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## Morphological, biogeographical and molecular evidence of *Carpocoris mediterraneus* as a valid species (Hemiptera: Pentatomidae)

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### Abstract

*Carpocoris mediterraneus* Tamanini, 1958, synonymized with *Carpocoris fuscispinus* (Boheman, 1851) by Ribes *et al.* (2007), is restored to the species level. The shape of the pronotum is a good diagnostic character to distinguish the two species. The existence of two valid species is supported by geographical distribution patterns in Western Europe: Mediterranean-Atlantic for *C. mediterraneus*, and Continental for *C. fuscispinus*. In France and Spain, in some areas, the two species are found in sympatry (sometimes even on the same plant). Morphological observations are confirmed at the molecular level by sequencing of the mitochondrial Cytochrome c oxidase I standard barcode fragment. Indeed, inter-specific divergence largely exceeded intra-specific divergence and our phylogenetic reconstructions reveal that *Carpocoris mediterraneus* and *Carpocoris fuscispinus* form two reciprocally monophyletic genetic lineages. A morphological identification key is proposed for all the European species of the genus *Carpocoris*, to facilitate identification. *Carpocoris fuscispinus* is first time recorded from Portugal.

**Key words:** Hemiptera, Heteroptera, Europe, France, Spain, Portugal, morphological characters, biogeography, barcoding, mitochondrial DNA, Cytochrome oxidase, taxonomy, identification, *Carpocoris*

### Résumé

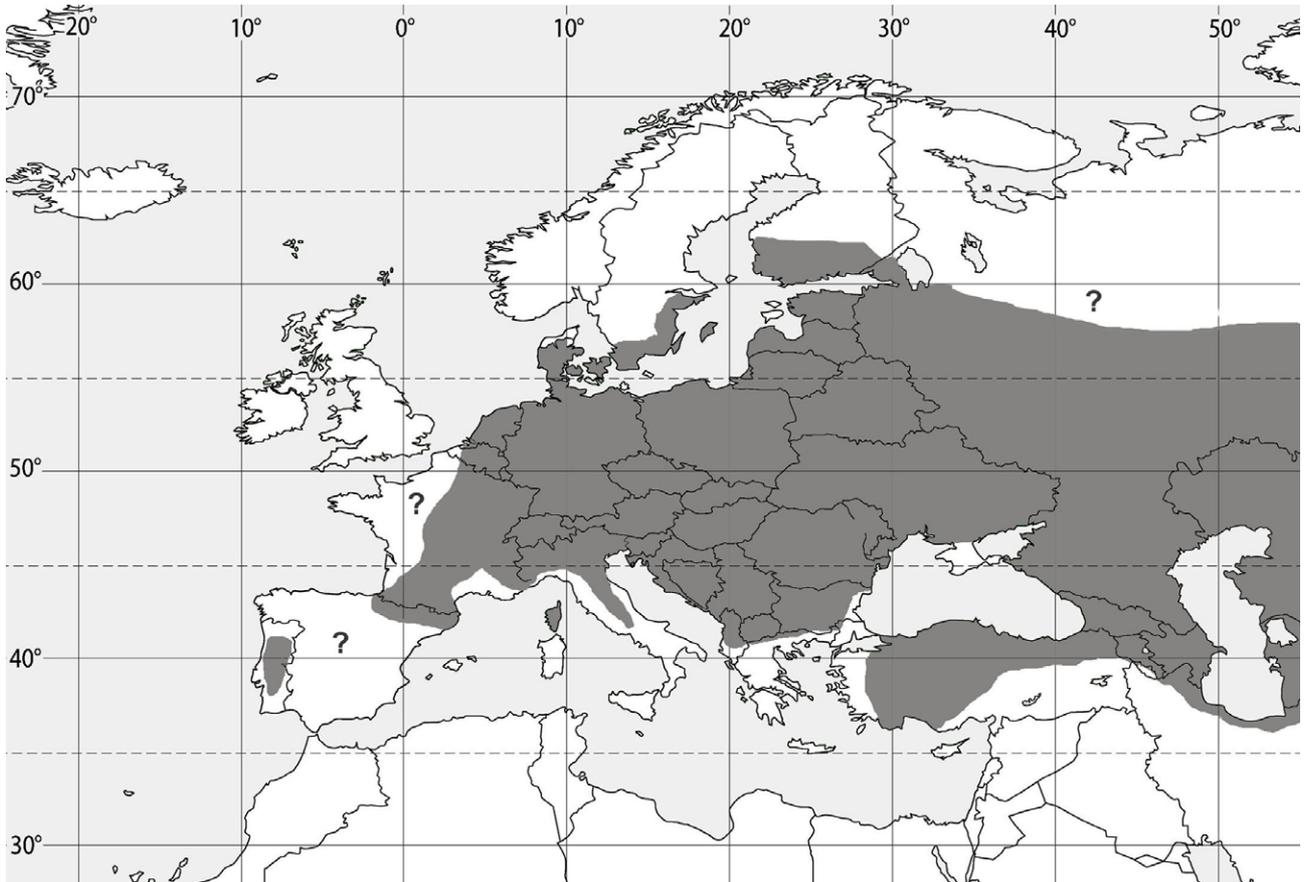
*Carpocoris mediterraneus* Tamanini, 1958 mis en synonymie par Ribes *et al.* (2007) avec *Carpocoris fuscispinus* (Boheman, 1851), est réhabilité au rang d'espèce valide. La forme du pronotum est un bon caractère diagnostique pour séparer ces deux espèces. L'existence de deux espèces valides est soutenue par les patrons de distribution géographique observés dans l'ouest de l'Europe : méditerranéo-atlantique pour *C. mediterraneus*, et continental pour *C. fuscispinus*. En France et en Espagne, dans certaines localités, les deux espèces sont sympatriques (parfois sur la même plante). Les observations morphologiques sont confirmées au niveau moléculaire par le séquençage d'un fragment du gène mitochondrial codant pour la sous-unité I de la Cytochrome oxydase, utilisé comme code barre standard. En effet, les divergences inter-spécifiques sont nettement supérieures aux divergences intra-spécifiques et sur les reconstructions phylogénétiques, *Carpocoris mediterraneus* et *Carpocoris fuscispinus* apparaissent comme deux lignées génétiques réciproquement monophylétiques. Une clé d'identification morphologique est proposée pour l'ensemble des espèces européennes du genre *Carpocoris* afin d'en faciliter l'identification. *Carpocoris fuscispinus* est mentionné pour la première fois au Portugal.

**Mots clés:** Hémiptères, Hétéroptères, Europe, France, Espagne, Portugal, caractères morphologiques, biogéographie, code barre, ADN mitochondrial, Cytochrome oxydase, taxonomie, identification, *Carpocoris*.

## Introduction

Up until 1958, the genus *Carpocoris* Kolenati, 1846 included four species in Western Europe: *Carpocoris purpureipennis* (De Geer, 1773), *Carpocoris fuscispinus* (Boheman, 1851), *Carpocoris pudicus* (Poda, 1761) and *Carpocoris melanocerus* (Mulsant & Rey, 1852). A fifth species, *Carpocoris mediterraneus* Tamanini, 1958 was then described as closely related to *C. fuscispinus* (Tamanini, 1958). *Carpocoris mediterraneus* was further divided into two subspecies: *Carpocoris mediterraneus mediterraneus* and *Carpocoris mediterraneus atlanticus*. Tamanini (1958) divided the genus into two “groups”: a first one comprising *C. purpureipennis*, *C. mediterraneus* and *C. fuscispinus*, which have two teeth on the parameres, and a second one including *C. pudicus* and *C. melanocerus*, which have only one tooth on parameres (see figures 8 and 9). All the following authors accepted the validity of those taxa: Stichel (1961), Ribes (1965), Wagner (1966), Fuente (1974), Josifov (1981), Kis (1984), Derzhanski (1990), Vinokurov & Kanyukova (1995), Belousova (2004), Dusoulier & Lupoli (2006), and some identification keys were proposed.

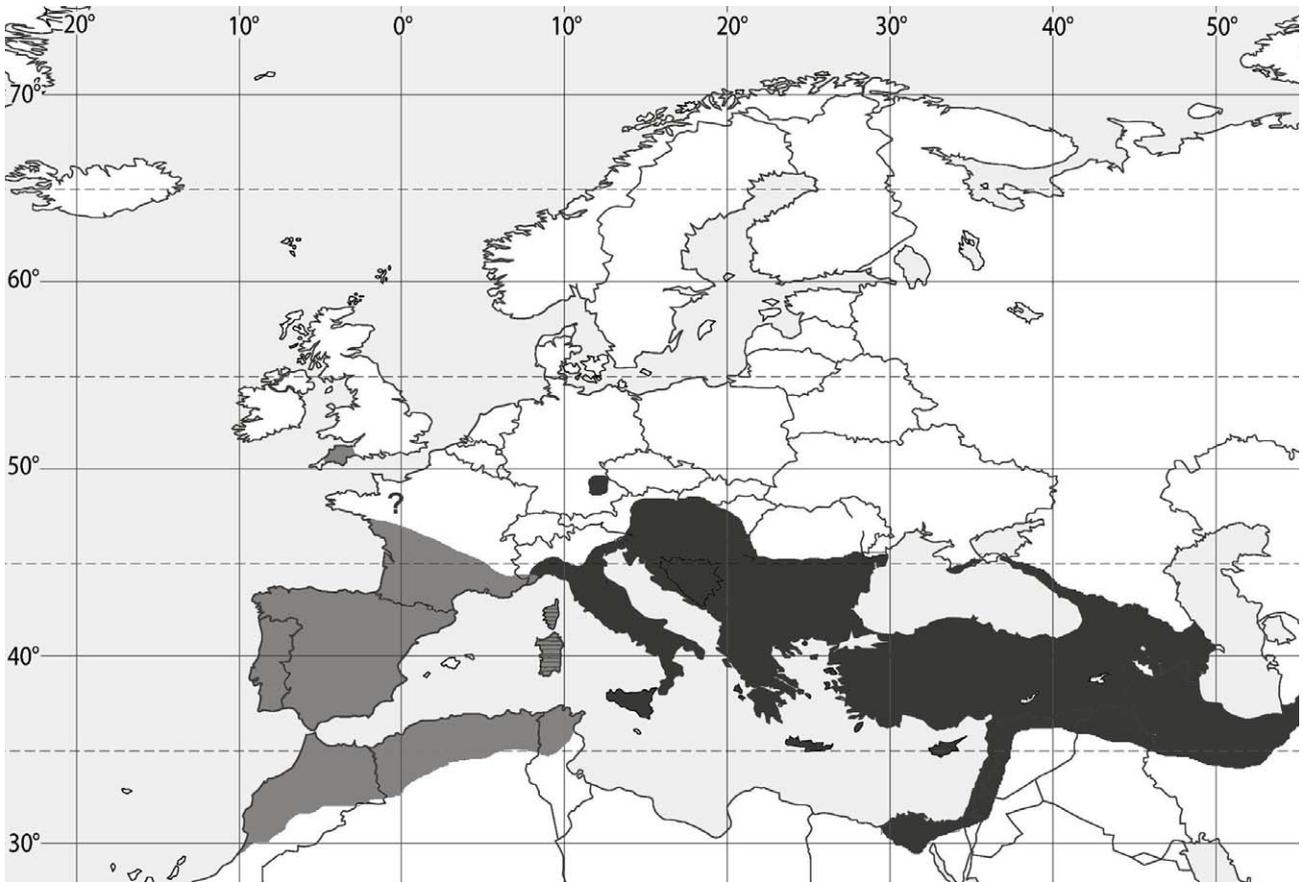
The original species description by Tamanini (1958) also included distribution maps of the European *Carpocoris* species. The distribution area of *C. fuscispinus* (figure 1) extends to the northeast of Europe above the 60° north latitude, and has a continental Eurosiberian distribution. This species is not encountered along the Mediterranean Sea, except in mountainous places where it does not occur further south than the 36° north latitude. *C. mediterraneus* (figure 2) does not extend to the north above the 50° north latitude and has a Mediterranean-Atlantic distribution.



**FIGURE 1.** European distribution of *Carpocoris fuscispinus* (Boheman, 1851) according to Tamanini (1959). The shaded part represents the area where the species was found. Question marks from Tamanini where the distribution was questionable.

This species is found on the edge of the Mediterranean Sea, and also more widely in inland Italy, the Balkans, Turkey and up to Egypt. The subspecies *C. mediterraneus mediterraneus* is found more to the east. To the west, on the Iberian Peninsula and in North Africa, the subspecies *C. mediterraneus atlanticus* spreads south to the 30° north latitude. The two species *C. fuscispinus* and *C. mediterraneus* therefore have partially overlapping

distributions representing highly differentiated chorotypes. Tamanini (1958) acknowledged some ambiguities on species distributions (indicated by question marks on his maps). Those ambiguities are as follows: one regarding the distribution limits of these two species in France, another for the exact distribution area of *C. fuscispinus* north of Russia and a last one for the distribution limits of *C. fuscispinus* in the Iberian Peninsula.



**FIGURE 2.** European distribution of *Carpocoris mediterraneus* Tamanini, 1958 according to Tamanini (1959). The lighter shaded part represents the area where *Carpocoris mediterraneus atlanticus* was found, and the darker represents *Carpocoris mediterraneus mediterraneus*. Tamanini observed in Corsica and Sardinia the presence of both subspecies (dark and light shaded areas). Question mark from Tamanini where the distribution was questionable.

*C. mediterraneus mediterraneus* can easily be distinguished from *C. fuscispinus* because *C. fuscispinus* has humeral angles of pronotum sharp and protruding, while *C. mediterraneus mediterraneus* has not (table 1, figures 8B & C). However, *C. mediterraneus atlanticus* has humeral angles of pronotum sharp and protruding similar to *C. fuscispinus* (table 1, figures 8A & C). This morphological similarity has led to some confusion between these two taxa.

Ribes *et al.* (2007) revised morphological characters for distinguishing *C. mediterraneus atlanticus* from *C. fuscispinus*. They re-analyzed previously published characters used to separate these species, and noticed several issues. They mentioned that all characters showed intermediate forms (hemelytra and pronotum punctation, spots on the pronotum and shape of the pronotum), were too variable (size of the head and antennal segments, spots on the scutellum and parasternites), or were poorly observed (paramere of male genitalia) and thus, did not clearly distinguish the two species. The authors also looked for other characters by observing the aedeagus of males in inflation or the spermathecal capsules of females, without finding any difference between the two species. They concluded that the characters used by Tamanini (1958) and the authors who followed him, were variable, poorly observed or identical and proposed to consider *C. mediterraneus* as a synonym of *C. fuscispinus*. Later, Ribes *et al.* (2008) and Ribes & Pagola-Carte (2009) proposed new morphological characters to diagnose the genus *Carpocoris*, and provided an identification key of the Euromediterranean species.

In the current study, we examined the species limits between *C. fuscispinus* and *C. mediterraneus*. To do so, we 1) observed morphological characters of specimens from collections and specimens collected in alcohol for

molecular analysis, 2) studied the geographical distribution of the two taxa, and 3) conducted molecular analyses (distances and phylogenetic analyses) on the mitochondrial Cytochrome c oxidase I standard barcode fragment to test whether specimens from *C. fuscispinus* and *C. mediterraneus* formed reciprocally monophyletic genetic lineages. Following our observations, a morphological identification key is proposed for all the European species of the genus *Carpocoris*, to facilitate identification

## Material and methods

**Specimens from collections.** Altogether, we observed in the collections 1 683 specimens of *C. mediterraneus atlanticus* corresponding to 816 occurrences (one occurrence = a sample event: one locality, one date) (1 490 from France (717 occurrences), 193 from Spain (83 occurrences), 27 from Portugal (16 occurrences)), and 324 specimens of *C. fuscispinus* (307 from France (182 occurrences), 16 from Spain (8 occurrences), 1 from Portugal (1 occurrence)). We also observed 136 specimens of *C. mediterraneus mediterraneus* (47 occurrences) from Greece and Cyprus. All specimens were identified following the identification key provided in this paper (table 1).

We examined the holotype of *Carpocoris fuscispinus*, photographed at SMNH (Swedish Museum of Natural History of Stockholm). We did not examine the types of *C. mediterraneus mediterraneus* and *C. mediterraneus atlanticus*.

Apart from the personal collections of some of the authors (Roland Lupoli, François Dusoulier and Jean-Claude Streito), the examined specimens come from the following private and public collections: Yvon Boissonnot (La Verrie, Vendée); Yoan Braud (Thèze, Alpes-de-Haute-Provence); Mickaël Buord (Quimper, Finistère); Jacques Cadou (Saint-Jean-de-Monts, Vendée); Georges Chauvin (Noyal-sur-Vilaine, Ille-et-Vilaine); Robert Constantin (Saint-Lô, Manche); Patrick Dauphin (Bordeaux, Gironde); Jean-François Elder (La Meauffe, Manche); Michel Fauchoux (Nantes, Loire-Atlantique); Claude Favet (Cabrières-d'Aigues, Vaucluse); Antoine Foucart (Montpellier, Hérault); Philippe Fouillet (Morlaix, Finistère); Philippe Magnien (Paris); Jean-Michel Maldès (Montpellier, Hérault); Armand Matocq (Paris); Jean-Philippe Maurel (Ramonville-Saint-Agne, Haute-Garonne); Philippe Meunier (Le Mans, Sarthe); Pierre Moulet (Avignon, Vaucluse); Claire Mouquet (Couvains, Manche); Jean Péricart (Saint-Lunaire, Ille-et-Vilaine, now in MNHN); Gérard Tiberghien (Rennes, Ille-et-Vilaine); Academy of Natural Sciences of Philadelphia (ANSP); INRA, Centre de biologie pour la gestion des populations Montpellier (CBGP formerly known as EAMF); Conservation des musées de Vendée (CMV); Centre de valorisation des collections scientifiques de Poitiers (CVCSP); Faculté Universitaire des Sciences Agronomiques de Gembloux, Belgique (FUSAG); Institut royal des sciences naturelles de Belgique (ISNB); Musée départemental des Hautes-Alpes (MDHA); Musée universitaire d'histoire naturelle de Rennes (MUHNR); Muséum d'histoire naturelle d'Aix-en-Provence (MHNA); Muséum d'histoire naturelle de Dijon (MHND); Muséum d'histoire naturelle d'Elbeuf (MHNE); Muséum d'histoire naturelle de Genève (MHNG); Muséum d'histoire naturelle de Marseille (MHNM); Muséum d'histoire naturelle de Nantes (MHNNA); Muséum d'histoire naturelle de Nice (MHNNI); Muséum d'histoire naturelle Requien d'Avignon (MHNRA); Muséum d'histoire naturelle de Toulon et du Var (MHNTV); Muséum national d'histoire naturelle de Paris (MNHN); Natural History Museum of London (BMNH); Siberian Zoological Museum, Novosibirsk (SZMN); Zoologische Staatssammlung, München (ZSMC).

It should be noted here that distributional information of *C. fuscispinus* published before Tamanini's work (1958), and not confirmed by the examination of specimens in collections, is useless.

**Specimens collected for molecular analysis.** A total of 69 *Carpocoris* specimens were collected alive in the field and fixed directly in 95% ethanol (table 2). Sampling of *C. mediterraneus atlanticus* specimens was performed from France and Morocco, and two specimens of *C. mediterraneus mediterraneus* were collected in Greece. Sampling of *C. fuscispinus* was only made in France, in relatively distant localities (Hautes-Alpes, Alpes-de-Haute-Provence, Aisne, Drôme). In one of these sites (Ventavon), *C. fuscispinus* was sympatric to *C. mediterraneus atlanticus*. All specimens were identified following the identification key provided in this paper (table 1).

**DNA extraction, amplification and sequencing.** Total genomic DNA was isolated from one leg of each specimen using Qiagen DNeasy 96 Blood & Tissue extraction kit following standard protocols. DNA was stored at -20°C. Voucher specimens were deposited at CBGP, Montferrier-sur-Lez, France.

**TABLE 1.** Key for the identification of European species of the genus *Carpocoris*.

1 (2)	- Abdomen as wide or broader than the pronotum. - Scutellum with a triangular depression in a semicircular Y-shape, bound laterally by a high ridge (red arrow in figure 8F). - Male: apical process of parameres with only one tooth (figure 9F). <i>Carpocoris melanocerus</i> (Mulsant & Rey, 1852) (figure 8F) Distribution in France: relatively common altitudinal species in the Alps and Pyrenees mountains from 1400m.	
2 (1)	- Abdomen narrower than the pronotum. - Scutellum without a marked depression, or with only a few superficial flat marks. - Male: apical process of parameres with one tooth or two (figures 9A-E) . . . . .	3
3 (4)	- Lateral margins of scutellum with a clear and deep notch in the middle (red arrow in figure 8E) - Male: apical process of parameres with only one tooth (figure 9E). <i>Carpocoris pudicus</i> (Poda, 1761) (figure 8E) Distribution in France: southern species common in southern and southeastern France up to Besançon.	
4 (3)	- Lateral margins of scutellum almost straight, without a clear and deep notch in the middle. - Male: apical process of parameres with two teeth (figure 9A-D) . . . . .	5
5(8)	- Humeral angles of pronotum rounded and only slightly protruding (figure 8B, D). . . . .	6
6 (7)	- Posterolateral margin of pronotum slightly convex. - Base of scutellum without contrasting black spots. - Legs not orange <i>Carpocoris purpureipennis</i> (De Geer, 1773) (figure 8D) Distribution in France: species common everywhere excepted in thermo- and meso-Mediterranean areas.	
7 (6)	- Posterolateral margin of pronotum nearly straight. - Base of scutellum with contrasting black spots. - Legs orange <i>Carpocoris mediterraneus mediterraneus</i> Tamanini, 1958 (figure 8B) Distribution in France: never found in France (East Palaearctic distribution, figure 2).	
8 (5)	- Humeral angles of pronotum sharp and protruding (figure 8A, C) . . . . .	9
9 (8)	- Anteroposterior distance between a line joining humeral angles of pronotum, and the anterior margin of pronotum, usually larger than a third of the length of pronotum (figure 3B). - Anterolateral margins of pronotum with a marked concavity (figure 3B). - Posterolateral margin of pronotum nearly straight (figure 3B). - Base of scutellum and connexivum often with contrasting black spots. - Legs sometimes orange. - Apex of the scutellum exceptionally truncated. <i>Carpocoris mediterraneus atlanticus</i> Tamanini, 1958 (figure 8A) Distribution in France: Thermophilic common species, only found in the Mediterranean area and along the Atlantic coast northwards until Normandy (figures 4 & 5).	
10 (9)	- Anteroposterior distance between a line joining humeral angles of pronotum, and the anterior margin of pronotum, usually smaller than a third of the length of pronotum (figure 3A). - Anterolateral margins of pronotum raised, with a slightly marked concavity (figure 3A). - Posterolateral margin of pronotum always distinctly convex (figure 3A). - Base of scutellum and connexivum exceptionally bearing contrasting black spots. - Legs never orange. - Altitudinal populations (Alps) often with the end of scutellum truncated and black. <i>Carpocoris fuscispinus</i> (Boheman, 1851) (figure 8C) Distribution in France: common in eastern and central France, except in thermo- and meso-Mediterranean areas. Becomes scarce from east to west and from north to south (figures 4 & 5).	

We amplified the mitochondrial Cytochrome c oxidase I standard barcode fragment using LCO1490Puc (5' TTTCAACWAATCATAAAGATATTGG 3') and HCO2198Puc (5' TAAACTTCWGGRTGWCCAAARAATCA 3') (Cruaud *et al.*, 2010). PCRs were performed as 25 µl volume reactions with a quantity of 2 µl of 25 ng/µl DNA template per reaction. Fragments were amplified in reactions containing 0.7 µM of each primer, 2.5 mM MgCl<sub>2</sub>, 0.05 mM dNTPs, and 0.025 U/µl Taq polymerase (Qiagen, Hilden, Germany). PCR conditions were: 94°C for 3 min followed by 37 cycles of 94°C for 30 s, 50°C for 60 s, 72°C for 90 s, with a final extension at 72°C for 10min.

PCR products were purified using Exonuclease I and Phosphatase, and sequenced directly using the BigDyeTerminator V3.1 kit (Applied Biosystems) and an ABI 3730XL sequencer at Genoscope, Evry, France. Both strands for each overlapping fragment were assembled using the sequence editing software Geneious v4.6.2 (Drummond *et al.*, 2010). All sequences were deposited in GenBank (table 2).

**TABLE 2.** Specimens used for molecular study. The collectors are identified by their initials (CC: Christian Cocquempot, FD: François Dusoulier, RL: Roland Lupoli, JCS: Jean-Claude Streito).

Voucher	Locality	Country	Date	Collector	GenBank accession Nos
<i>Carpocoris mediterraneus mediterraneus</i>					
CCOC_3288 to CCOC_3289	Thesprotia, Agios-Georgios	Greece	15.V.2005	CC	JN871537 to JN871538
<i>Carpocoris mediterraneus atlanticus</i>					
CCOC_3285	Hérault, St-Jean-de-Buèges	France	6.VIII.2005	JCS	JN871536
CCOC_3290 to CCOC_3292	Var, Montauroux	France	19.IX.2005	JCS	JN871539 to JN871541
CCOC_3295	Rabat	Morocco	6.VII.2007	JCS	JN871542
CCOC_3309	Hautes-Alpes, Ventavon	France	7.IX.2007	FD	JN871543
CCOC_3312	Hérault, Ste-Croix-de-Quintillargues	France	27.IX.2007	CC	JN871544
CCOC_3313	Hérault, Mireval	France	18.V.2005	JCS	JN871545
CCOC_10961 to CCOC_10963	Var, Aiguines	France	14.VI.2010	RL	JN871546 to JN871548
CCOC_10964	Var, Les Salins-d'Hyères	France	12.VI.2010	RL	JN871549
<i>Carpocoris fuscispinus</i>					
CCOC_3298	Hautes-Alpes, Champoléon	France	9.IX.2007	FD	JN871550
CCOC_3307 to CCOC_3308	Hautes-Alpes, Ventavon	France	7.IX.2007	FD	JN871551 to JN871552
CCOC_10925 to CCOC_10930	Hautes-Alpes, Mont-Dauphin	France	6.VII.2010	RL	JN871553 to JN871558
CCOC_10931	Alpes-de-Haute-Provence, Montagne de Lure	France	11.VI.2010	RL	JN871559
CCOC_10967 to CCOC_10968	Aisne, Mezy-Moulins	France	25.VIII.2010	RL	JN871560 to JN871561
CCOC_10969	Drôme, Eyroles	France	6.VII.2010	RL	JN871562
<i>Carpocoris melanocerus</i>					
CCOC_3303, 3304, 3306	Hautes-Alpes, Champoléon	France	9.IX.2007	FD	JN871563 to JN871565
<i>Carpocoris purpureipennis</i>					
CCOC_3272 to CCOC_3277, 3282, 3283	Pyrénées-Orientales, Prats-de-Mollo	France	18.VI.2005	CC	JN871510 to JN871517
CCOC_3296 to CCOC_3297	Paris	France	1.IX.2007	RL	JN871518 to JN871519
CCOC_3310 to CCOC_3311	Hautes-Alpes, Champoléon	France	9.IX.2007	FD	JN871520 to JN871521
CCOC_10889 to CCOC_10891	Moselle, Vernéville	France	17.VII.2010	RL	JN871522 to JN871524
CCOC_10920 to CCOC_10924	Aisne, Mezy-Moulins	France	25.VIII.2010	RL	JN871525 to JN871529
CCOC_10949 to CCOC_10950	Côte-d'Or, Messigny-et-Vantoux	France	15.VI.2010	RL	JN871530 to JN871531
CCOC_10951 to CCOC_10954	Essonne, Abbéville-la-Rivière	France	15.VII.2010	RL	JN871532 to JN871535
<i>Carpocoris pudicus</i>					
CCOC_3278 to CCOC_3281	Hérault, Vailhauques	France	11.VI.2005	JCS	JN871566 to JN871569

.....continued on the next page

TABLE 2. (Continued)

Voucher	Locality	Country	Date	Collector	GenBank accession Nos
CCOC_3284	Hérault, Saint-Jean-de-Buèges	France	06.VIII.2005	JCS	JN871570
CCOC_3287	Alpes-Maritimes, Cipières	France	26.VI.2005	JCS	JN871571
CCOC_3293 to CCOC_3294	Var, Montauroux	France	18.IX.2005	JCS	JN871572 to JN871573
CCOC_3299 to CCOC_3302	Hautes-Alpes, Ventavon	France	7.IX.2007	FD	JN871574 to JN871577
CCOC_10965	Drôme, Eyroles	France	6.VII.2010	RL	JN871578
<i>Codophila varia</i>					
CCOC_3314	Hautes-Alpes, Ventavon	France	7.IX.2007	FD	JN871579
CCOC_10937 to CCOC_10938	Var, Aiguines	France	14.VI.2010	RL	JN871580 to JN871581
<i>Anthemina lunulata</i>					
CCOC_10917 to CCOC_10918	Hautes-Alpes, Mont-Dauphin	France	6.VII.2010	RL	JN871582 to JN871583

**Sequence data analyses.** Sequences were aligned with ClustalW (1.81) (Thompson *et al.*, 1997) using default parameters. Alignment was then translated to amino acids using Mega 5.05 (Tamura *et al.*, 2011) to detect frameshift mutations and premature stop codons, which may indicate the presence of pseudogenes. Pairwise nucleotide sequence divergences were calculated using a Kimura 2-parameter model of substitution (Kimura, 1980) in MEGA 5.05.

Phylogenetic trees were estimated using maximum likelihood (ML) and Bayesian methods. All analyses were conducted on a 150 cores Linux Cluster at CBGP. The most appropriate model of evolution for the *COI* fragment was identified using the Akaike information criterion implemented in MrAIC.pl 1.4.3 (Nylander, 2004). We performed ML analyses using MPI-parallelized RAxML 7.2.8. (Stamatakis, 2006a). GTRCAT approximation of models was used for ML bootstrapping (Stamatakis, 2006b) (1000 replicates).

Bayesian analyses were conducted using a parallel version of MrBayes v. 3.1.2. (Huelsenbeck & Ronquist, 2001). Parameter values for the model were initiated with default uniform priors and branch lengths were estimated using default exponential priors. To improve mixing of the cold chain and avoid it converging on local optima, we used Metropolis-coupled Markov chain Monte Carlo (MCMC), with each run including a cold chain and three incrementally heated chains. The heating parameter was set to 0.02 in order to allow swap frequencies from 20% to 70% (Huelsenbeck & Ronquist, 2001). We ran two independent runs of 10 million generations. All values were sampled every 1000 generations. For the initial determination of burn-in, we examined the plot of overall model likelihood against generation number to find the point where the likelihood started to fluctuate around a constant value. The points sampled prior to convergence of the chains were then discarded. Convergence was also evaluated using Tracer v1.5 (Rambaut & Drummond, 2007). The results were based on the pooled samples from the stationary phases of the two independent runs.

Two specimens of *Anthemina lunulata* (Goeze, 1778) and three specimens of *Codophila varia* (Fabricius, 1787) were used as outgroups (table 2).

## Results

### Morphology

The examination of *C. fuscispinus* holotype showed that we had the same species interpretation as Tamanini (1958). Unfortunately we did not get the opportunity to examine the types of *C. mediterraneus mediterraneus* and *C. mediterraneus atlanticus*. Nevertheless, the interpretation of Tamanini (1958) was never discussed by former authors, nor it was by Ribes *et al.* (2007). Furthermore, descriptions and drawings by Tamanini (1958) allow us to be confident about the interpretation of both taxa. Our results are then fully congruent with those of Tamanini (1958) and we do not propose any modification of the taxonomy established by this author.

We found that the most important morphological character to distinguish *C. fuscispinus* from *C. mediterraneus atlanticus* was the general shape of the pronotum. Specifically, we propose to compare more precisely the anterolateral and posterolateral margins of the pronotum in those two taxa.

Apart from the overall shape of the pronotum, we agree with Ribes *et al.* (2007) that the other characters do not always individually allow to unambiguously distinguish *C. fuscispinus* and *C. mediterraneus atlanticus* at least based on the specimens we observed.

In order to compare the shape of the pronotum to distinguish these two taxa, we show in figure 3A the antero-dorsal view of *C. fuscispinus* and in figure 3B the antero-dorsal view of *C. mediterraneus atlanticus*.

*Anterolateral margins of the pronotum.* The anterolateral margins of the pronotum are more raised in *C. fuscispinus* and concavity is slightly marked (red arrow in figure 3). The humeral angles of the pronotum are wider than in *C. mediterraneus atlanticus* (figure 3A).

In *C. mediterraneus atlanticus*, anterolateral margins are less raised, and the concavity is then more pronounced (red arrow in figure 3), with a declivity of pronotum more pronounced near the head. The humeral angles of the pronotum are more slender than in *C. fuscispinus*.

By drawing a line (straight interrupted red line in figure 3) between the humeral angles of the pronotum, we note that the distance between the anterior margin of the pronotum and this line is shorter in *C. fuscispinus* (it is usually less than a third of the length of pronotum) than in *C. mediterraneus atlanticus*.

*Posterolateral margins of the pronotum.* In *C. fuscispinus*, the posterolateral margins of the pronotum are always distinctly convex. When drawing a line between the humeral angle of the pronotum and the anterolateral corner of the scutellum, the convexity of the pronotum largely and distinctly exceeds the line (shown by a green line in figure 3A). In *C. mediterraneus atlanticus*, the posterolateral margins of the pronotum seem almost straight. When drawing a line between the humeral angle of the pronotum and the anterolateral corner of the scutellum, the posterolateral margin does not exceed it or to a lesser extent than *C. fuscispinus* (shown by a green line in figure 3B).

Some secondary characters may be used to supplement those we have described above, though individually they do not completely discriminate between taxa. Hence, the amount of contrasting color on the pronotum and connexivum, and leg pigmentation might be useful in combination. If the contrasting spots on the scutellum and connexivum are not present in all *C. mediterraneus atlanticus*, they are almost always absent in *C. fuscispinus*. The presence of these contrasting spots on the scutellum and connexivum therefore allows effortless identification of *C. mediterraneus atlanticus*; in their absence, the shape of the pronotum will always be the main character to be taken into account. Similarly, we observed that the orange color of the legs or the body was a character to be considered in the same way, as *C. fuscispinus* almost never has orange legs or body. It is likely that these criteria are related to environmental conditions; but they are nevertheless typical of *C. mediterraneus atlanticus*.

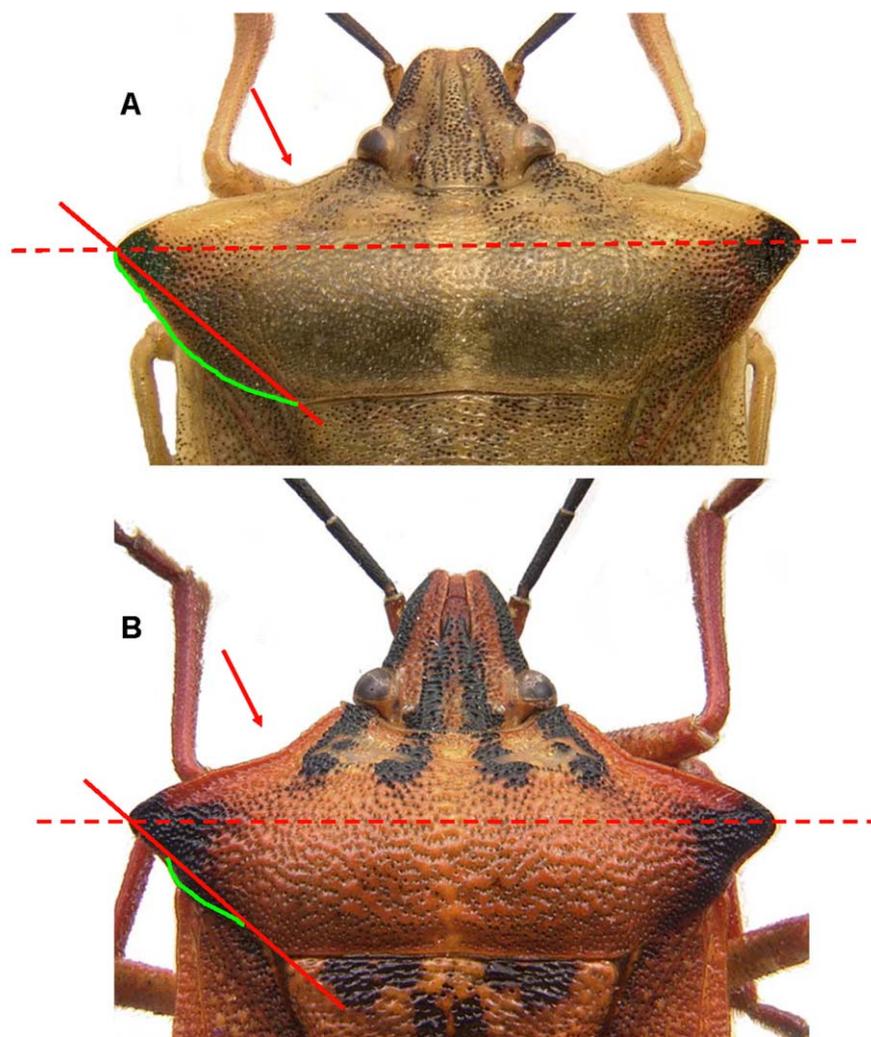
Following our morphological observations, we propose in table 1 an original key to the European species of the genus *Carpocoris*. This key allows identification of both sexes, identification of males can be completed by the Ribes & Pagola-Carte (2009) key based on male parameres.

We also reviewed the identification of the specimens illustrated in Ribes *et al.* (2007) using our morphological key. Ribes *et al.* (2007) observed a large number of *Carpocoris* specimens from several European countries. They mentioned collection localities for 38 specimens, and showed photographs of the left half of 24 of them. To illustrate the existence of intermediate forms in the pronotum, they show 12 photographs (their Fig. 3 a–l) of specimens harvested in the province of Navarre (figure 7). Following our morphological study, these 12 intermediate specimens can all be attributed to *C. mediterraneus atlanticus*. The observed variability in the shape of the pronotum is indeed intraspecific there, and does not represent intermediate forms. The 12 other specimens (Fig. 2 a–l) show seven specimens of *C. fuscispinus* (Fig. 2 a, b, c, d, e, j, k), four specimens of *C. mediterraneus atlanticus* (Fig. 2 f, g, h, i) and one specimen of *C. purpureipennis* (Fig. 2 l).

## Biogeography

The most accurate European distribution of *C. mediterraneus atlanticus* and *C. fuscispinus* was given in the work of Tamanini (1958) (figures 1 and 2). Later, Tamanini's data were included in the Catalogue of Palearctic Heteroptera by mentioning the countries where those two taxa were collected (Rider, 2006).

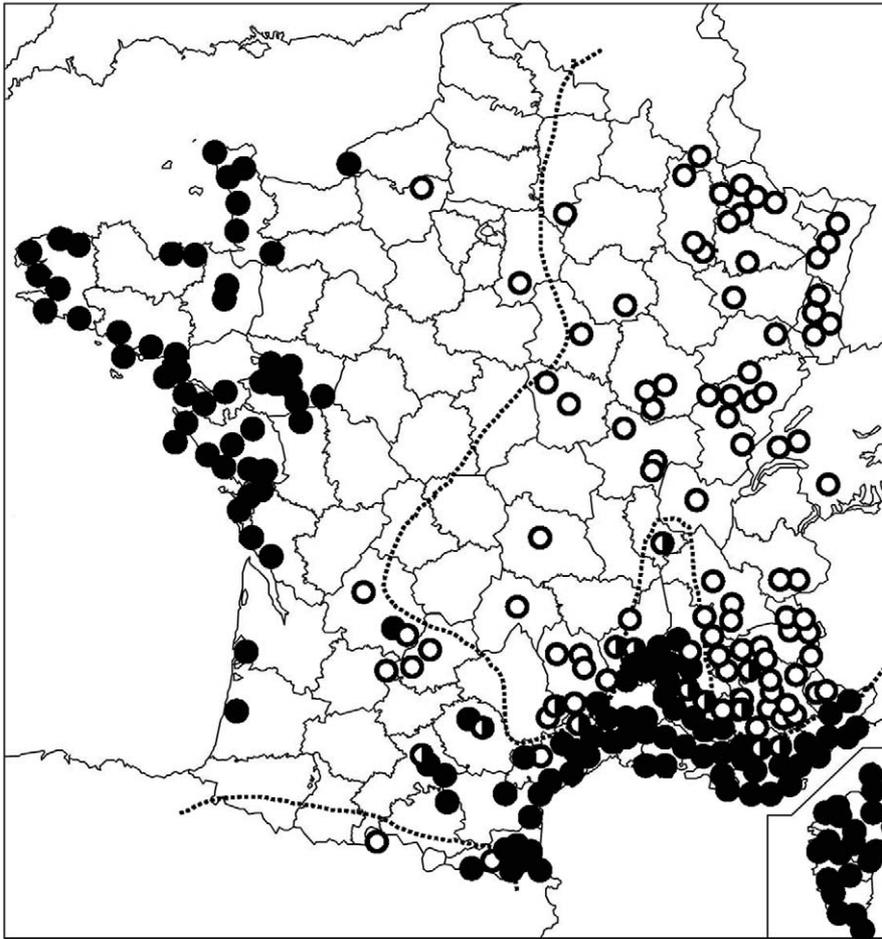
Distributions of *C. mediterraneus atlanticus* and *C. fuscispinus* in France. According to the morphological criteria mentioned above, we present in figure 4 the distribution in France of *C. mediterraneus atlanticus* and *C. fuscispinus* from the specimens we have seen in collections. From the 717 occurrences of *C. mediterraneus atlanticus* listed in France, 569 (79 % i.e. 1 297 specimens) are located in the Mediterranean area, 129 (18 % i.e. 174 specimens) in the Atlantic area, and 19 (3 % i.e. 19 specimens) outside. *C. fuscispinus* is rarely found in the French Mediterranean area, despite a greater exploration pressure in this region. From the 182 occurrences (307 specimens) of *C. fuscispinus* listed in France, 19 (10 %, 36 specimens) of these are located in the Mediterranean area, including 10 in Aiguines, Var (26 specimens). To show this more precisely, we expanded the French Mediterranean area (excluding Corsica) in figure 5. The shaded area is not considered part of the Mediterranean area (i.e., thermo-, sub- and meso-Mediterranean only) (Ozenda, 1994). *C. fuscispinus* was found once in Hérault in the commune of Mireval in the Gardiole hills. This is the only locality in the sub-Mediterranean area where the species has been found. Hence, the probability of encountering *C. fuscispinus* in the French Mediterranean area is very low.



**FIGURE 3.** Antero-dorsal views of A) *C. fuscispinus*. B) *C. mediterraneus atlanticus*. Red arrows indicates a more pronounced concavity of the anterolateral margin of the pronotum in *C. mediterraneus atlanticus*. The posterolateral margin is more convex in *C. fuscispinus*. Hence, the convexity (shown by green lines) further exceeds in *C. fuscispinus* the red line drawn between the humeral angle of the pronotum and the humeral angle of the scutellum. The distance between the anterior margin of the pronotum and the dotted red line, drawn between the humeral angles, is shorter in *C. fuscispinus* (usually less than a third of the length of pronotum).

*C. fuscispinus* and *C. mediterraneus atlanticus* are sometimes found in France in the same locality. We observed that the localities where we encountered the two species together are found in supra-Mediterranean areas at altitudes of about 700 to 900m: north of Ardèche, south of Hautes-Alpes, Mont Ventoux in Vaucluse, Causse de Blandas in Gard, and Aiguines (Causse de Canjuers) in Var. In the latter locality, the two species were found multiple times on the same day, in the same place and sometimes on the same host plant (6.viii.1990, 14.v. & 9.viii.1994, 7.v. & 21.vi.1998).

*C. fuscispinus* was collected in France mainly in areas that are subjected to approximately two months or more of frost per year (on the french coasts, the number of frost days per year varies from 2 to 30). We have drawn a dotted line in figure 4 that includes the regions exposed to more than 60 days of frost per year (period 1951–1980) in central and eastern France and in the Pyrenees (Kessler & Chambraud, 1986).



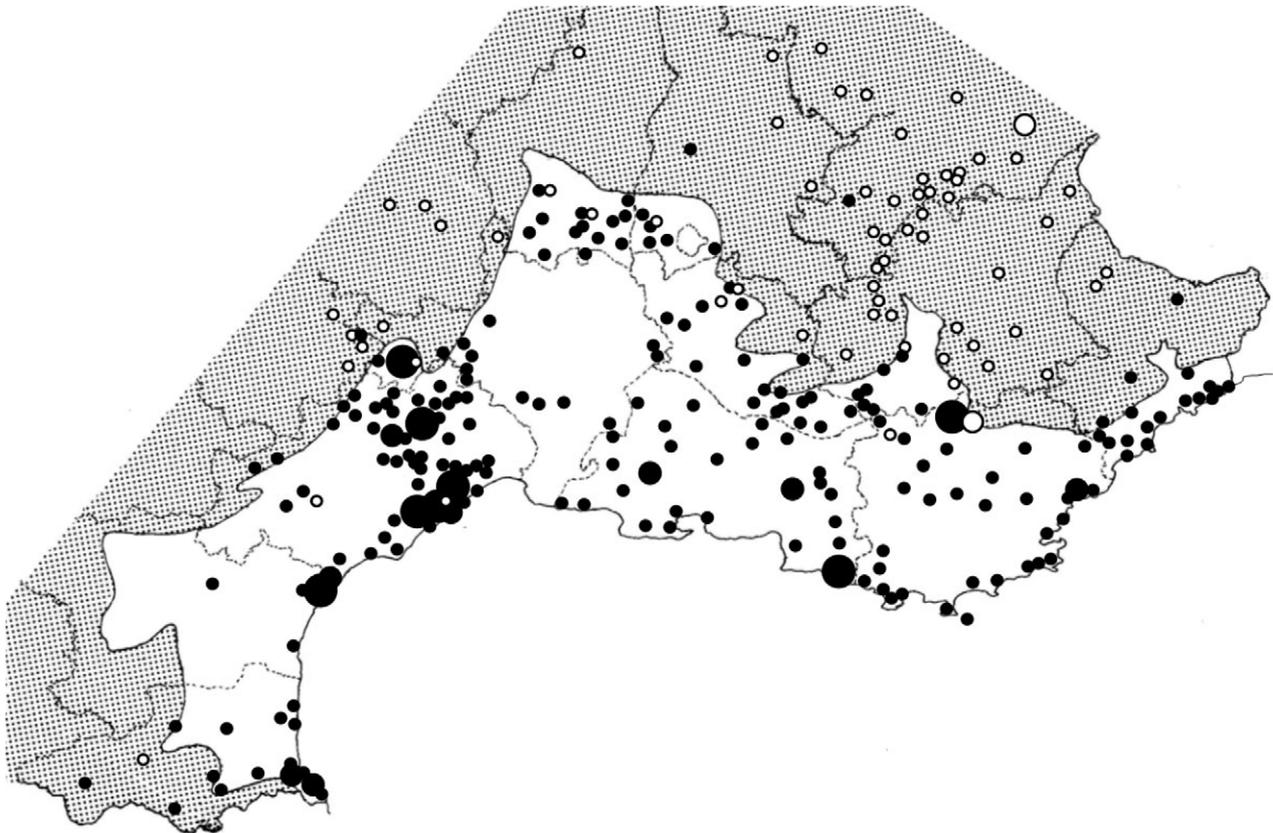
**FIGURE 4.** Localities in France where *C. fuscispinus* (white spots) and *C. mediterraneus atlanticus* (black spots) were observed. The localities where the two species were observed together are represented by two-tone black and white spots. The dotted lines encompass the areas that encounter more than 60 days of frost per year, in Northeast France and the Pyrenees.

In the Dordogne and Quercy, *C. fuscispinus* seems to live in areas where the length of the freezing period is shorter, as well as in the supra-Mediterranean area (the latter is defined by a degraded Mediterranean climate with a very strong continental influence). Local cold spots could explain these incursions outside the favored biogeographical realm of this species. In the French Pyrenees, we have identified only one occurrence of *C. fuscispinus* in Fuilla (Pyrénées-Orientales).

The distribution of *C. mediterraneus atlanticus* in France is not only a function of latitude. It follows a typical Mediterranean-Atlantic distribution pattern, as shown in other species including the Pentatomidae *Ancyrosoma leucogrammes* (Gmelin, 1790), and is probably also related to the number of hours of annual sunshine (Lupoli, 1993). *Carpocoris mediterraneus atlanticus* is not found in northeastern France, but is found in the northwest to Brittany and Cotentin (western Normandy).

Distributions of *C. mediterraneus atlanticus* and *C. fuscispinus* in Iberian Peninsula.—*C. mediterraneus atlanticus* is a very common and abundant species throughout all the Iberian Peninsula. Specimens of *C. fuscispinus* we have noted in the Iberian Peninsula only from cold spots near to mountain areas (table 3). As cold spots are rarer in Spain and Portugal, *C. fuscispinus* was logically much less collected than *C. mediterraneus atlanticus* which is one of the most abundant stink bugs in the Iberian Peninsula.

It is interesting to note that we have collected recently and several times, *C. fuscispinus* in the Montes Universales from Albarracín and Tramacastilla in Aragón. In Albarracín, we also collected *C. mediterraneus atlanticus* in the same place, on the same day.



**FIGURE 5.** Localities in Mediterranean mainland France where *C. fuscispinus* (white spots) and *C. mediterraneus atlanticus* (black spots) were observed. A solid line delineates the Mediterranean area (including thermo-, meso- and supra-Mediterranean areas) according to Ozenda (1994). The shaded area is not considered to have a Mediterranean climate. The small spots represent 1 to 4 occurrences per station, the medium spots from 5 to 9 and the large spots more than 10 occurrences.

In the Iberian Peninsula, Tamanini (1958) considered the presence of *C. fuscispinus* as questionable. Indeed, the species is quite rare there. All the 20 recorded specimens from collections corresponding to 11 occurrences are shown in table 3 and in figure 7. In the map of the Iberian Peninsula, localities where *C. fuscispinus* was effectively collected are shown as white dots. The black dots correspond to the specimens photographed by Ribes *et al.* (2007) that we identify as *C. mediterraneus atlanticus* (their Fig. 2f, g, h, i, and Fig 3a, b, c, d, e, f, g, h, i, j, k, l). The three specimens of *C. fuscispinus* photographed by Ribes *et al.* (2007) in Spain come from 2 localities—Huesca and Begos—from the Spanish Pyrenees (their Fig 2e, j, k). A narrow dotted line indicates the distribution of *C. fuscispinus* assumed by Tamanini on the Iberian Peninsula.

Tamanini (1958) predicted the presence of *C. fuscispinus* in Portugal without mentioning any locality. To our knowledge, our observation of *C. fuscispinus* is then the first mention of this species in Portugal.

**TABLE 3.** Global list of the *Carpocoris fuscispinus* specimens observed in the Iberian Peninsula.

Department	City	Locality	Date	Collector	Collection	Identification
Spain: 10 occurrences of <i>Carpocoris fuscispinus</i> (19 specimens)						
Cataluña	Begos	Val d'Aran	19.IX.1922 (2 specimens)	F. Léon-Hilaire	MZBS	L. Tamanini Fig. 2j & 2k in Ribes <i>et al.</i> (2007)
Léon	Astorga	Peredo	31.V.1970	A. Comellini	MHNG	F. Dusoulrier
Aragón	Huesca	San Juan de la Peña	28.VII.1964	J. Ribes	J. Ribes	J. Ribes Fig. 2e in Ribes <i>et al.</i> (2007)
Aragón	Huesca	San Juan de la Peña	03.VIII.1974 (2 specimens)		FUSAG	Linnavuori
Aragón	Albarracín	Gea de Albarracin	11.VI.2003	R. Lupoli	R. Lupoli	R. Lupoli
Aragón	Albarracín	road toward Moscardon	01.VIII.2004 (8 specimens)	R. Lupoli	R. Lupoli	R. Lupoli
Aragón	Tramacastilla		08.VI.2006	F. Dusoulrier, R. Lupoli & J.-C. Streito	R. Lupoli	F. Dusoulrier, R. Lupoli & J.-C. Streito
Castilla y León	Espinama	Picos de Europa	01.IX.1935	K.G. Blair	BMNH	F. Dusoulrier
Castilla y León	Humada	10 km N., 1000 m	07.IX.1993	J.H. Martin	BMNH	F. Dusoulrier
Andalucía	Lanjarón		18.V.1961	G. Fagel	ISNB	R. Lupoli
Portugal: One occurrence of <i>Carpocoris fuscispinus</i> (1 specimen)						
Serra da Estrela	Guarda			Wattison	BMNH	F. Dusoulrier

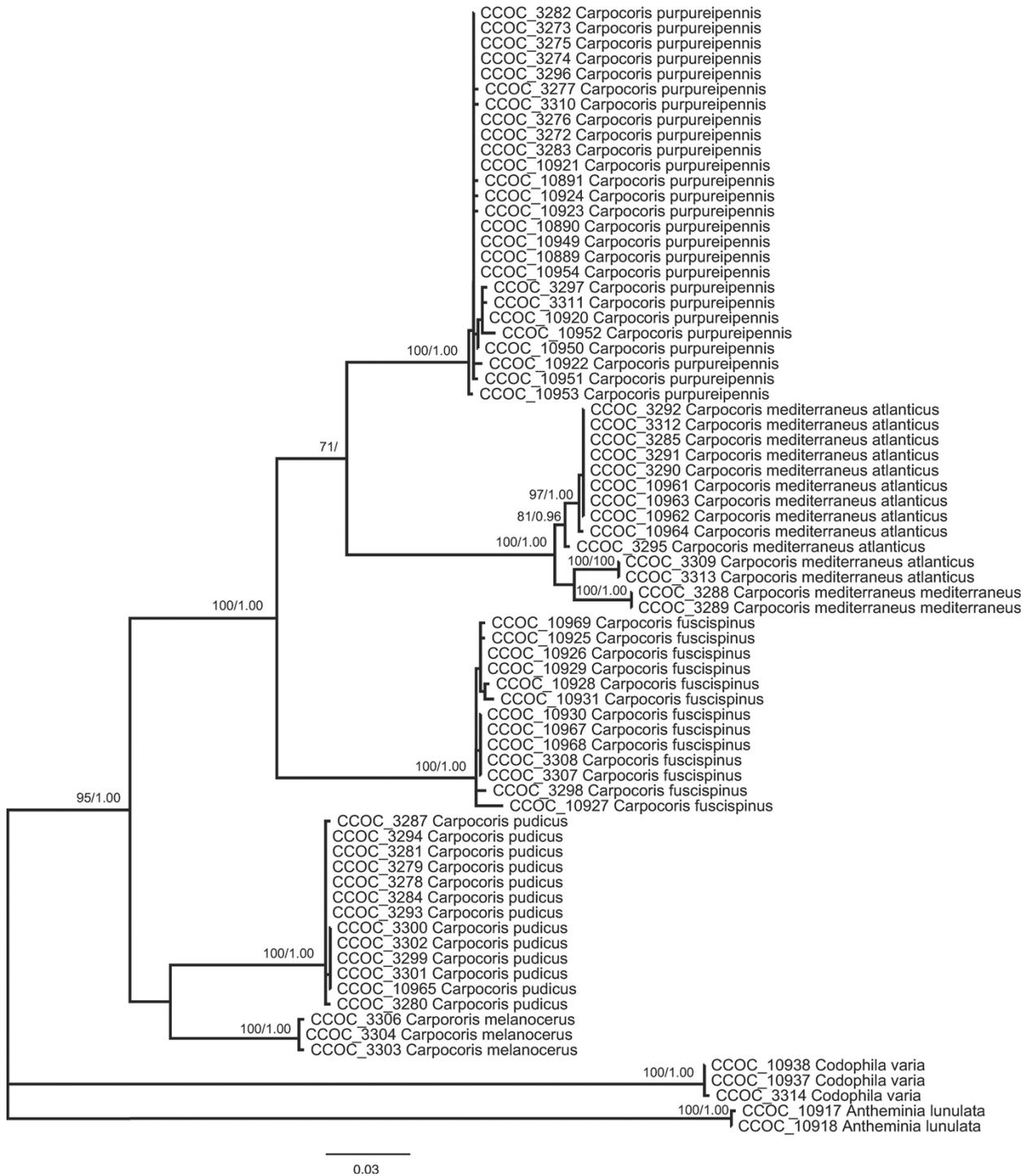
## Molecular analyses

*COI* (660 bp) was successfully amplified from all specimens. Alignment was straightforward due to a lack of length variation and no stop codons or frame shifts were detected.

Intraspecific K2P distances varied from  $0.3 \pm 0.09\%$  for *C. purpureipennis* to  $1.61 \pm 0.33\%$  for *C. mediterraneus*, with  $0.54 \pm 0.16\%$  for *C. fuscispinus*. Interspecific divergence varied from  $8.1 \pm 1.3\%$  between *C. melanocerus* and *C. pudicus* to  $14.2 \pm 1.7\%$  between *C. mediterraneus* and *C. fuscispinus*. Intergeneric divergences (*Carpocoris* / *Codophila* / *Anthemina*) exceeded 16%. The genetic distances between our specimens of *C. mediterraneus* versus *C. fuscispinus* varied from 13.6% to 14.9%. For all species, inter-specific divergence largely exceeded maximum intra-specific divergence.

Model chosen by MrAIC was GTR + I +  $\Gamma$ . Given that  $\alpha$  and the proportion of invariable sites can not be optimized independently from each other (Gu, 1995) and following Stamatakis' personal recommendations (RAxML manual, 2006a), we used GTR +  $\Gamma$ .

ML and Bayesian analyses recovered the same well-supported clusters of sequences (BP=100, PP=1.00), which corresponded to morphologically delineated species (figure 6). Especially, specimens from *C. fuscispinus* and *C. mediterraneus* formed reciprocally monophyletic genetic lineages. Specimens from *C. mediterraneus mediterraneus* and *C. mediterraneus atlanticus* grouped together but did not form clearly differentiated clusters, which suggest that further studies are needed to clarify the statut of these subspecies. While, species having paramers with two teeth (*C. fuscispinus*, *C. mediterraneus* and *C. purpureipennis*) formed a strongly supported clade (BP=100, PP=1.00), the clade grouping species exhibiting paramers with one tooth (*C. melanocerus* and *C. pudicus*) was only weakly supported.



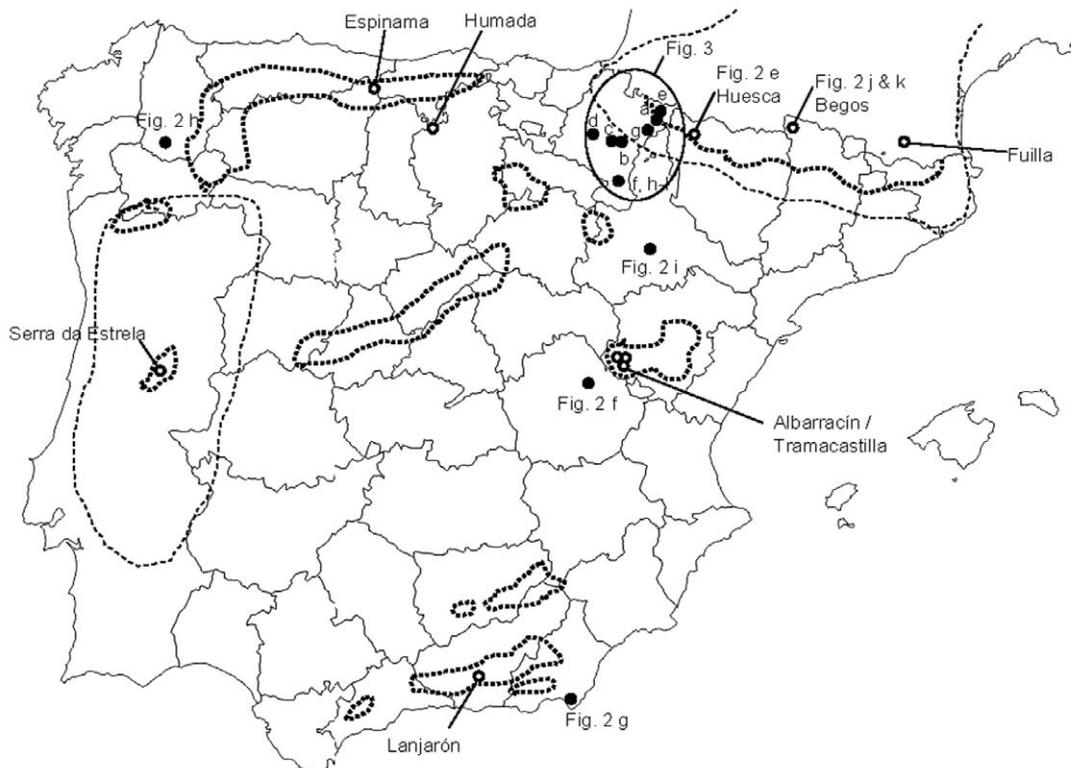
**FIGURE 6.** Phylogram of relationships among the European *Carpocoris* species (ML tree). Maximum likelihood and Bayesian reconstructions produced similar topologies. Node support (Bootstrap percent > 70 / Posterior probabilities > 0.95) are indicated at nodes.

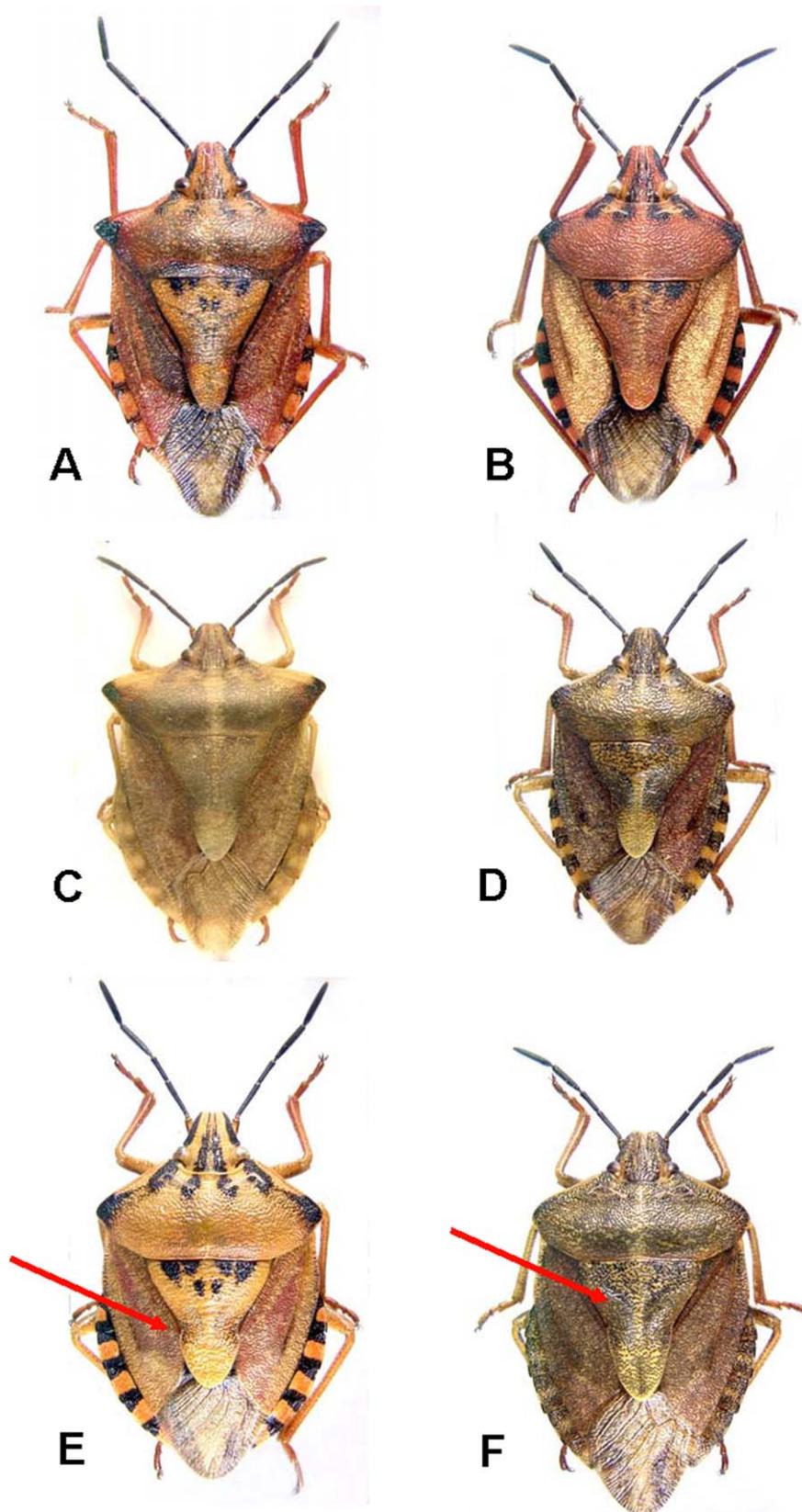
**TABLE 4.** COI mtDNA intraspecific divergence based on pair-wise comparison for European *Carpocoris* species. *Codophila varia* and *Anthemina lunulata* as outgroups.

	Number of specimens	Divergence %	Standard error
<i>Carpocoris purpureipennis</i>	26	0.30	0.09
<i>Carpocoris mediterraneus</i>	14	1.61	0.33
<i>Carpocoris fuscispinus</i>	13	0.54	0.16
<i>Carpocoris melanocerus</i>	3	0.12	0.12
<i>Carpocoris pudicus</i>	13	0.12	0.10
<i>Codophila varia</i>	3	0.12	0.12
<i>Anthemina lunulata</i>	2	0.18	0.17

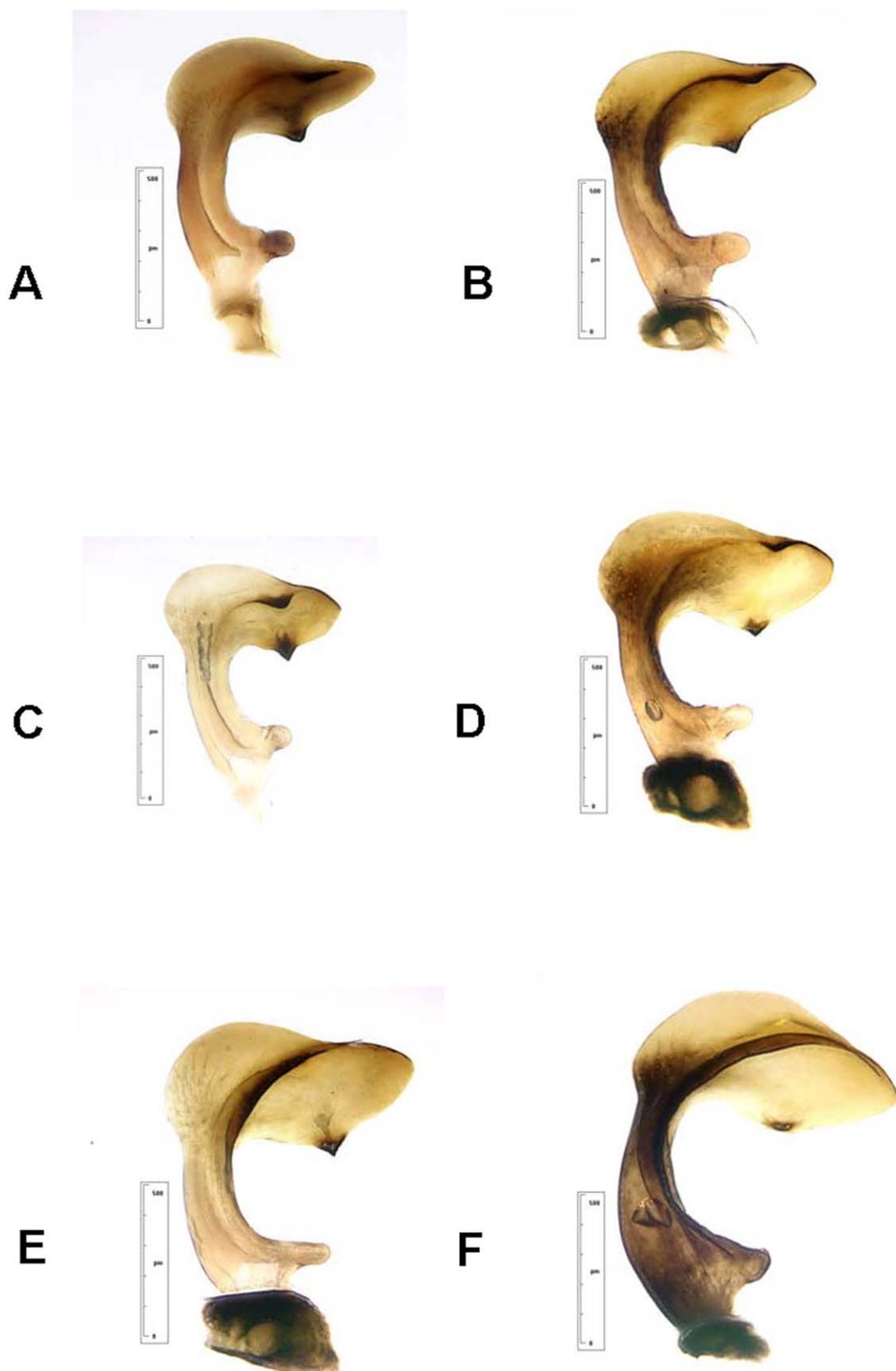
**TABLE 5.** COI mtDNA divergence based on pair-wise comparison among European *Carpocoris* species. *Codophila varia* and *Anthemina lunulata* as outgroups. Below the diagonal divergence (%); above the diagonal standard error (%).

	C. pur	C. med	C. fus	C. mel	C. pud	Co. var	An. lun
<i>Carpocoris purpureipennis</i>		1.3	1.5	1.5	1.7	1.9	2.1
<i>Carpocoris mediterraneus</i>	9.9		1.7	1.5	1.7	1.9	1.9
<i>Carpocoris fuscispinus</i>	10.9	14.2		1.5	1.7	2.2	2.1
<i>Carpocoris melanocerus</i>	12.8	12.9	11.2		1.3	1.9	1.9
<i>Carpocoris pudicus</i>	13.8	13.5	12.9	8.1		2.0	2.0
<i>Codophila varia</i>	17.3	17.6	19.9	16.2	17.9		2.1
<i>Anthemina lunulata</i>	20.6	16.9	19.7	16.7	18.3	19.6	

**FIGURE 7.** Localities on the Iberian Peninsula where *C. fuscispinus* was collected according to our criteria (white spots). The black spots correspond to the original localities of the specimens photographed by Ribes *et al.* (2007) that we identify as *C. mediterraneus atlanticus*. We indicate on this map the figure numbers (their Fig 2e, f, g, h, i, j, k and their Fig. 3a, b, c, d, e, f, g, h, i, j, k, l) corresponding to the photographs shown in Ribes *et al.* (2007). Narrow dotted lines show the distribution assumed by Tamanini (1959) of *C. fuscispinus* on the Iberian Peninsula (see also figure 1). Thick dotted lines surround the areas of high mountain climate.



**FIGURE 8.** Habitus of the six European species and subspecies of the genus *Carpodoris*: A. *Carpodoris mediterraneus atlanticus* Tamanini, 1958 (from Madrid, Spain); B. *Carpodoris mediterraneus mediterraneus* Tamanini, 1958 (from Nicosia, Cyprus); C. *Carpodoris fuscispinus* (Boheman, 1851) (from Avançon, Hautes-Alpes, France); D. *Carpodoris purpureipennis* (De Geer, 1773) (from Malzéville, Meurthe-et-Moselle, France); E. *Carpodoris pudicus* (Poda, 1761) (from Cheval-Blanc, Vaucluse, France); F. *Carpodoris melanocerus* (Mulsant & Rey, 1852) (from Ristolas, Hautes-Alpes, France). Red arrows show characters described in table 1.



**FIGURE 9.** Right parameres of the six European species and subspecies of the genus *Carporcoris*: A. *Carporcoris mediterraneus atlanticus* Tamanini, 1958; B. *Carporcoris mediterraneus mediterraneus* Tamanini, 1958; C. *Carporcoris fuscispinus* (Boheman, 1851); D. *Carporcoris purpureipennis* (De Geer, 1773); E. *Carporcoris pudicus* (Poda, 1761); F. *Carporcoris melanocerus* (Mulsant & Rey, 1852). Photographs obtained with an inverted microscope Leica DMIL magnification x100, a digital camera JVC 3CCD KY-F75U, and Perfect image software.

## Discussion

Morphological study on a large number of specimens shows that the shape of the pronotum, associated with other criteria, allows distinction between *C. fuscispinus* and *C. mediterraneus*. Morphological evidences are confirmed by our molecular analyses. Indeed, inter-specific divergence largely exceeded intra-specific divergence and all species were recovered as reciprocally monophyletic by our phylogenetic analyses. To us, *C. mediterraneus* and *C. fuscispinus* should then be considered as two valid species.

Ribes *et al.* (2007) and our studies show that the distribution of *C. fuscispinus* observed in France and Spain is typically continental. The species is found in cold areas in Northeastern Europe, as well as in the south. This species is not found either on the Atlantic coast or across the Northwestern part of France. *C. fuscispinus* is found on plains in Northeastern France, but also further south on mountainous regions. Cold spots are rarer in Iberian Peninsula, and as such *C. fuscispinus* is only found in mountain areas, probably as relict populations.

In figure 7, thick dotted lines surround the areas of high mountain climate on the Iberian Peninsula. Populations of *C. fuscispinus* are well established in this region, and it is likely that it can be found in other mountain areas in Spain. It would be interesting to check collections that include specimens from these regions, and/or to conduct a field study to look specifically for *Carpocoris* species in mountain areas to make a more precise mapping.

*C. mediterraneus atlanticus* and *C. fuscispinus* share some of their range in Western Europe with multiple contact zones. These areas correspond to the overlapping range of these two distinct species. We observed several times in France and Spain, areas where the two species were found in sympatry, sometimes even on the same day and on the same plant. Sympatric areas where the two taxa coexist, when local needs can be met, also reinforce the hypothesis of two distinct species rather than forms or subspecies. Also, the rare occurrence of *C. fuscispinus* in Spain and its relationship to cold mountain areas, compared to the abundance of *C. mediterraneus atlanticus* throughout the Iberian Peninsula, is a sign of a separation of these two species by the end of a previous ice age (e.g. Ozenda, 1994; Blondel & Aronson, 1999).

Several points remain to investigate for a better understanding of the genus *Carpocoris*. First it may be interesting to collect more specimens in the eastern part of Europe, especially from southern countries to confirm the validity of the two *C. mediterraneus* subspecies. Then, sequencing several nuclear genes could allow studying more precisely phylogenetic relationships between *Carpocoris* species. Especially those studies could confirm the existence of two morphological groups of species *fuscispinus* / *mediterraneus* / *purpureipennis* (paramers with two teeth) and *melanocerus* / *pudicus* (paramers with one tooth). Furthermore, breeding experiments should be conducted to explore reproductive barriers between the taxa. Beyond taxonomy, phylogeographic studies of *C. fuscispinus* would be particularly interesting, to estimate how long relict altitudinal populations have been isolated in Iberian Peninsula compared to the last glaciations. This isolation, studied on vegetal models, also explains the important levels of endemism observed in the Spanish mountains (Mota *et al.*, 2002).

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We would like to dedicate this paper to the memory of Jean Péricart, who encouraged and progressed the work on Western Palearctic Heteroptera systematics for more than half a century, particularly through its publications of 10 books in the "Faune de France" series.

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