cluster (PP = 0.85 and 0.81, geometric means of three workers each). Without running samples as wild-cards, the overall classification success is 96.3% using all variables in the analysis.

The *t*-tests were calculated to assess significant differences (P) of body-size ratios between specimens of the two different clusters, resulting in significant differences for seven ratios ([Table 2](#)). Unfortunately, there is not a single numeric body-size ratio available for reliable separation of these clusters on individual level ([Table 2](#)): the most reduced multivariate function that can reach the goal of attaining an acceptably high rate of classification success (> 95%) requires a minimum of six morphometric characters achieved via backward stepwise method.

The simplest D(6) function yielding 4.3% of error rate at the individual level is as follows:

$$D(6) = 0.03501 * CW - 0.03384 * SL - 0.03144 * HTL - 0.01762 * ML + 0.03653 * PeSH + 0.07458 * EL + 16.61469$$

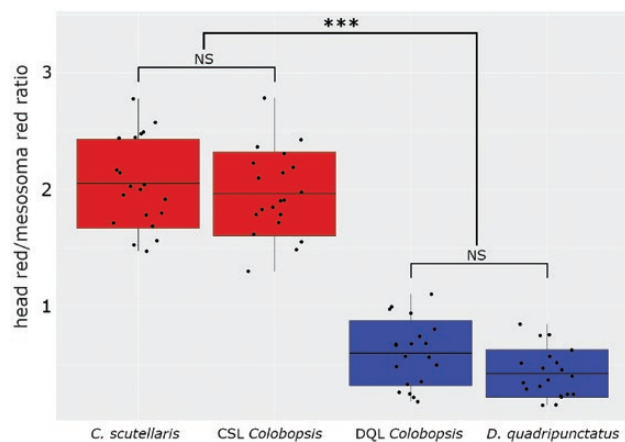


Figure 3. Chromatic ratios calculated from pictures of the *Colobopsis* CSL and DQL patterns and from pictures of the two putative model species *Cr. scutellaris* and *D. quadripunctatus* ($N = 20$ for each species or chromatic form). Boxplots show mean and standard deviation, while whiskers represent minimum and maximum values. Dots correspond to measured individuals. Their dispersal on the X-axis is a randomized graphic effect to avoid overlaps.

D(6) scores for CSL pattern cluster ($N = 55$) = mean -1.59 [$-4.17, +0.66$] ± 1.05

D(6) scores for DQL pattern cluster ($N = 60$) = mean -1.54 [$-0.37, +3.76$] ± 0.96

GENETICS: MITOCHONDRIAL *COI* SEQUENCES

The maximum likelihood phylogenetic analysis on mtDNA *COI* sequences identifies four main clusters with good nodal support (Fig. 6). Clade A is formed by specimens exhibiting the CSL pattern collected from Andalusia (Spain) and Morocco. The specimens of clade B exhibited the DQL pattern and were sampled from Bulgaria, Castilla La Mancha (Spain), Hungary, mainland Italy and the Occitanic region of France. Clade C groups specimens with the DQL pattern, sampled in the Spanish regions of Aragona and Catalonia, and from the Balearic Islands. Finally, clade D is formed by all the specimens from Sicily, showing the CSL pattern, and one of the Spanish specimens from Catalonia, actually exhibiting the DQL pattern.

ECOLOGY: COEXISTENCE WITH MODEL SPECIES

Crematogaster scutellaris is present in 97% of the trees investigated occupied by CSL *Colobopsis*, and in 20% of the ones occupied by DQL *Colobopsis*. The difference is statistically significant ($\chi^2_1 = 26.23$, $P < 0.001$). *Dolichoderus quadripunctatus* was never detected in trees occupied by CSL *Colobopsis*. On the other hand, *D. quadripunctatus* occurs on 40% of

the trees investigated occupied by DQL *Colobopsis* (6% of which also hosted *Cr. scutellaris*). Results are illustrated in Fig. 7, and detailed data are provided in the Supporting Information, File S1.

ETHOLOGY: INTERSPECIFIC TRAIL-FOLLOWING BEHAVIOUR

Trail-following behaviour was never observed during field observations in *Colobopsis* colonies exhibiting the DQL pattern (neither *Cr. scutellaris* nor *D. quadripunctatus* trails). Conversely, 77% of the *Colobopsis* colonies observed exhibiting a CSL pattern had workers following the *Cr. scutellaris* trails (Fig. 8). Detailed data are shown in the Supporting Information, File S1.

CSL *Colobopsis* followed *Cr. scutellaris* trails by either walking on them directly (more scarcely populated worker trails with considerable gaps) or slightly sideways (crowded trails with no or small gaps only). If coming into contact with a *Cr. scutellaris* worker, they immediately performed sudden accelerations and evasive movements. Trail-following often began shortly after the *Colobopsis* worker had left its nest and encountered a *Cr. scutellaris* trail on the tree trunk, and ended with the *Colobopsis* worker leaving the trail and heading towards specific twigs, no longer following *Cr. scutellaris* workers.

BIOGEOGRAPHY, EVIDENCE DISCUSSION AND FINAL SPECIES HYPOTHESIS

CSL and DQL chromatic patterns are found to effectively separate the Mediterranean *Colobopsis* into two populations that occur allopatrically, with each covering a vast geographic region (Fig. 9). The DQL pattern characterizes almost the entire European distribution of *Colobopsis*, in addition to Western Asia, while the CSL pattern occurs mainly in the Maghreb region (north-western Africa), a well-recognized ant biodiversity hotspot (Borowiec, 2014), and in the European regions with greater biogeographic proximity to it (Sicily and Southern Iberia) (e.g. Alicata & Schifani, 2019; Tinaut & Ruano, 2021). In particular, this distribution mirrors strikingly well that of some camponotine ant species such as *Camponotus barbaricus* Emery, 1905, *Ca. micans* (Nylander, 1856) and *Ca. ruber* Emery, 1925 (Fig. 9; for their distribution see: Forel, 1890, 1905; Santschi, 1925; Finzi, 1940; Menozzi, 1940; Cagniant, 1968, 1996; Collingwood & Yarrow, 1969; Baroni Urbani, 1971; Cagniant & Espadaler, 1993; Schembri & Collingwood, 1995; de Haro *et al.*, 1996; Janicki *et al.*, 2016; Guénard *et al.*, 2017; Schär *et al.*, 2020) and, to a slightly lesser extent, the distribution of myrmicine ants such as the *Aphaenogaster crocea* species group, *A. sardoa* Mayr, 1855 or the *Temnothorax algericus–mediterraneus*

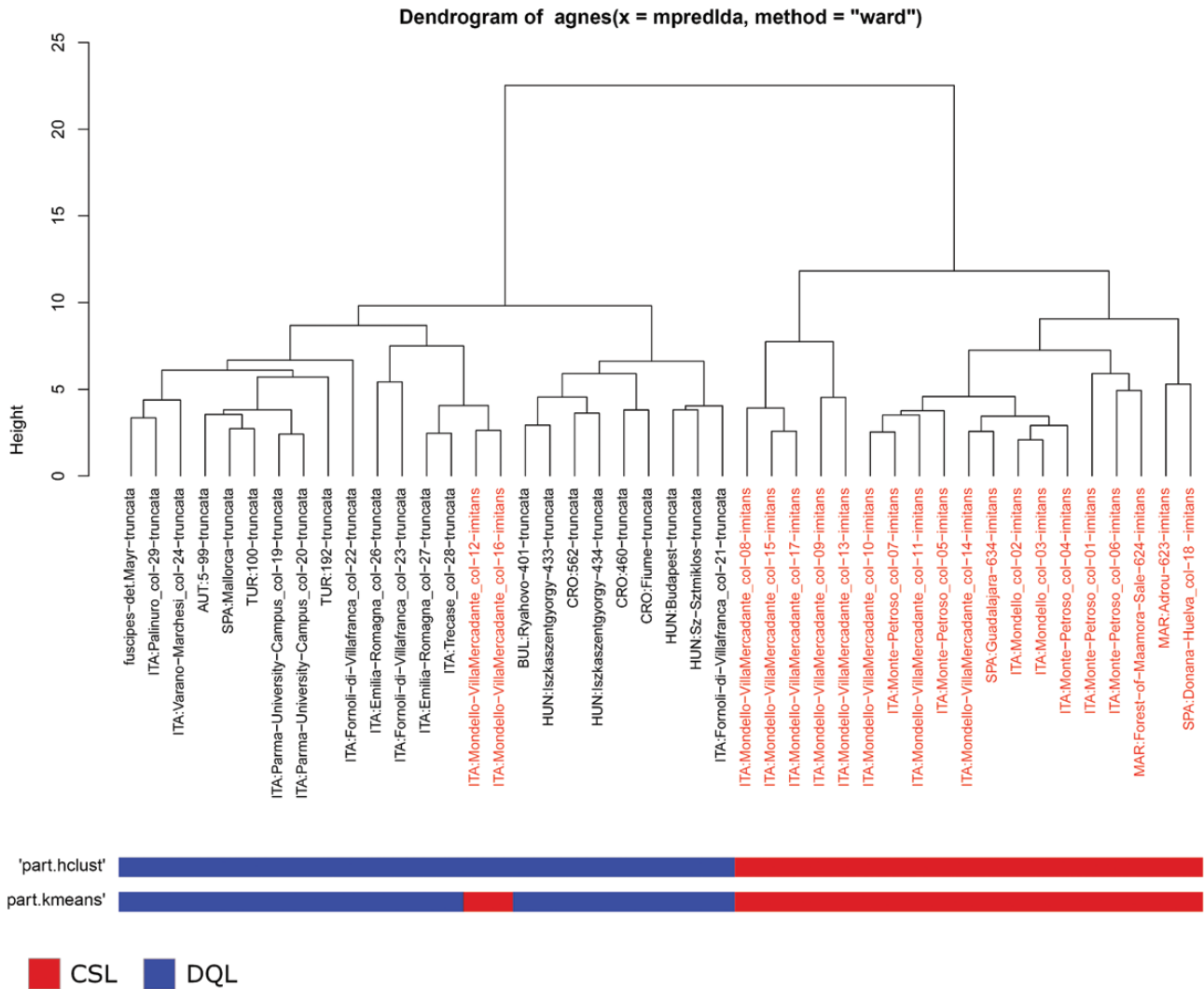


Figure 4. Dendrogram comparing the results of 'kmeans', and 'hclust' in NC Clustering of *Colobopsis* morphometric raw data. Two samples (4.5% of the total) are misplaced by both the dendrogram and one of the partitioning analyses, NC-part. kmeans; partially different samples being affected in each of the three analyses. The other partitioning analysis, NC-part. hclust returned the same sample assignment as the LDA did.

complex (see: [Mayr, 1853](#); [Emery, 1880](#); [Forel, 1909](#); [Santschi, 1929](#); [Galkowski & Cagniant, 2017](#); [Alicata & Schifani, 2019](#)). Ecological and behavioural field surveys across different Italian regions suggest that the two chromatic patterns are related to different lifestyles: the CSL pattern often coexists with *Cr. scutellaris* and often follows its trails, while the DQL pattern is associated with *D. quadripunctatus* without the involvement of frequent trail-following. The CSL pattern characterized specimens from the Spanish locality where mimicry, close nesting association and extensive trail-following of *Cr. scutellaris* were described by [Carpintero et al. \(2001, 2005\)](#). The DQL pattern instead characterized samples from the Austrian region where [Wagner \(2019\)](#) based his suggestions of close association and mimicry

between *Colobopsis* and *D. quadripunctatus*. A survey that was conducted in the region of Vienna (Austria) and employed similar methodologies to ours estimated that 36% of the *Colobopsis* colonies investigated ($N = 110$) nested on trees occupied by *D. quadripunctatus*, confirming the trend observed in our study ([Wagner, pers. comm.](#)). However, interspecific trail-following between DQL pattern *Colobopsis*, and either *D. quadripunctatus* or *Cr. scutellaris*, as reported in [Wagner \(2019\)](#) (personal communication), was never observed during our surveys, and seems to represent a considerably less frequent phenomenon. Such differences between the two groups in chromatic pattern, biogeography and life-history traits could arguably be sufficient to suggest a division of the West-Palaeartic *Colobopsis* into

Table 2. Mean of morphometric ratios calculated for CSL pattern and DQL pattern clusters based on individuals (raw data). Morphometric traits are divided by cephalic size (CS), namely the arithmetic mean of CL and CW. The upper row in each data field gives arithmetic mean \pm standard deviation; the lower one, in square brackets, the lower and upper extremes. Significant differences are highlighted in bold

Character	CSL ($N = 55$)	P	DQL ($N = 60$)
CS	897 \pm 51 [725, 1025]	0.924	898 \pm 55 [803, 1042]
PreOc/CL	0.546 \pm 0.01 [0.517, 0.571]	0.000	0.537 \pm 0.01 [0.517, 0.570]
CL/CW	1.152 \pm 0.02 [1.113, 1.198]	0.052	1.145 \pm 0.02 [1.106, 1.189]
dAN/CS	0.387 \pm 0.01 [0.345, 0.431]	0.128	0.391 \pm 0.02 [0.358, 0.426]
SL/CS	0.872 \pm 0.03 [0.798, 0.931]	0.000	0.846 \pm 0.02 [0.798, 0.906]
MW/CS	0.682 \pm 0.02 [0.648, 0.717]	0.476	0.680 \pm 0.02 [0.648, 0.723]
PeW/CS	0.332 \pm 0.02 [0.268, 0.372]	0.257	0.336 \pm 0.02 [0.306, 0.395]
HTL/CS	0.931 \pm 0.02 [0.888, 0.978]	0.000	0.902 \pm 0.02 [0.845, 0.957]
ML/CS	1.485 \pm 0.03 [1.395, 1.543]	0.000	1.460 \pm 0.02 [1.410, 1.526]
NOL/CS	0.139 \pm 0.01 [0.117, 0.165]	0.008	0.134 \pm 0.01 [0.112, 0.157]
PeSH/CS	0.239 \pm 0.02 [0.193, 0.284]	0.000	0.253 \pm 0.02 [0.203, 0.293]
EL/CS	0.312 \pm 0.01 [0.290, 0.335]	0.000	0.321 \pm 0.01 [0.304, 0.343]

two species, even according to a conservative classic taxonomical approach. Moreover, specimens examined from the two chromatic patterns are also classified into two morphometric clusters whose separation reaches a significant threshold, thereby indicating heterospecificity according to the current procedures of cryptic ant species separation (Seifert, 2020). At the same time, the morphometric separation between the two clusters is relatively narrow, possibly indicating that the two species may have separated recently. As for the mtDNA phylogenetic analysis, each clade is unambiguously monophyletic as regards morphometric and chromatic evidence (A and D = CSL pattern; B and C = DQL pattern), with only one misplaced DQL specimen (a 2.4% error rate). However, mtDNA lineages of CSL and DQL patterns resulted polyphyletic. This can be explained with possible retention of ancestral polymorphisms and/or introgression of mtDNA (see: Chan & Levin, 2005; Willis *et al.*, 2014). Indeed, these

phenomena appear to be largely responsible for the actual estimate of parphyly emerging from mtDNA phylogenies analyses in about 20% of animal species (Funk & Omland, 2003; Ross, 2014). As in other eukaryotic groups, mitochondrial DNA introgression is frequent in ants (e.g. Darras & Aron, 2015; Beresford *et al.*, 2017; Seifert, 2018), and coalescence during speciation commonly results in species undergoing phases of polyphyly and parphyly – on average longer in arthropods than in other groups – before normally reaching monophyly due to the stochastic process of complete lineage sorting (Avice, 2004; Ross, 2014). In the presently analysed taxa, this would support the hypothesis of recent divergence. Due to their geographic origins, the ambiguous placement of a few specimens during morphometric or genetic analyses also seems better supported by this hypothesis than by hybridization (despite the latter being relatively frequent in European ants, e.g. Steiner *et al.*, 2011; Seifert, 2018, 2019b).

In conclusion, the CSL and DQL *Colobopsis* clusters can be considered separate species in agreement with the good practices of ant alpha-taxonomy. All sources of evidence available suggest monophyly with the exception of mtDNA, whose advisory role in inferring species boundaries may be relatively weak in comparison with multiple nuclear genes or their expression products, as per Seifert (2020). As a result, the formal naming of CSL and DQL *Colobopsis* species holds a key informative value over their biology and life-history traits. The type material of *Co. truncata*, consisting of a single queen, could not be part of the morphometric or genetic analyses, but shows the DQL pattern clearly, and its geographic origin is unambiguous (with the type locality at the Orero mountains, near Genoa, in Liguria, Italy, placed in the centre of a highly investigated area within the DQL *Colobopsis* geographic range, about 780 km from the closest area inhabited by CSL *Colobopsis*). The same arguments of safe chromatic identification apply to *Co. fuscipes*; in this case they are supported by an even stronger biogeographic argument. Consequently, the *Colobopsis* characterized by the CSL pattern is an undescribed species. Accordingly, a formal description is provided below.

COLOBOPSIS IMITANS SP. NOV.

Zoobank registration: urn:lsid:zoobank.org:act:6B513EC6-6319-406F-97D0-82C0EF67E626

Etymology: *imitans* is the present participle of the Latin verb *imitor*, meaning ‘imitating’, and is here used in apposition. It refers to the interpretation that this species resembles *Cr. scutellaris*.

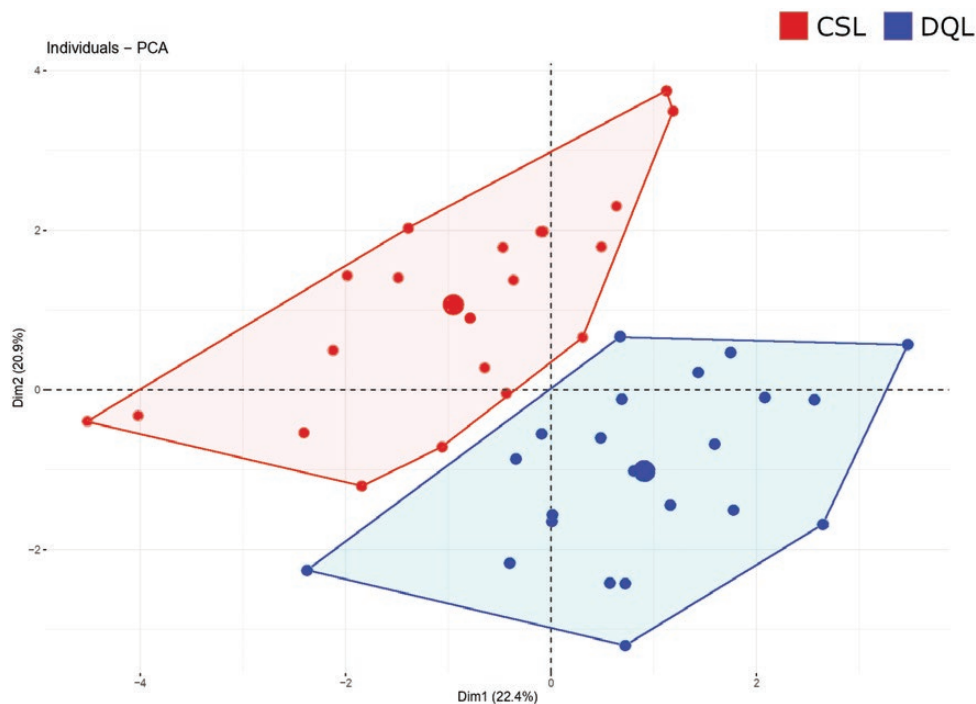


Figure 5. Principal component analyses of morphometric data of *Colobopsis* nest samples according to the two clusters evidenced by NC-PART clustering. Each small dot represents a colony sample. Large dots represent centroids.

Type series: One holotype worker (Fig. 10) and 14 paratype workers from Mondello, Sicily (Italy), 38.1953, 13.3354, 5 m, 14.X.2018, E. Schifani leg. The holotype is stored in the Hungarian Natural History Museum collection.

Worker description: Morphometric indexes are shown in Table 2. Subrectangular head, rounded on all sides. A straight, central furrow runs from the frontal triangle to the level where the frontal carinae end. Large eyes, extremely reduced ocelli. Antennae of 12 segments, without a distinct antennal club. Pronotum significantly wider than the rest of the mesosoma. In lateral profile, pronotum and mesonotum gently convex, propodeum profile often showing a central concavity, thus having a saddle-like appearance. Petiolar scale profile anteriorly roundly concave and posteriorly straight, its dorsal crest excavated in frontal view. Well-visible promesonotal and mesoepinotal sutures, as well as metathoracic and propodeal spiracles. All legs with well-developed tibial spurs, more so in the anterior legs, which are characterized by strikingly large femurs (identical to *Co. truncata*, function unknown). Pigmentation as described in the CSL model. Fine alveolate to areolate sculpture covering the whole body and appendages. Few erect hairs near the posterior margin of the vertex, between the frons and on the

clypeus, and few others on the gaster tergites. See Figures 1, 8, 10, 13.

Soldier (= phragmotic major worker) description: Measurements (two specimens from Sicily): CL = 1327, 1419; CW = 1262, 1470; SL = 865, 942; ML = 1752, 1774; MW = 921, 1103; EW = 264, 331; EL = 409, 459; CS = 1294, 1444; CL/CW = 0.96, 1.05; SL/CS = 0.65, 0.67; ML/CS = 1.23, 1.35. Large cylindrical phragmotic head, and a flattened anterior part formed by the mandibles, part of the clypeus and of the genae. Characterized by strong areolate-rugose sculpture and dense coverage of short thick erect hairs in the distalmost half. Rest of the shape, sculpture and pigmentation generally similar to the worker but white dots or stripe on the first gastral tergite sometimes evident. See Figure 10.

Queen description: Measurements (three specimens from Sicily): CL = 1437–1531; CW = 1281–1406; SL = 1156–1218; ML = 2687–3031; MW = 1281–1312; EW = 325–362; EL = 525–537; CS = 1359–1468; CL/CW = 1.08–1.11; SL/CS = 0.83–0.87; ML/CS = 1.93–2.22. Large phragmotic head, similar to the soldier in shape and size (despite larger body size), but well-developed ocelli, much larger eyes and much longer scapi. Immediately distinguishable by the larger, dorsally flatter mesosoma, which is largely

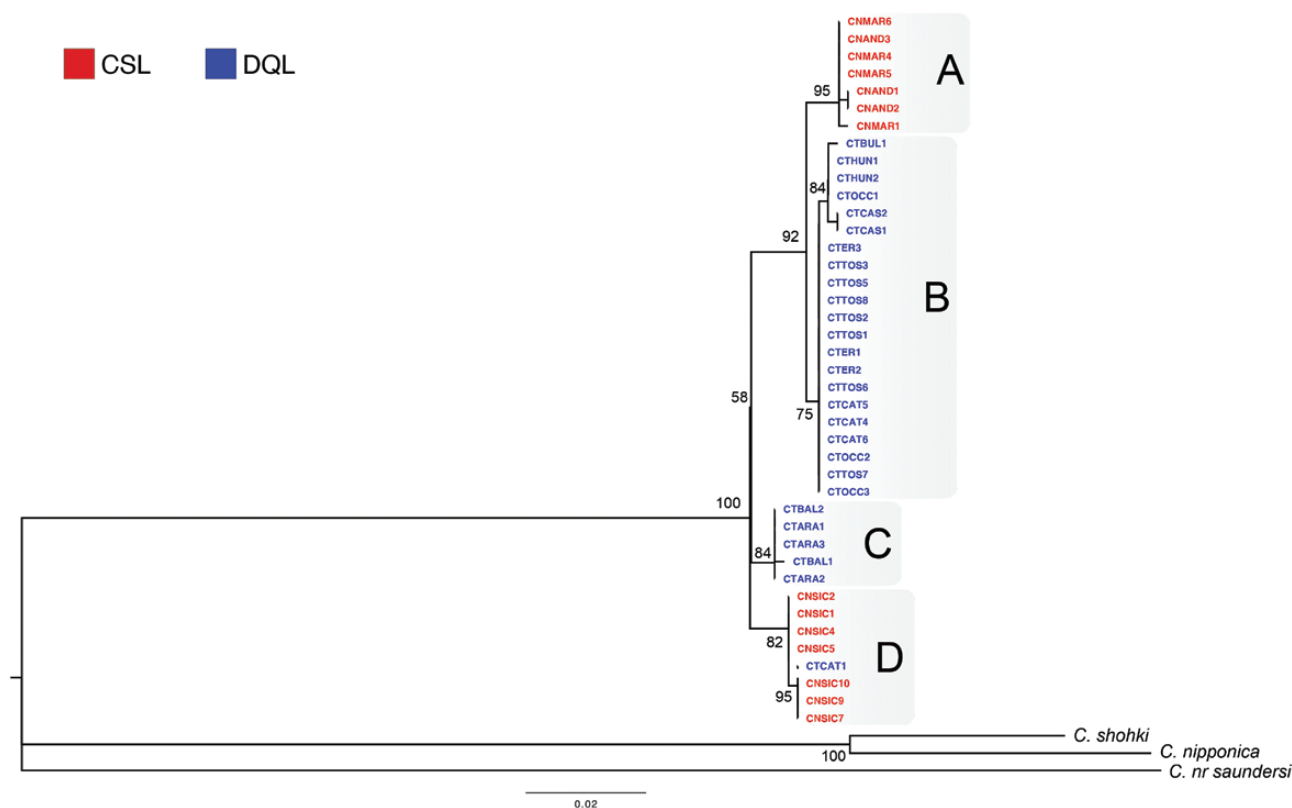


Figure 6. Maximum likelihood phylogenetic tree based on the barcode fragment of the mtCOI gene from the *Colobopsis* specimens sequenced.

unsculptured and shiny. Propodeum profile similar to the end of soldiers' propodeum. Head red, as in the worker, but the mesosoma is brownish and the white dots or stripe on the first gaster tergite are/is evident. See [Figure 11](#).

Male description: Measurements (three specimens from Sicily): CL = 875–1093; CW = 781–1000; SL = 937–1001; ML = 2281–2437; MW = 1062–1218; EW = 300–387; EL = 462–525; CS = 828–1046; CL/CW = 1.09–1.17; SL/CS = 0.92–1.20; ML/CS = 2.32–2.75. Small, subrectangular head with large ocelli and large eyes protruding laterally. Toothless mandibles. Relatively large mesosoma, propodeum more gently rounded than in queens or workers. Low, round petiolar node. Weak sculpture, shiny mesosoma. Hairy mandibles, other hairs on clypeus and gaster. Entire body ferruginous or brownish, gaster blackish. See [Figure 11](#). Genitalia as in [Figure 12](#).

Diagnosis: Generally easy to determine on the basis of worker material due to strong chromatic differentiation from *Co. truncata* and allopatric distribution (although further investigation is required for possible contact regions in southern Iberia and southern Italy). Some

small worker specimens may appear almost completely black, therefore lacking the typical chromatic pattern; workers with entirely red mesosoma seldom observed. Tentative identifications of isolated soldier or queen specimens should be much more cautious, although their chromatic appearance may sometimes appear to be explicit. The low number of males and the lack of particularly evident distinctive characters from *Co. truncata* do not allow a safe species-level identification of this caste based on morphology. As for workers, the morphometric linear discriminant function provided in the Results section should help with dubious cases, including decoloured specimens. Finally, DNA barcoding, which can be used for the same purpose and can also be employed on the other castes, shows relatively low error rate but may present risks due to the polyphyletic pattern that we observed.

Biological, ecological and phenological notes: Relatively thermophilous; occurring in Sicily from a few meters above sea level to at least 1015 m elevation, ascending up to 1290 m a.s.l. in Morocco, and so far known from coastal lowland areas in Spain. Probably common but also heavily under-recorded due to its cryptic arboreal lifestyle, low colony population

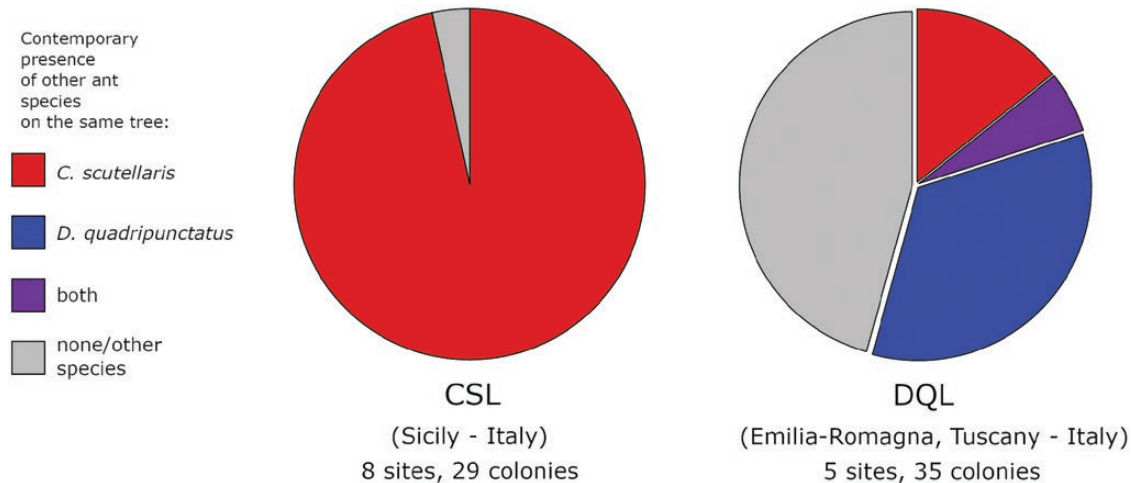


Figure 7. Coexistence between *Colobopsis* and their putative model species on the same tree.

(most-likely monogynous), effective mimicry and long periods of inactivity during the most arid and coldest times of the year. Workers and soldiers are unlikely to descend to the ground but were observed to do so at least once, following a sparsely populated *Cr. scutellaris* trail. Soldiers, in general, are difficult to find outside the nest and are usually seen acting as gatekeepers of the nest entrance. Founding queens were observed to do the same. Nests are hidden in minute holes on the dead parts of arboreal trunks, where *Co. imitans* often seems to act as secondary user of cavities excavated by xylophagous insects. It exploits *Andricus quercustozae* (Bosc, 1792) oak galls as nests [occupying about 15% of galls collected in Bosco della Ficuzza (Sicily) in a recent survey, authors' unpublished data], in a similar way to *Co. truncata* (see: [Giannetti et al., 2019, 2021](#); [Fürjes-Mikó et al., 2020](#)). Polydomy appears probable due to repeated findings of groups of workers with no queen within oak-galls. Nesting observed on several diverse plant species, including at least: *Citrus reticulata* Blanco, *Ci. sinensis* (L.) Osbeck, *Laurus nobilis* L., *Olea europaea* L., *Quercus ilex* L., *Q. pubescens*-group, *Q. suber* L., *Pyrus communis* L. and *Pittosporum tobira* (Thunb.) W.T.Aiton. Dense populations appear to have been found in old *Citrus* orchards, relatively sparse cork oak (*Q. suber*) woods and also in deciduous oak forests. However, *Co. imitans* occurs in a broad range of habitats, from cities to agricultural lands to natural forest habitats, but information available has so far been insufficient to depict a satisfactory picture of habitat preferences. Despite the earlier claim by [Carpintero et al. \(2005\)](#), there is currently no evidence backing the fascinating hypothesis that *C. imitans* foundress queens prefer trees hosting *Cr. scutellaris* to found their colonies. A focused investigation on this topic would be interesting. Nuptial flights for *Co.*

imitans occur approximately in the same period as *Co. truncata* (alates in Sicily observed from June 30 to July 13, $N = 5$, see [Supporting Information, File S1](#)). Winged queens and males were repeatedly seen to be attracted by artificial light at night.

DISCUSSION

Body pigmentation pattern is the only qualitative character that allows identification of *Co. imitans* without having to resort to quantitative data, as it is otherwise morphologically extremely similar to *Co. truncata* up to a significant level of crypsis (see: [Wagner et al., 2018](#)). These pigmentation differences among West-Palaeartic *Colobopsis* have so far gone completely unnoticed, the sole exception being a brief statement by [Santschi \(1929\)](#) noting that the chromatic aspect of the Moroccan *Co. truncata* differs from typus one in its lighter head colour. The case we documented can be considered one of the few where such an element is important for species discrimination in European ants. While body pigmentation was used with no scientific rigor by some past ant taxonomists [see the example described by [Boer \(2008\)](#)], it can be important for the morphological identification of species such as *Formica clara* [Forel, 1886](#) and *F. cunicularia* Latreille, 1798 or even fundamental for many *Temnothorax* spp. ([Seifert & Schulz, 2009](#); [Seifert, 2018](#)), and should not be overlooked in multi-character approaches for taxonomic purposes. Under these conditions, checking pictures uploaded on citizen science platforms and social media proved to be significantly helpful in obtaining data on these species distribution, evidencing once more the uncovered potential of citizen science in the study of ant distribution (e.g. [Lucky et al., 2014](#); [Zhang et al.,](#)

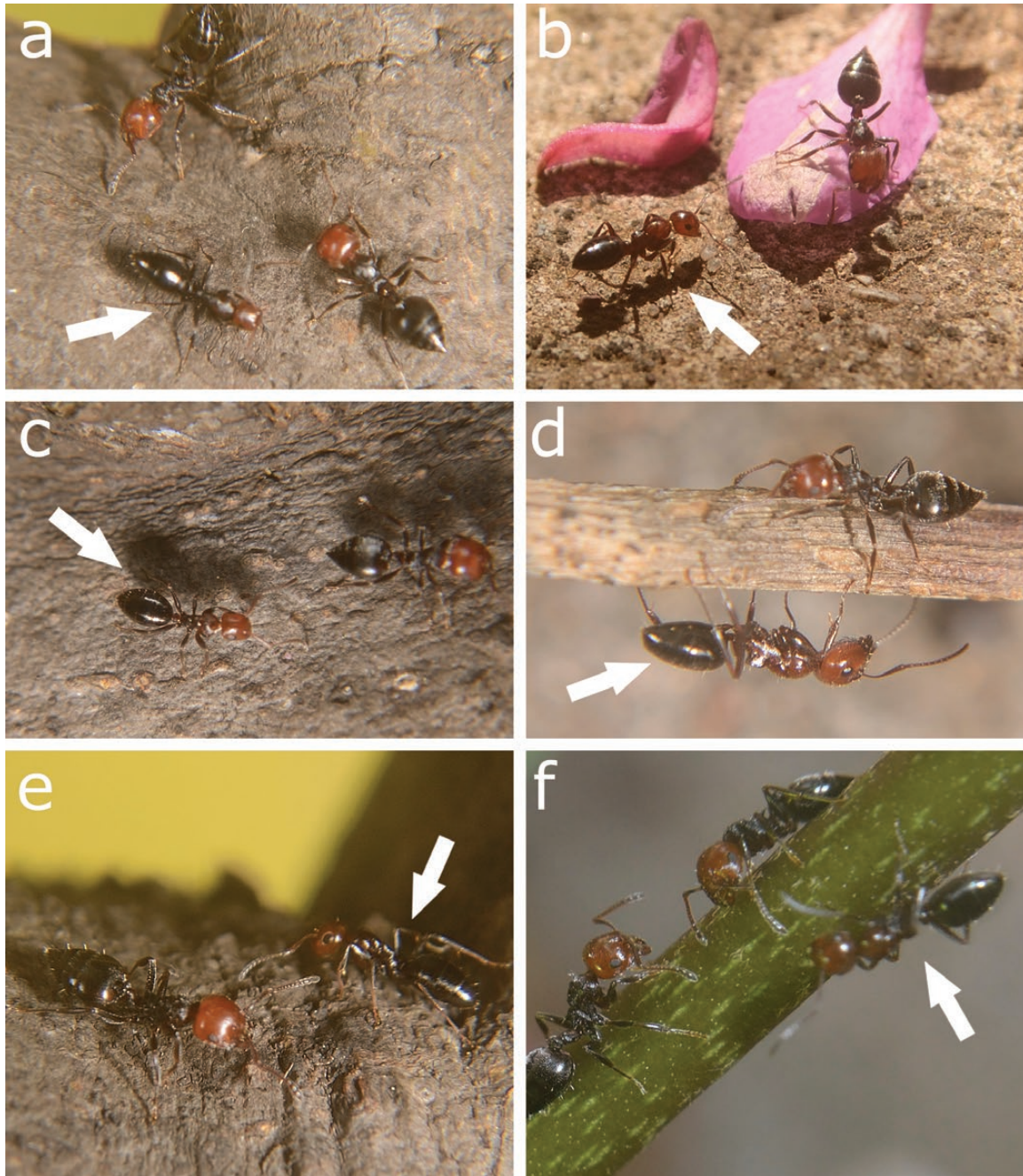


Figure 8. Trail-following behaviour on *Crematogaster scutellaris* trails by other ants (indicated with arrows). On the left (A, C, E) CSL *Colobopsis*; on the right (B, D, F) *Camponotus lateralis* observed in the same locality performing the same behaviour (photos taken in Palermo (Sicily) during field surveys).

2019; Castracani *et al.*, 2020; Sheard *et al.*, 2020) and more, in general, of platforms hosting these kinds of data in the study of insect distribution (e.g. Schifani & Paolinelli, 2018; Hochmair *et al.*, 2020; Ruzzier *et al.*, 2020; Winterton, 2020). Moreover, behavioural data are seldom considered in integrative taxonomic approaches dealing with ants, but they may prove valuable in some

cases (see also: Ronque *et al.*, 2016). Finally, while mtDNA has decent identification performance, our data clearly support the idea that it should not be used as a primary source of information to make taxonomic decisions on species delimitation (see: Seifert, 2020).

The taxonomic status of the West-Palaeartic *Colobopsis* populations appears to be now well

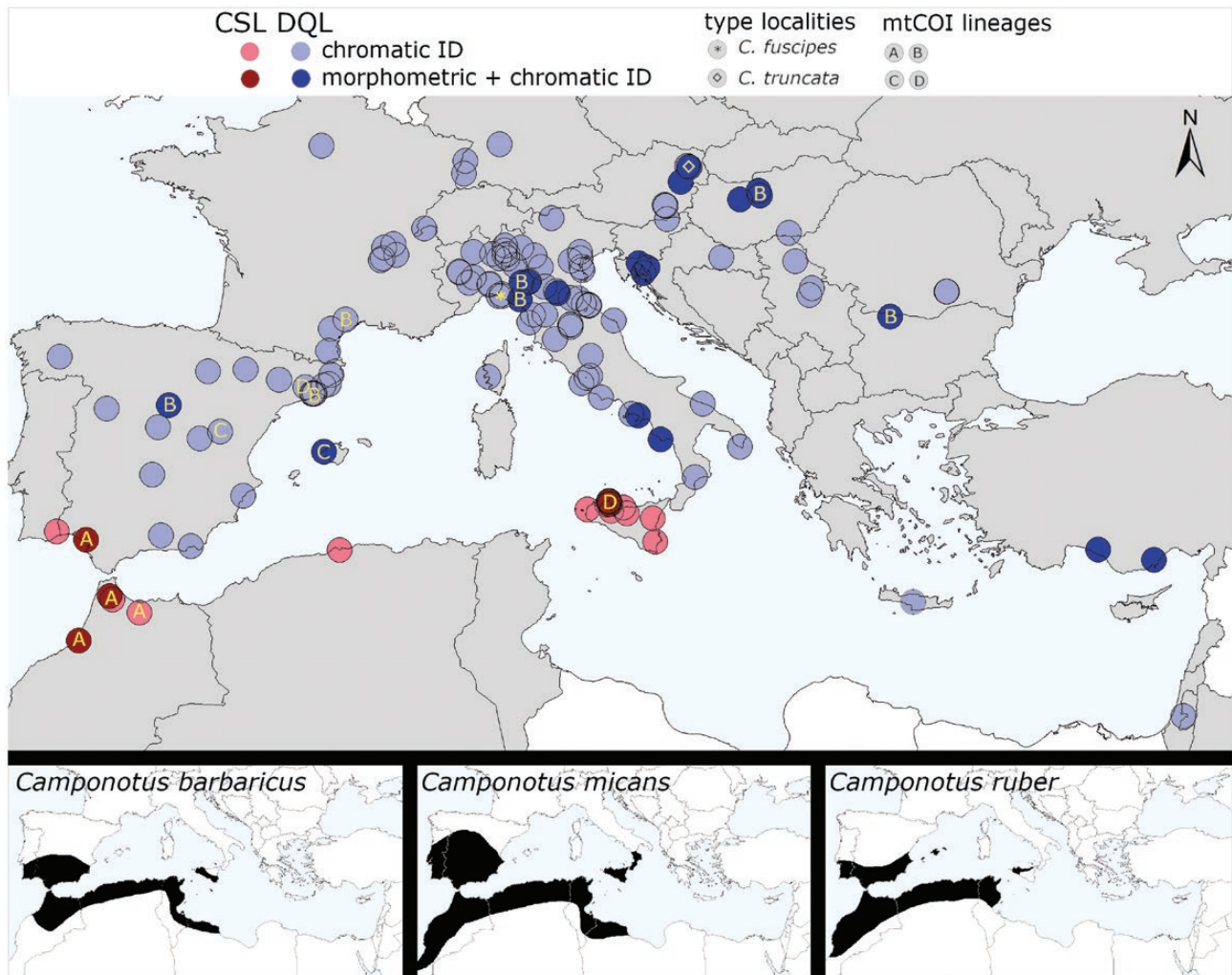


Figure 9. Above: distribution map of *Colobopsis* samples examined – countries where *Colobopsis* presence is known from the literature are highlighted in grey. Below: approximate distributions of other *Camponotini* (*Camponotus barbaricus*, of *C. micans* and of *C. ruber*) which resemble that of CSL *Colobopsis*.

resolved. However, our analyses lacked data from what the existing literature describes as the easternmost distribution of *Co. truncata*, east of the Mediterranean region, which reaches the Kopet Dag in Turkmenistan (Dlussky *et al.*, 1990; Gratiashvili & Barjadze, 2008; Dubovikoff & Yusupov, 2018; Bračko, 2019; Samin *et al.*, 2020). In biogeographic terms, they are extremely unlikely to represent a disjunct *Co. imitans* population, while conspecificity with *Co. truncata* appears likely due to the existence of several ant species with similar distributions (e.g. Wagner *et al.*, 2017; Seifert, 2018). Within the Mediterranean, the range limits of *Co. imitans* and *Co. truncata*, or their possible sympatry in contact zones, should be appropriately investigated in areas of biogeographic transition (southern Iberia, Sicily, Calabria and perhaps Sardinia; see: Alicata & Schifani, 2019;

García, 2020; Schifani *et al.*, 2020, 2021; Tinaut & Ruano, 2021).

The fact that *Co. imitans* and *Co. truncata* differ greatly from a chromatic perspective is interesting if one considers that phylogenetics and morphometry suggest recent differentiation. In evolutionary terms, the most likely interpretation is to link such differentiation to a shared strategy based on ant-mimicry modulated according to the presence or absence of certain good models across different Mediterranean regions. Both *D. quadripunctatus* and *Cr. scutellaris* have much more populous colonies than *Co. imitans* and *Co. truncata*, while both are probably less palatable for predators and are armed with effective toxic substances (Cavill & Hinterberger, 1960; Wagner, 2019). Therefore, even though *Cr. scutellaris* only is truly recognized as an aggressive dominant species (Santini *et al.*, 2007;

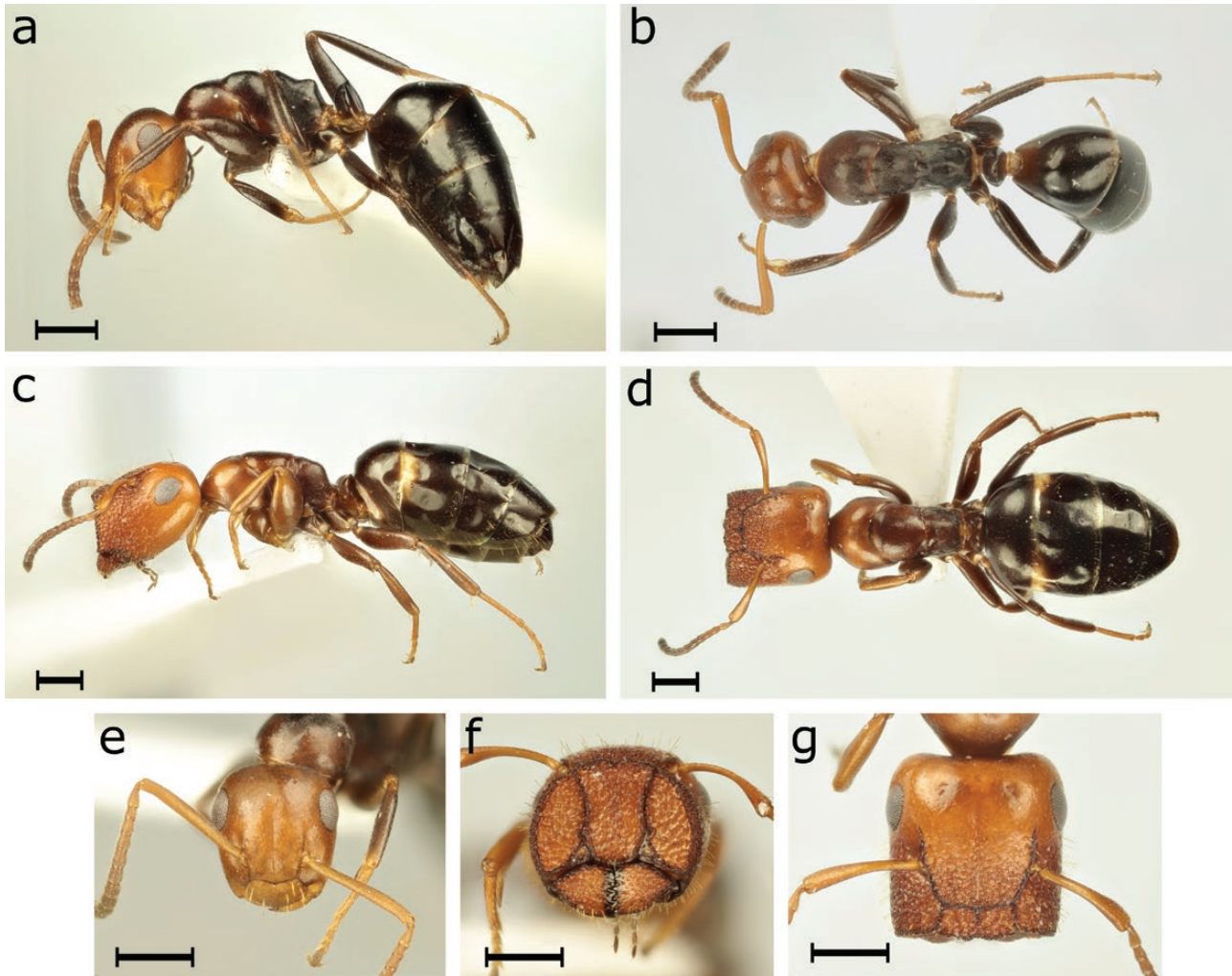


Figure 10. *Colobopsis imitans*. A, B, E, worker (holotype); C, D, F, G, soldier (specimen from the type locality). Scale bars: 0.5 mm. Pictures also available on AntWeb.org database, specimen codes: ANTWEB1041481 and ANTWEB1041482.

Frizzi *et al.*, 2015; Castracani *et al.*, 2017; Seifert, 2018), both appear to possess the traits required to be considered good Batesian models to the non-aggressive and relatively unarmed *Colobopsis* (which still possesses some formic acid). However, *D. quadripunctatus* is almost completely absent across the distribution range of *Co. imitans*: it does not inhabit the Maghreb, its Iberian distribution is concentrated to the North and in Sicily it is considered rare (Schifani & Alicata, 2018; Cabanillas *et al.*, 2019). However, it is interesting to note that the opposite is not the case for *Co. truncata*: the latter is not only sympatric with *D. quadripunctatus* along its entire range (including in the hypothesis that easternmost *Colobopsis* are *Co. truncata*; see: Reznikova, 2003; Ghahari *et al.*, 2015), but also sympatric with *Cr. scutellaris* in south-western Europe and with *Cr. schmidtii* in the east. Finally, it is worth noting that the white dots

or stripe linked to mimicry of *D. quadripunctatus* in *Co. truncata* (Forel, 1886; Wagner, 2019) are/is absent or hardly visible in *Co. imitans* workers but at the same time well-visible in at least a few soldiers and especially the queens that we inspected. Following the mimicry interpretation of the chromatic patterns, this character can be considered an ancestral remnant, but selective (predatory) pressures leading to perfect mimicry can be seen as stronger on workers than on queens or soldiers, which rarely leave the safety of their nest.

Mimicry may be considered a third defensive strategy of *Colobopsis*, which is unique or rare among ants after suicidal autothysis and phragmosis (Emery, 1925; Maschwitz & Maschwitz, 1974; Davidson *et al.*, 2012; Shorter & Rueppel, 2012; Ward *et al.*, 2016; Laciny *et al.*, 2018). Apart from the two species we treated, the only existing claims of mimicry in the genus come

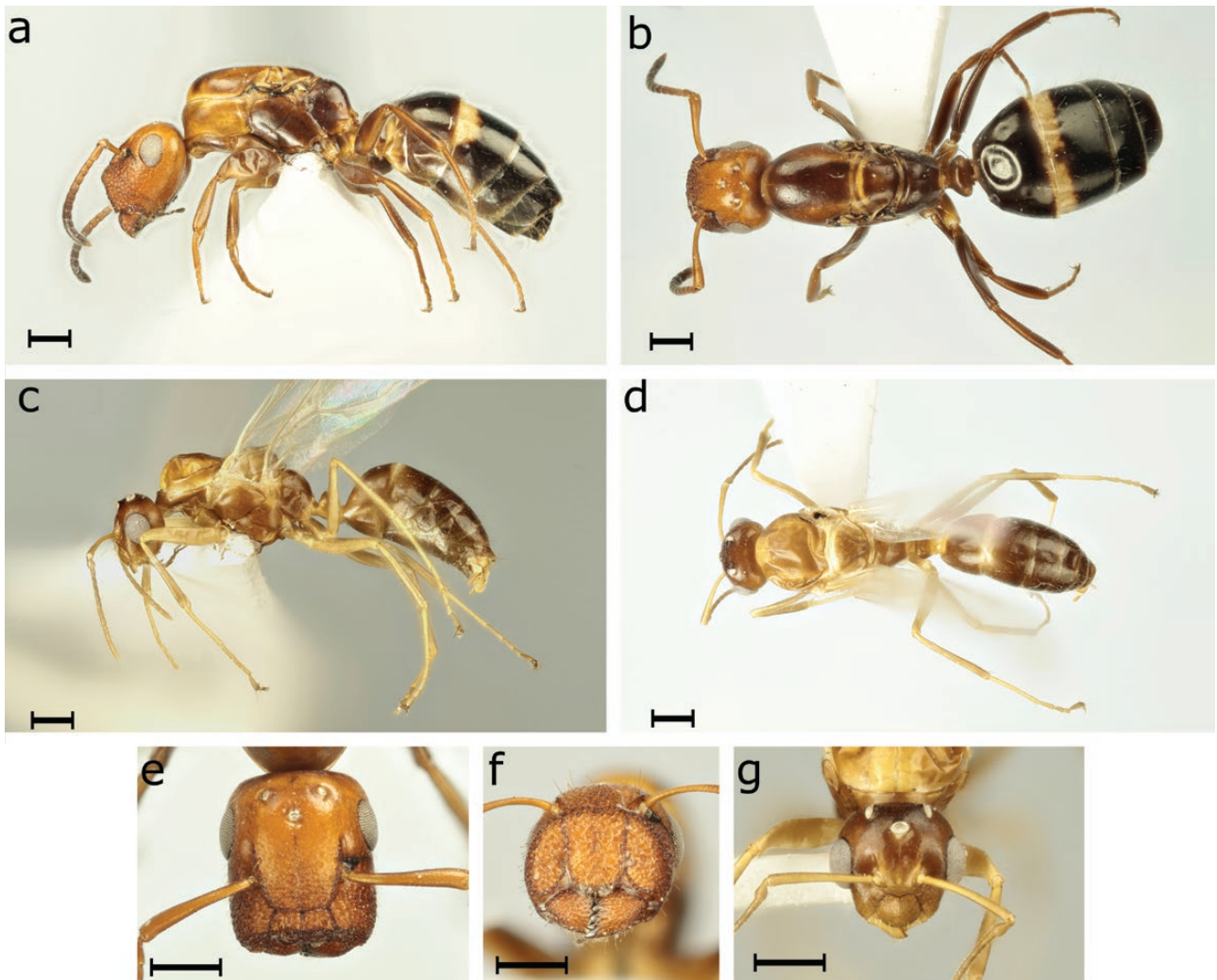


Figure 11. *Colobopsis imitans*. A, B, E, F, queen; C, D, G, male. Specimens from the type locality. Scale bars: 0.5 mm. Pictures also available on AntWeb.org database, specimen codes: ANTWEB1041483 and ANTWEB1041484.

from morphologically different and probably unrelated species from the Fiji Islands (Santschi 1928; Wheeler 1934). However, since several other Palaearctic *Colobopsis* species share a general morphological similarity with *Co. imitans* and *Co. truncata*, probably belonging to the same evolutionary lineage, it is possible that some of them represent yet undiscovered mimics. It also appears that mimicry may have played a powerful role driving phenotypic diversification of West-Palaearctic *Colobopsis*: *Co. imitans* and *Co. truncata* can be considered the only well-documented example among ants that suggests mimicry-driven phenotypic divergence of sister-species. In comparison, the intraspecific case of mimicry pattern divergence in *Ca. lateralis* is an instance of much more modest differentiation (Wagner, 2014; Seifert, 2019a). Similar accounts are not particularly common in other

organisms either, but an interesting scenario of strong diverging aposematic patterns coupled with minimal genetic differentiation has recently been described for a group of frogs (Tarvin *et al.*, 2017).

It is still not known which visual predators may have been responsible for the selective pressures that led to the emergence of ant-mimicry across different ant lineages. Birds and lizards appear to be good candidates to start with (Ito *et al.*, 2004; Wagner, 2014). Our surveys around the colonies and trails of *Cr. scutellaris* and *D. quadripunctatus* led us to find several possible mimics of either species that belong to different insect and spider groups already known for ant-mimicry (Fig. 13). In particular, Santschi (1919) has suggested the existence of an association similar to the one between *Ca. lateralis* and *Cr. scutellaris* in the Canarian relative of *Ca. ruber*



Figure 12. Male genitalia of *Colobopsis imitans* in ventral and dorsal view, specimen from the type locality. Scale bars = 0.25 mm.

(*Ca. guanchus* Santschi, 1908), while Harvey *et al.* (2018) described the anti-predatory function of ant-mimicry in *Gelis* spp., Komatsu (1961) reported on *Phrurolithus*–ant associations, Corcobado *et al.* (2016) reported on *Leptorchestes*–ant associations and, finally, Chandler (2010) mentions myrmecomorphism among Anthicidae. Although some of these findings may have been coincidental and deserve further investigation, *Co. imitans* and *Co. truncata* may each be considered part of a larger cohort of different arthropods that evolved mimicry to resemble *Cr. scutellaris* or *D. quadripunctatus* in response to visually hunting generalist insectivores, similarly to the ‘golden mimicry complex’ described by Pekár *et al.* (2017). Further investigation is also required to understand whether the advantages of mimicry for *Co. imitans* and *Co. truncata* may lie in a dilution effect, if Batesian mimicry is truly implied, and if Müllerian mimicry also plays a role (see: Speed, 1999; Pekár *et al.*, 2017), bearing in mind that different evolutionary relations may exist between the same prey and different predators.

Interspecific but intrageneric trail-following described for some ants may be related to phylogenetic proximity, and morphofunctional and behavioural similarities or similar foraging strategies among species (e.g. Grasso *et al.*, 2002 and references therein). On the other hand, the significance of the *Colobopsis*–*Crematogaster* trail-following recorded is not yet fully clear to us. Similar cases (such as that of *Ca. lateralis*) have often been referred to as parabiosis, but we have avoided this term since different interpretations of its meaning coexist, thereby creating ambiguity: it

is sometimes used to simply indicate trail-following, while in other occasions it also implies nest-sharing (see: Forel, 1898; Swain, 1980; Vantaux *et al.*, 2007; Menzel *et al.*, 2008, 2010, 2014a, b; Seifert, 2018). Besides *Co. imitans* and *Co. truncata*, many other camponotine ants follow *Crematogaster* trails without necessarily acting as mimics (Ito *et al.*, 2004; Vantaux *et al.*, 2007; Menzel *et al.*, 2008, 2014a, b). Baroni Urbani (1969) and Menzel *et al.* (2014) speculated that the compounds used as trail pheromones by *Crematogaster* are generally easily perceived by *Camponotus*, this capacity representing an important pre-adaptation to trail-following. During our field surveys, we unexpectedly observed several workers of *Camponotus piceus* (Leach, 1825) (a relative of *Ca. lateralis* with no resemblance to *Cr. scutellaris*; see: Seifert, 2019a) easily following part of a *Cr. scutellaris* trail to the canopy of a tangerine tree while avoiding *Cr. scutellaris* attacks. It may be possible that other similar camponotine ants rarely perform the same without possessing specific mimicry adaptation, which can partly explain the occasional observations of trail-following between *Co. truncata* and *Cr. scutellaris* (Zimmermann, 1934; Goetsch, 1942; Baroni Urbani, 1969; Wagner, 2014). However, in the overwhelming majority of the cases documented, interspecific trail-following is associated with either mimicry (as for *Colobopsis imitans*; see: Gobin *et al.*, 1998; Ito *et al.*, 2004; Menzel *et al.*, 2010; Powell *et al.*, 2014) or nest-sharing (Vantaux *et al.*, 2007; Menzel *et al.*, 2008, 2014). For mimics, it appears to be primarily a way to better hide within the ranks of the model species, an example of dilution effect (Lehtonen & Jaatinen, 2016),

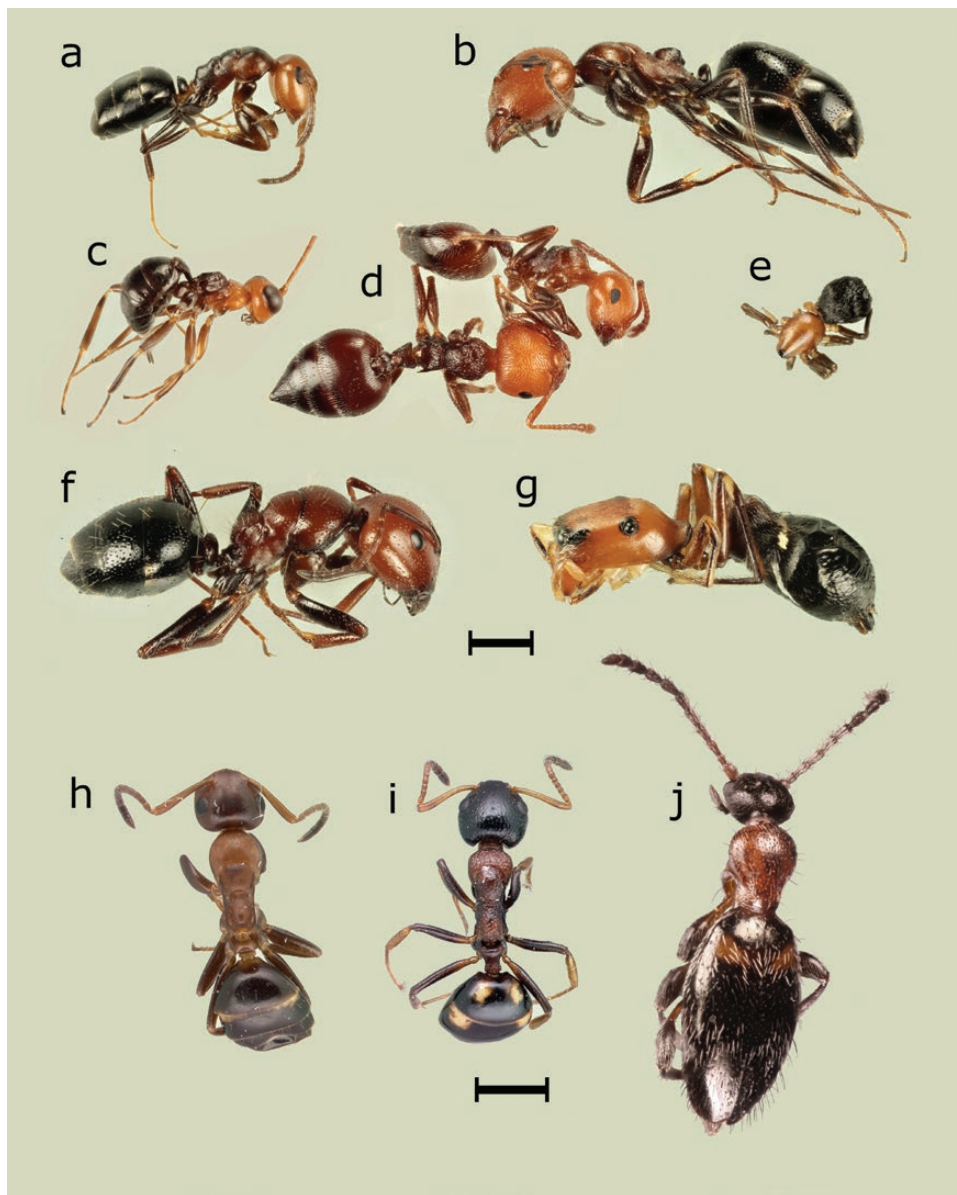


Figure 13. Above *Cr. scutellaris* and species showing a similar chromatic pattern that were collected near *Cr. scutellaris* trails in Sicily (Italy): A, *Co. imitans* (worker from Mondello); B, *Ca. lateralis* (worker from Monte Pellegrino); C, *Gelis* sp. (Hymenoptera: Braconidae) from Monte Petroso; D, *Cr. scutellaris* from Levanzo island; E, *Phrurolithus* sp. (Araneae: Phrurolitidae) from Mondello; F, *Ca. ruber* (worker from Monte Pellegrino); G, *Leptorchestes* sp. (Araneae: Salticidae) from Monte Petroso. Below, *D. quadripunctatus* and species with a similar chromatic pattern collected near its trails or in the same trees in mainland Italy: H, *Co. truncata* (specimen from Bulgaria, AntWeb code CASENT0280000, photographer Michele Esposito); I, *D. quadripunctatus* (specimen from Czech Republic, AntWeb code CASENT0179916, photographer Michele Esposito); J, *Formicomus pedestris* (Rossi, 1790) (Coleoptera: Anthicidae) from Parma (Italy).

which to a certain degree may also apply to non-mimetic ant species as well. However, the trail-followers may be able to obtain additional benefits in their success of locating trophic resources, sometimes even establishing somewhat parasitic relationships

(see: [Vantaux et al., 2007](#); [Menzel et al., 2010, 2014a, b](#)). It is unclear whether *Colobopsis* ants may also benefit from a similar mechanism, although a parasitic aspect of its trail-following behaviour has been suggested by [Baroni Urbani \(1969\)](#).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site. File S1. List of examined material, GenBank accession numbers, raw morphometric data, data on trail following and on the coexistence with the model species.