Original article

Ecological niche overlap in sister species: how do oil-collecting bees *Macropis europaea* and *Macropis fulvipes* (Hymenoptera: Melittidae) avoid hybridization and competition?

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Abstract – Oil-collecting bees are found worldwide and always in association with particular oil-producing flowers. In the Western Palearctic, three oil-collecting bee species within the genus *Macropis* (Hymenoptera, Melittidae) interact in a tight pollination mutualism with species of the only European oil-producing plant genus *Lysimachia* L. (Myrsinaceae). Two of these oil-collecting bees (*Macropis europaea* and *Macropis fulvipes*) show overlapping geographic distributions, comparable morphologies, and similar ecological characteristics (e.g., habitat type, floral preferences). In view of these similarities, we presume that hybridization should occur between the two species unless potential variation among the species' ecological niches prevents it, simultaneously decreasing competition for resources. Using modern genetic analyses and ecological niche modeling on a large bee sampling throughout Europe, we discuss new perspectives on the ecology and evolutionary history of this mutualism.

Macropis / mutualism / *Lysimachia* / ecological niche / hybridization

1. INTRODUCTION

Mutualisms between insects and plants are widely recognized as indispensable components in ecosystems functioning (Bronstein et al. 2006). Among the most fascinating plant-insect mutualisms, many nursery pollination systems have been documented, notably in the fig/fig wasps and the yucca/yucca moths interactions (e.g.,

Pellmyr 2003; Cook and Rasplus 2003; Machado et al. 2005). In contrast, specific pollination systems involving oil-producing plants and oil-collecting bees have been much less studied (Vogel 1988). In such systems, oil-offering flowers, found worldwide in 11 different plant families (Renner and Schaefer 2010), are pollinated by specifically adapted oil-collecting bees distributed in a few genera within Melittidae and Apidae families. Such bees use oil combined with pollen as larval food, for water-resistant lining of larval cells, or both (Renner and Schaefer 2010). Oil-collecting bees have developed special branched hairs on the legs or abdomen to facilitate oil transportation (Rasmussen and Olesen 2000), an adaptation observed on the 53-Mya-old fossil species *Palaeomacropis*

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eocenicus (Michez et al. 2007), which attests to the ancient nature of coevolutionary processes within this interaction.

In temperate habitats, only a few species have developed such adaptations. Among them are species within the genus *Macropis* Panzer 1809 (Hymenoptera, Melittidae), one of the 14 genera included in Melittidae (Michener 2000). The 16 species of the genus are subdivided into three subgenera (*Macropis* s. str., *Paramacropis* Popov and Guiglia 1936, and *Sinomacropis* Michener 1981), spanning the Holarctic (Michez and Patiny 2005). All *Macropis* taxa visit, collect oil, and pollinate the yellow flowers of *Lysimachia* species (Myrsinaceae; Vogel 1976). Evidence of floral oil was found in at least 75 among the 191 species of *Lysimachia* (Vogel 1986, 1988; Hao et al. 2004). Here, the oil is produced by elaiophores (oil-secreting trichomes) located at the basal part of petals and anther filaments (Simpson et al. 1983). These plants are native either of temperate Eurasia (subgenus *Lysimachia* s. str.) or northern America (subgenus *Seleucia*; Hao et al. 2004). As in the great majority of mutualistic interactions, strict one-to-one relationships between oil-collecting bees and *Lysimachia* species do not represent the general rule; hence, each plant species is generally visited by more than one species of oil-collecting bees, depending on localization and period of flowering (Cane et al. 1983; Simpson et al. 1983; Pekkarinen et al. 2003; Celary 2004).

Three species of *Macropis* are found in the western Palearctic ecozone: *Macropis europaea* Warncke 1973, *Macropis frivaldszkyi* Mocsary 1878, and *Macropis fulvipes* Fabricius 1805 (Michez and Patiny 2005). All three species can be found together in sympatric populations (Celary 2004; Pekkarinen et al. 2003; Bassin and Triponez, personal observation). Among them, *M. frivaldszkyi* (for which very little documentation is currently available relative to its ecological characteristics) shows the narrowest distribution (Balkans, Anatolia, Syria). In contrast, *M. europaea* and *M. fulvipes* are widely distributed and hold rather similar ranges across Europe, although the former species is more restricted eastwards (in Russia) and even

absent from Anatolia or Caucasus, and the latter does not reach the Scandinavian Peninsula (i.e., Sweden and Norway) and the British Islands (Michez and Patiny 2005). These two species display a high level of similarity in their morphology, behavior, habitat, and floral preferences. For instance, a comparative study established in southern Poland (Celary 2004) showed that all features concerning the nest's architecture and the larval stages differ almost only in size (always slightly bigger for *M. fulvipes*). The most obvious difference between the two species seems to stand in their respective phenologies, with a flight period delayed of about 10 days to 3 weeks (independently observed in different regions of Europe; Westrich 1990; Michez 2002; Pekkarinen et al. 2003; Celary 2004). Such an offset in flight periods might be meaningful for resource partitioning in sympatric *Macropis* populations, especially in places where only one *Lysimachia* species is available (Celary 2004). The floral preferences of bees (reviewed by Michez and Patiny 2005) reveal that *Lysimachia vulgaris* seems to be the main resource of oil for both *M. europaea* and *M. fulvipes*. It might even be the only one for the strictly monolectic *M. europaea*. *M. fulvipes* was additionally observed on *Lysimachia nummularia* (sometimes presumed as its preferred plant species [Westrich 1990; Michez 2002; Pekkarinen et al. 2003]) and on *Lysimachia punctata* (especially in its native area in Anatolia and Caucasus; Triponez, personal observation).

In the current study, our aim was to address whether or not *M. europaea* and *M. fulvipes* are able to efficiently avoid excessive competition and counter-selected hybridization, despite their similar ecologies and frequent syntopy on *L. vulgaris*. Based on a large-scale sampling all over Europe, we compared the bees' ecological niches and analyzed the sampled specimens using both gene sequencing and Amplified Fragment Length Polymorphism (AFLP) genotyping in order to answer the following questions. (1) How distantly related are these two species from a genetic point of view? (2) Can hybrids between *M. europaea* and *M. fulvipes*

be detected, and how frequently? (3) On which ecological properties do the ecological niches of the two species differ? (4) How do these potential ecological differences affect their co-existence and allow them to avoid competition for resources and hybridization? So far, no study has provided objective data to answer these questions.

2. MATERIAL AND METHODS

2.1. Field work

As a first step to identify potential populations of *Macropis*, we used international and national freely accessible databases to locate sites of *L. vulgaris*, *L. nummularia*, and *L. punctata* (in its native area) across Europe. The sampling and the observations of *Macropis* in Europe were performed mainly during summers 2006 to 2008, each year between June and August depending on the region. Because of the tight interaction between the plant and the bee, as soon as a *Lysimachia* population was found, *Macropis* females could be catch easily while pollinating the flowers. If active, patrolling males were also collected in flight with an insect net. We aimed at capturing between five and ten bees per population. All insects were conserved in 70% ethanol. The three *Macropis* species were determined following Michez and Patiny (2005). A complementary study describing the pollinators of *L. nummularia* was carried out in parallel in June and July 2008 (see Supplementary material).

2.2. Genetic distance between *M. europaea* and *M. fulvipes* and detection of putative hybrids

Total genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) from the wing muscles of the bees carefully removed and rinsed, in order to avoid plant DNA contamination via pollen grains stuck to the bees' exoskeleton. To estimate the genetic distance between *M. europaea* and *M. fulvipes*, we chose four individuals (from distant origins) for each bee species and sequenced three mtDNA regions classically used

for barcoding: (1) 16s ribosomal RNA (*16s rRNA*, using the primers LR-N-13398 and LR-J-12883 from Simon et al., 1994), (2) partial cytochrome oxidase I (*COI*, with the primers C1-J-1751 and C1-N-2191 from Simon et al., 1994), and (3) partial cytochrome B (*CytB*, using primers designed by Belshaw and Quicke 1997). Fragments were amplified using a standard 30 µl PCR mix. The PCR were run in a TGradient thermocycler (Biometra, Goettingen, Germany) using the same program for all mtDNA regions (the detailed composition of PCR mix and conditions of PCR amplification steps are available on request to the last author). The PCR product purification and sequencing was carried out by Macrogen Inc. (Seoul, South Korea). Sequencing was performed using forward primers for all mtDNA regions under BigDye TM terminator cycling conditions, purifying the products using ethanol precipitation and running them into an automated sequencer 3730xl (Applied Biosystems, Foster City, USA). Sequences were manually corrected using the software Chromas Pro 1.34 (Technelysium, Helensvale, Australia). Alignment was carried out for each mitochondrial region using ClustalW Multiple Alignment (Thompson et al. 1997) as implemented in the software BioEdit 7.0.5.3 (Hall 1999), followed by minor manual corrections. Kimura two-parameter genetic distances between species and general statistics were calculated using MEGA 4 (Kumar et al. 2008).

In order to detect potential hybridization among *Macropis* species, we used Amplified Fragment Length Polymorphism (AFLP) genome fingerprinting. AFLP analyses were performed following the classical method described by Vos et al. (1995), with slight modifications (detailed protocol available upon request to the last author). Reactions were conducted in 96-well plates, in which samples were randomly distributed. We used restriction enzymes *EcoRI* and *MseI* for DNA digestion. A primer trial on a small number of samples was conducted using six different primer combinations to identify pairs of selective primers that were reproducible and polymorphic enough. Each individual sample was subsequently fingerprinted with the two primer combinations *EcoRI*-ACA/*MseI*-CAA and *EcoRI*-ACG/*MseI*-CAA. Final selective PCR products were analyzed using the GeneScan technology with an automated capillary sequencer (ABI 3730XL, Applied Biosys-

tems, Foster City, CA; service provided by Macrogen Inc., Seoul, South Korea). Resulting fluorescent AFLP patterns were scored using GeneMapper 3.7 (Applied Biosystems) with some changes in default parameters (maximum bin width=1 bp, light peak smoothing, peak threshold=200 RFU). We scored the presence or absence of each AFLP marker in all specimens. The matrices of the two scored primer pairs were concatenated into one single binary matrix where individuals and bands were stored as lines and columns, respectively. Multivariate ordinations using the principal coordinate analysis (PCoA) approach were produced on this distance matrix to investigate genetic relationships among specimens and taxa using the software Ginkgo 1.5.8 (Bouxin 2005). The PCoA relied on the Jaccard similarity coefficient (Jaccard 1908) to avoid grouping terminals on the basis of shared absences.

2.3. Ecological niche modeling in *M. europaea* and *M. fulvipes*

We modeled the current distribution of *M. europaea* and *M. fulvipes* using seven modeling techniques implemented in the BIOMOD (Thuiller et al. 2009) R package (see detailed protocol provided as Supplementary material). Models were calibrated from presences collected during the field sampling pooled with occurrences from the data collected by Michez and Patiny (2005) and using seven climatic layers from Worldclim (Hijmans et al. 2005). An ensemble forecasting approach (Marmion et al. 2009) was then used to project a central tendency from a combination of the models obtained by the different niche-based modeling techniques into current climate conditions in Europe. Ecological niches of both species were displayed on the same referential, a multi-dimensional scale represented by the two first axes of a principal component analysis (PCA; see detailed method of Broennimann et al. (in press) provided in Supplementary material). We finally used the geographical data recorded during field work to calculate the correlation between the presence of each bee species and both altitude and latitude, and we tested if the two species showed significant differences regarding both factors by performing either a *t* test or a one-way ANOVA for altitude, or a Mann–Whitney rank sum test for latitude. All statistical

analyses were performed with the program SigmaStat version 2.03 (SPSS Inc., Chicago, USA).

3. RESULTS

3.1. *Macropis* sampling

In total, 94 *Macropis* populations, comprising one or two species, were collected in Europe, Turkey, and Georgia during bees' main period of activity (Table I; Figure 1). *Macropis europaea* was the most frequently observed, as illustrated by our total sampling of 419 individuals collected in 74 populations between 25 June and 19 August. It was followed by *M. fulvipes*, represented by 195 individuals collected in 34 populations between 25 June and 10 August. *Macropis frivaldszkyi* was the least frequently sampled, with only eight individuals from three populations restricted to Serbia and Turkey, between 9 July and 12 July. All specimens are deposited at the Department of Ecology and Evolution from the University of Lausanne, Switzerland. Fifty-eight populations comprised only *M. europaea* (in white on Figure 1), 18 only *M. fulvipes* (in gray), and one single only *M. frivaldszkyi* (in black). Sympatric populations were also found: in 15 sites from Western and central Europe, both *M. europaea* and *M. fulvipes* were sampled; *Macropis frivaldszkyi* was observed in sympatry with *M. europaea* in a single location in Serbia (population KRU) and with *M. fulvipes* in a single location in Turkey (population ERF). Sympatry cannot be excluded in several sites comprised in the western distribution range of *M. fulvipes*, in which only *M. europaea* was collected, as ten French and Spanish locations (ASP, BAZ, BOV, CAM, CAP, CAR, CRU, JUG, MAV, and PUE; see Table I) that were visited later than August 10 (i.e., the latest appearance of *M. fulvipes* in our sampling). Although *M. fulvipes* females seem to be active until late August (Westrich 1990; Celary 2004), an earlier sampling in July would confirm or invalidate its actual absence in these sites. Regarding floral preferences, *M. europaea* was

Table I. *Macropis* populations sampled in Europe, Anatolia, and Caucasus, showing the number of individuals collected per species, as well as the corresponding geographical and ecological data.

Population information		*Macropis* species collected (number of individuals)			Geographical and ecological information (Latitude, longitude: decimal degrees/elevation: meters above sea level)					
Code	Date	*M. europaea*	*M. fulvipes*	*M. frivaldszkyi*	Country	Place	Latitude	Longitude	Elevation	Habitat
ARI	27 July 2008	2	7	–	Spain	Arija-Llano	42.991	−3.975	835	L
ASP	12 August 2007	6	–	–	Spain	Aspurz	42.715	−1.158	560	R
BAG	05 July 2008	–	2	–	Italy	Bagnasco	44.312	8.048	481	F
BAK	18 July 2008	–	15	–	Georgia	Bakuriani	41.765	43.484	1,552	F
BAL	08 July 2007	8	–	–	Switzerland	Balgach	47.402	9.619	317	F
BAU	11 July 2002	3	–	–	Belgium	Baudour	50.489	3.822	65	C, W
BAZ	19 August 2008	7	–	–	France	Bazouges	47.680	−0.206	34	C, R
BDL	03 August 2007	6	–	–	France	Bout du Lac (d'Annecy)	45.771	6.242	464	S
BEC	08 August 2007	–	9	–	Spain	Becedas	40.403	−5.627	1,094	F
BEG	03 August 2007	10	–	–	Switzerland	Begnins	46.427	6.242	477	S
BOK	05 August 2008	4	–	–	Sweden	Boekholm	56.403	13.600	113	F
BOL	08 July 2008	7	–	–	Switzerland	Bolle di Magadino	46.161	8.863	200	S
BON	20 July 2008	5	–	–	Switzerland	Bonfol	47.469	7.174	441	S
BOV	11 August 2008	11	–	–	France	Boves	49.855	2.378	405	L, S
CAM	12 August 2007	13	–	–	Spain	Sierra de Camero Nuevo	42.226	−2.627	793	R
CAP	13 August 2007	12	–	–	France	Captieux	44.301	−0.255	95	C, W
CAR	14 August 2008	7	–	–	Spain	Carrion de Los Condes	42.335	−4.617	820	C, F
CAS	25 July 2007	7	–	–	Italy	Castelvecchio di Compito	43.777	10.628	2	C, F
CER	10 August 2007	1	13	–	Spain	Cervera de Pisuerga	42.947	−4.492	1,090	R
CHA	16 July 2008	4	1	–	Switzerland	Chavanne-des-bois	46.313	6.138	459	F
CML	15 July 2007	4	–	–	France	Camélas	42.629	2.684	310	R
CRU	12 August 2008	7	–	–	Spain	Santa Cruz de Campezo	42.675	−2.361	558	C, F
CUE	08 August 2008	7	–	–	Spain	Cuenca	40.192	−2.113	960	R
DAD	10 July 2008	–	6	–	Turkey	Daday	41.481	33.603	1,015	F, R

Table I. (continued)

Population information		Macropis species collected (number of individuals)			Geographical and ecological information (Latitude, longitude: decimal degrees/elevation: meters above sea level)					
Code	Date	M. europaea	M. fulvipes	M. frivaldszkyi	Country	Place	Latitude	Longitude	Elevation	Habitat
DAG	12 July 2008	–	–	5	Turkey	Dağyolu	39.574	39.864	1,296	R
DAM	05 July 2008	3	–	–	Switzerland	Damphreux	47.472	7.112	425	S
DES	15 July 2007	5	2	–	Serbia	Despotovac	44.042	21.537	311	F
DIO	11 July 2008	5	–	–	Hungary	Diosjeno	47.932	19.062	207	L
DIP	03 August 2008	6	1	–	Germany	Dippoldiswalde	50.919	13.681	432	S, W
DOM	06 July 2008	1	5	–	Slovakia	Domasa Dobra	49.016	21.673	240	L
DZE	19 July 2008	6	4	–	Latvia	Dzelmes	56.655	24.933	48	C, F
ECO	19 July 2008	3	1	–	Switzerland	Ecogia	46.291	6.137	440	F
ERF	11 July 2008	–	10	2	Turkey	Erfelek	41.859	34.744	682	S, W
FEL	07 July 2008	–	5	–	Romania	Feleacu	46.701	23.590	670	S
FUN	18 July 2008	7	–	–	Switzerland	Poutafontana-Uvrier	46.246	7.410	498	C
GJE	11 July 2007	12	–	–	Slovenia	Gorni Jezero	45.727	14.408	566	S
GRA	15 July 2008	8	–	–	Switzerland	Les Grangettes	46.395	6.901	376	C
GRAN	18 July 2008	3	1	–	Switzerland	Granois	46.254	7.337	972	R, S
GRI	18 July 2008	5	–	–	Switzerland	Grimisuat	46.256	7.392	795	F
HAJ	06 July 2008	3	–	–	Hungary	Hajduhadhaz	47.695	21.655	184	C
HOD	14 July 2007	7	–	–	Hungary	Hodmezovasarhely	46.356	20.209	64	S
HOL	09 August 2008	3	–	–	Germany	Holvede	53.368	9.642	39	C, F
HOS	01 August 2008	7	–	–	Czech Republic	Hostka	49.687	12.582	515	S
ILG	10 July 2008	–	5	–	Turkey	Ilgaz Geçidi	41.140	34.064	1,471	F
JON	15 July 2008	3	–	–	Switzerland	Jongny	46.500	6.881	663	F
JUG	18 August 2008	2	–	–	France	Jugon-les-Lacs	48.405	-2.325	26	L
KBA	13 July 2007	9	–	–	Hungary	Kis-Balaton	46.660	17.126	123	L, S
KET	02 August 2008	5	1	–	Poland	Kety	49.844	19.214	398	F
KHA	17 July 2008	–	7	–	Georgia	Khashuri	41.999	43.656	689	C, F
KOZ	18 July 2007	9	–	–	Macedonia	Kozjak	41.056	21.036	877	C, S

Table I. (continued)

Population information		Macropis species collected (number of individuals)			Geographical and ecological information (Latitude, longitude: decimal degrees/elevation: meters above sea level)					
Code	Date	M. europaea	M. fulvipes	M. frivaldszkyi	Country	Place	Latitude	Longitude	Elevation	Habitat
KRU	09 July 2008	3	–	1	Serbia	Krupačko jezero	43.105	22.688	410	L
LAU	19 July 2008	3	13	–	Latvia	Lauciene	57.178	22.692	119	C, S
LEM	09 August 2008	3	–	–	Germany	Lembeck	51.723	6.995	46	L, S
LIP	08 August 2000	1	–	–	Romania	Lipova	46.716	27.234	230	F
LJU	06 August 2008	1	–	–	Sweden	Ljusfallshammar	58.802	15.452	54	S
LOC	11 July 2008	6	–	–	Switzerland	Etang du Loclat	47.018	6.998	437	L
LOD	08 July 2008	2	–	–	Switzerland	Loderio	46.379	8.973	348	C, F
LOZ	08 August 2007	2	1	–	Spain	Rio Lozoya	40.902	−3.863	1,125	R, W
LUG	20 July 2008	2	–	–	Switzerland	Lugnez	47.486	7.115	438	L, W
LUN	29 July 2008	–	11	–	Spain	Los Barrios de Luna	42.844	−5.862	1,030	R
MAR	05 August 2008	8	–	–	Denmark	Maribo	54.769	11.506	1	L
MARA	18 July 2008	5	–	–	Switzerland	Marais de la Brocassière	46.225	7.323	495	S
MAV	18 August 2008	2	–	–	France	Marais Vernier	49.421	0.533	0	S
MDV	19 July 2008	5	–	–	Switzerland	Moulin de Vert	46.180	6.027	356	F, W
MIN	09 August 2007	3	–	–	Spain	Rio Miño	41.974	−6.646	2	R
MOE	19 July 2008	4	–	–	Switzerland	Prévessins-Moëns	46.254	6.057	464	S
MOM	04 August 2008	–	1	–	Spain	Mombeltran	40.247	−5.018	530	F, R
MON	18 July 2008	7	–	–	Switzerland	Lac de Montorge	46.234	7.338	645	L
PAS	10 July 2008	–	1	–	Romania	Pasul Gutai	47.712	23.777	838	W
PIE	06 August 2008	–	5	–	Spain	Piedrabuena	39.048	−4.239	553	R
PRE	25 June 2008	2	2	–	Switzerland	Les Préhaies	47.285	7.429	620	F, S
PRE2	25 June 2008	–	2	–	Switzerland	Les Préhaies	47.287	7.431	637	F, S
PRT	12 July 2003	3	–	–	France	Portiragnes	43.302	3.332	3	R
PRW	29 July 2003	4	–	–	Belgium	Péruwelz	50.508	3.589	60	R, S
PUE	12 August 2008	14	–	–	Spain	La Puebla de Labarca	42.473	−2.593	399	R
RAS	08 August 2008	2	3	–	Spain	Rascafria-Lozoya	40.910	−3.870	1,133	S, W

Table I. (continued)

Population information		Macropis species collected (number of individuals)			Geographical and ecological information (Latitude, longitude: decimal degrees/elevation: meters above sea level)					
Code	Date	M. europaea	M. fulvipes	M. frivaldszkyi	Country	Place	Latitude	Longitude	Elevation	Habitat
REN	28 July 2008	6	–	–	Spain	Renodo	42.504	−4.729	900	C, R
RIE	23 July 2007	5	–	–	Italy	Rieti	42.510	12.753	373	L
RIL	17 July 2007	6	–	–	Bulgaria	Rila	42.102	23.091	470	F
RIO	08 August 2007	–	1	–	Spain	Rio Frio	40.324	−6.646	824	R, W
RUC	12 July 2007	11	–	–	Austria	Ruckersdorf	46.591	14.567	493	S
SAL	02 August 2008	5	–	–	Spain	Salamanque	40.957	−5.671	782	R
SAN	10 August 2007	–	20	–	Spain	Lago de Sanabria	42.115	−6.734	1,000	L
SCA	04 July 2007	9	–	–	France	Etang de Scamandre	43.606	4.336	−3	C
SON	27 July 2008	–	5	–	Spain	Soncillo-Herbosa	42.974	−3.805	825	S
SUC	02 August 2008	–	17	–	Czech Republic	Suchy	49.486	16.762	664	F
TKI	16 July 2008	–	1	–	Georgia	Tkibuli	42.377	43.037	1,229	S, W
TRO	03 August 2008	2	11	–	Germany	Trollblumenwiese	51.683	11.041	488	F, S
TUL	16 July 2007	4	–	–	Bulgaria	Tulovo	42.574	25.576	318	F, S
VAU	10 July 2008	–	6	–	Switzerland	Vauffelin	47.190	7.317	706	F, S
VIV	25 July 2007	14	–	–	Italy	Viverone	45.441	8.006	239	C, W
VRH	10 July 2007	9	–	–	Croatia	Vrh	45.334	13.920	1	S
WOL	31 July 2008	5	–	–	Germany	Wolbach/Woerbeschwang	48.421	10.599	437	L, S
ZAO	09 August 2008	19	–	–	Spain	Zaorejas	40.798	−2.155	943	R

Abbreviations for habitat are as follows: C canal/ditch, F field, L lakeshore, R riverside, S swamp, W wood/forest

only sampled on *L. vulgaris*, whereas *M. fulvipes* and *M. frivaldszkyi* were caught on both *L. vulgaris* and *L. punctata*. Furthermore, two individuals of *M. fulvipes* were observed on *L. nummularia* in one single site in Switzerland (see Supplementary material).

3.2. Genetic distance between *M. europaea* and *M. fulvipes*

Amplification of mitochondrial genes *COI*, *16s rRNA*, and *CytB* resulted in alignments of 423, 485, and 150 bp, respectively. The largest genetic distance between *M. europaea* and *M. fulvipes* was obtained with *CytB* (0.15±0.03), for which 24 nucleotides were variable (22 parsimony informative). *COI* was the second most variable gene (distance of 0.09±0.01), with 35 polymorphic sites (34 parsimony informative). Finally, *16s rRNA* resulted in the smallest distance among species (0.04±0.01) and showed 22 variable nucleotides (21 parsimony informative). The level of genetic divergence between *M. frivaldszkyi* and the two other bees has not been evaluated yet.

3.3. Hybridization in *Macropis*

In total, 288 individuals (198 *M. europaea*, 84 *M. fulvipes*, and six *M. frivaldszkyi*) were analyzed by AFLP genome fingerprinting in order to detect hybridization between *Macropis* species. The automatic scoring of the two primer pairs resulted in a total of 471 alleles (out of which 447 were parsimony informative), each individual yielding a minimum of 120 and a maximum of 219 fragments. The PCoA analysis (Figure 2) showed a strong partitioning of species when considering the first three axes (explaining respectively 37.9%, 7.9%, and 5.9% of total variance, i.e., 51.7% of cumulative variance). Hybridization was rare: only one single individual (a female, sampled in TUL, holding *M. europaea* morphology) showed an intermediate position in the PCoA, typical of hybrids, situated halfway between the scatter plots representative of *M. europaea* and *M. fulvipes*. This hybrid yielded 162 AFLP frag-

ments (a number included within the 5–95% confidence interval of the whole sampling); its intermediate position in the PCoA is thus not an artifact due, for instance, to contamination by two different DNAs, which would have yielded more than twice the minimum number of fragments per individual (i.e., 240 fragments).

3.4. Mean altitude and latitude comparison between *M. europaea* and *M. fulvipes*

Overall, *M. europaea* was collected at a significantly lower mean altitude (405 m. a.s. l.) than was *M. fulvipes* (732 m. a.s.l.) (t test, $t=$ 4.904, $P<0.001$). The ANOVA also showed that the mean altitudes of collection were significantly different ($df=2$, $P<0.001$; see Figure 3) between allopatric populations of *M. europaea* (360 m. a.s.l), of *M. fulvipes* (867 m. a.s.l) and sympatric sites (580 m. a.s.l.). As expected, sampling altitudes significantly decreased as latitude increased, with a strong negative correlation between altitude and latitude for both species (Pearson Product Moment Correlation, $r=-0.54$, $P<0.0001$ for *M. europaea* and $r=-0.66$, $P<0.0001$ for *M. fulvipes*). A Mann–Whitney rank sum test showed that there was no significant difference in the sampling latitude between the two species ($P=0.065$).

3.5. Ecological niches modeling comparison between *M. europaea* and *M. fulvipes*

The overlapping representation of the respective ecological niches of the two *Macropis* species on the two first PCA axes based on the Worldclim variables shows that the niche of *M. fulvipes* is slightly larger than that of *M. europaea* (presented in Supplementary material). The Schoener's D metric with its associated statistical tests (Warren et al. 2008) revealed that the ecological niches of each *Macropis* species were significantly different ($D=0.722$, $P=0.019$). Although in relation to the total available environment, the niches remained quite comparable, with the two bee species globally using analogous environmental space. Generally, *M. fulvipes* occupies a slightly larger and more
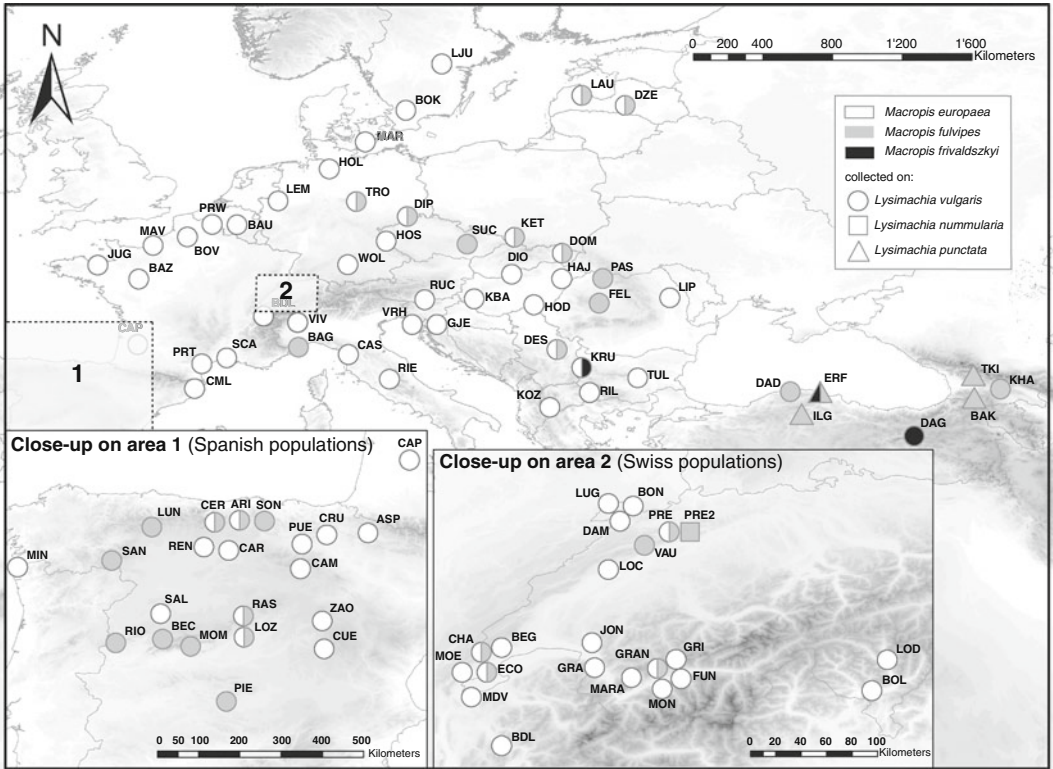
Figure 1. Map showing all *Macropis* populations sampled. Populations are displayed using different colors representing the three *Macropis* species, as well as different forms corresponding to the three *Lysimachia* species on which the bees were collected.

diffuse ecological niche, tolerating colder, more humid but also drier conditions than *M. europaea*. Consequently, differences in the patterns of potential European distribution could be observed for the two species. Figure 4 shows the current modeled distributions of both species. In general, *M. europaea* (Figure 4a) showed a more "temperate oceanic" pattern, with wide optimal areas in central Europe (north from the Alps) as well as in land zones adjacent to Atlantic, Baltic, and North Seas. The species also did not extend much further east than the Carpathians. In contrast, the potential distribution of *M. fulvipes* (Figure 4b) could rather be qualified of "temperate continental", showing a clear Eastern shift of its optimum, especially in the Balkans and the Carpathians, into Russia and around the Black Sea. Globally, suitable

areas (in different gray shadings) are more extended in *M. fulvipes* than in *M. europaea*, although optimal environments (in black) are rarer or, in any case, more diffuse. A close-up on the Alpine region confirmed the previous results forecasting *M. fulvipes* to be present at higher elevations (see Figure 4).

4. DISCUSSION

4.1. Important genetic distance as main post-zygotic barrier to hybridization between *Macropis* species

Even if hybridization between species might be counter-selected because hybrids tend to fall into adaptive valleys (Coyne and Orr 1998), different cases of hybridization have been
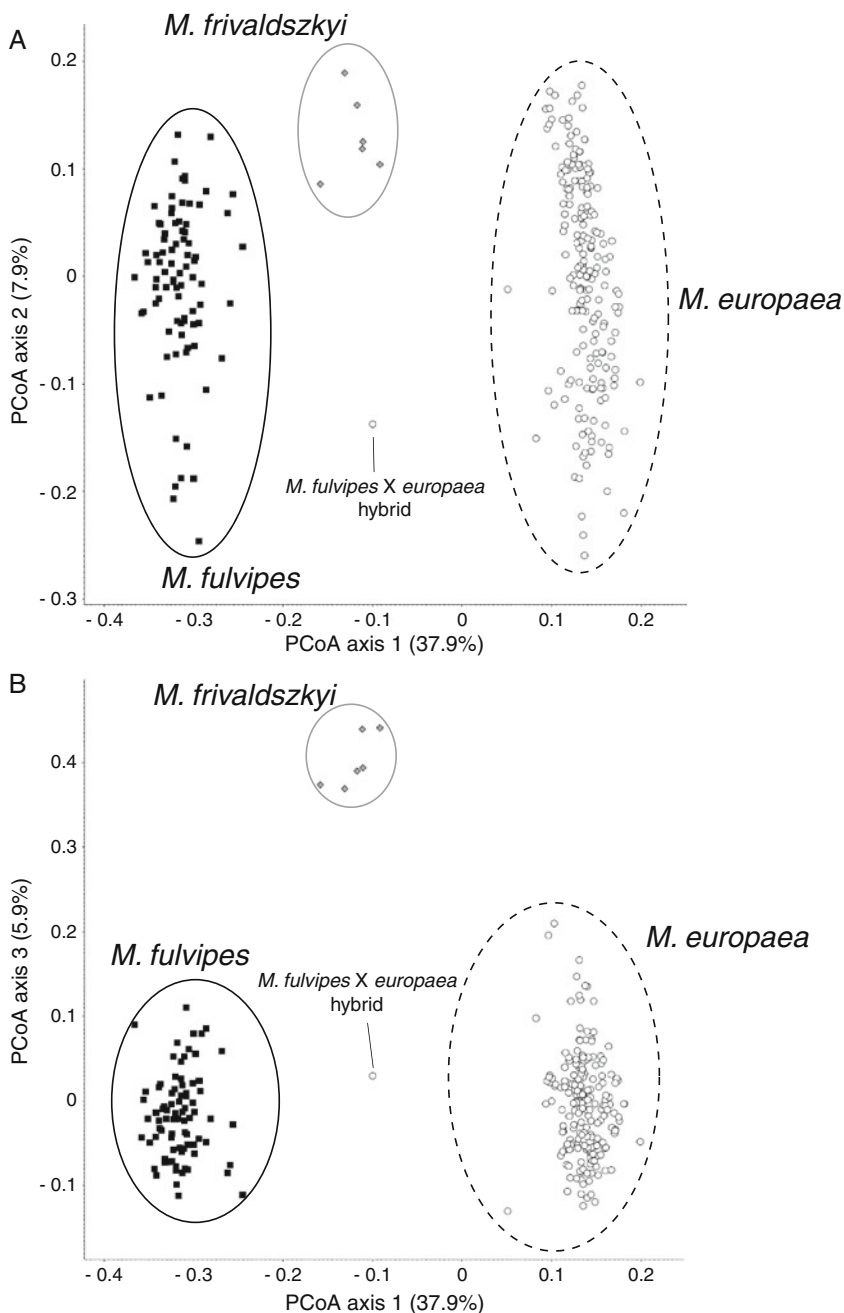
Figure 2. PCoA plot of all *Macropis* specimens genotyped, showing the potential hybrid detected. Each of the three phenotypic species is displayed on the first two axes (**a**) as well as on axes 1 and 3 (**b**).

described in bees (e.g., Ribble 1973; Hall 1990; Rinderer et al. 1991; Nascimento et al. 2000). Based on the assumptions that *M. europaea* and

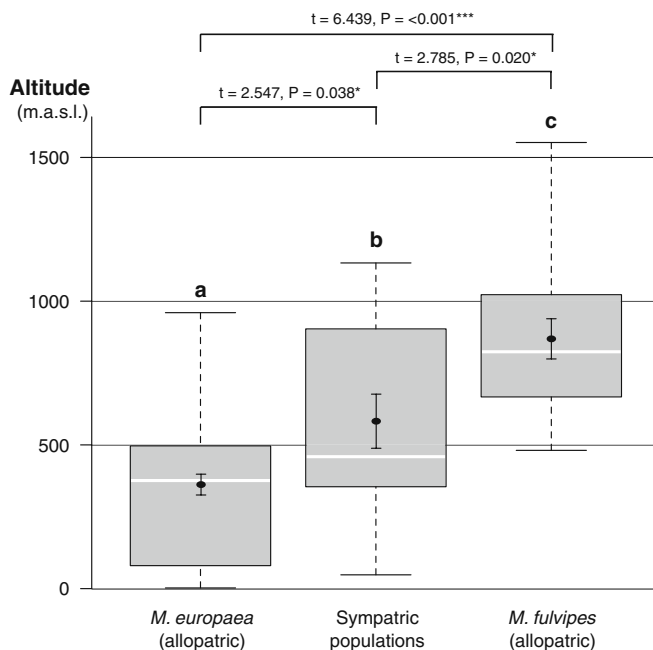*M. fulvipes* demonstrate (1) similar behaviors and ecologies (e.g., for pollination and reproduction), (2) comparable morphologies, and (3)

Figure 3. Range of sampling altitudes for the two bee species, either considering allopatric populations (*left and right*) or sympatric populations (*center*). For each treatment, the 5–95% confidence interval of data is shown as a *dotted line*. Within boxplots are represented the 50% of data (*in gray*) and the median (*white line*). The mean altitude value (with SE) is shown as a *black dot* over the boxplots. The results of the pairwise comparison (post hoc *t* test with Bonferroni correction) are shown above for the corresponding means compared.

frequent syntopy (i.e., they are often found in sympatry, collecting oil on the same plant), it could be expected that hybridization occurs between both species. In the current study, hybridization was shown to be rare, since among 291 individuals analyzed only one single confirmed hybrid was identified (0.3% of the total sampling).

The scarcity of hybrids can be partly explained by the high level of genetic divergence between these two taxa (e.g., 15% on *CytB*). Even if *M. europaea* and *M. fulvipes* have been shown to branch together as two sister terminal clades in an ongoing phylogenetic study on Mellittidae including six *Macropis* taxa (Michez, personal communication), their genetic divergence is at the upper limit of values observed for sister species within the genus (Sheffield et al. 2009). Such a high genetic distance indicates that these two species have diverged long before the Pleistocene, probably in the Pliocene or even

the Miocene. As a consequence, post-zygotic reproductive incompatibility between these species is probably well established.

4.2. Behavioral and ecological pre-zygotic barriers as local-scale impediments to hybrid formation

Although the high level of genetic divergence might explain the rarity of natural hybrids, the differences in the respective phenologies of *M. europaea* and *M. fulvipes* could still decrease their co-occurrence locally. Phenological studies available for *Macropis* report a slight offset (from 10 to 20 days) in the activity of the bee species (Westrich 1990; Pekkarinen et al. 2003; Celary 2004). Such an offset in the timing activity could thus represent a pre-zygotic barrier to interspecific reproduction, presumably with the larger the offset, the stronger the barrier. Stronger barriers to hybridization might rather affect
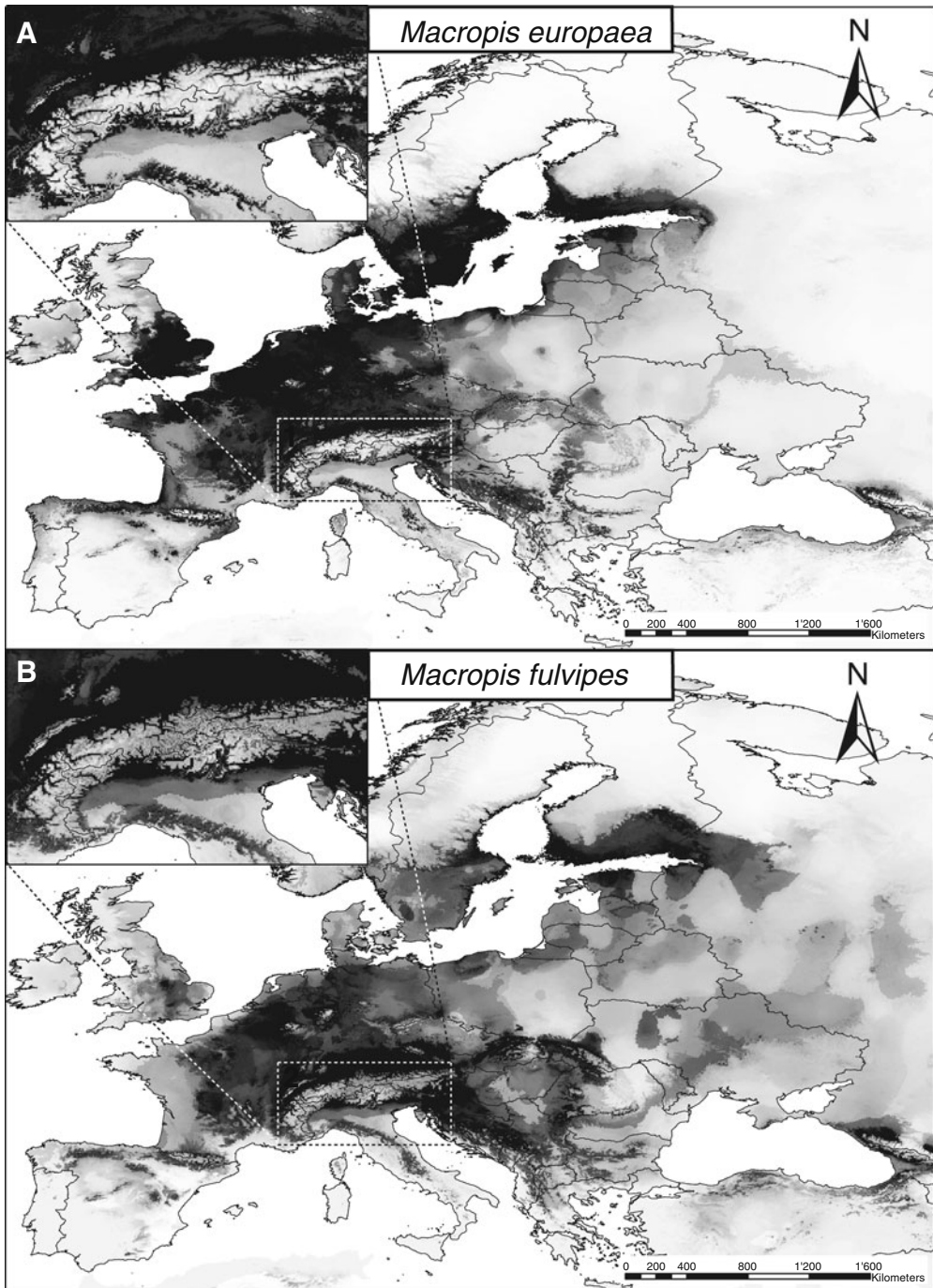
Figure 4. Modeling of the current potential European distributions of *M. europaea* (**a**) and *M. fulvipes* (**b**); the darker is an area, the higher is the probability that the ecological conditions in this area are optimal for the species. A close-up on the Alpine region is provided for each species, in order to compare the potential distributions in altitude.

southern populations, where the seasonal period of activity would start sooner and finish later, with a larger offset in the bees phenologies (although this is speculative since all available phenological studies were performed in northern European countries: Germany, Finland, and Poland, respectively). However, the same studies have shown that the two bee species co-exist for at least 1 month, especially during 1 or 2 weeks of shared intense activity. This period would be long enough to provide opportunities for the two species to meet and mate (notably between late-emerging *M. fulvipes* males and early-emerging *M. europaea* females). Because of the rare occurrence of natural hybridization, one might expect that the offset in respective phenologies locally reinforces the pre-zygotic reproductive barrier between the two species, although this feature is probably not a key factor in preventing hybridization.

Our large-scale study also revealed ecological trends that challenge previously reported observations on *Macropis*, notably regarding floral preferences or habitat associations. The presumed preference of *M. fulvipes* for *L. nummularia* (previously reported by Westrich 1990 or Pekkarinen et al. 2003) must be refuted. *Macropis fulvipes* certainly sometimes visits this plant (see Supplementary material), but it is principally dependant either on *L. vulgaris* in most of Europe or on *L. punctata* in southeastern Europe, Turkey and Caucasus. Previous conclusions concerning habitat associations should also be tempered. Some authors mentioned that *M. europaea* was mostly associated with open habitats (flood plains, wetlands) and *M. fulvipes* with forests (Westrich 1990). Even though such association might occur at a local scale (e.g., in Baden-Württemberg; Westrich 1990), evidence at a wider scale was lacking until the present study. In our results (see Table I), we observed that most sampled populations are located in open habitats such as swamps, wet fields, along rivers or lakeshores, and that both *M. fulvipes* and *M. europaea* are only rarely found in forests (probably because *L. vulgaris* and *L. punctata*, when growing in closed habitats, are generally less developed and harbor sparser inflorescen-

ces compared to plants from open locations). Hence, we cannot conclude that *M. fulvipes* is more associated with woody areas than is *M. europaea* at the European scale. Small-scale partitioning factors, such as floral or habitat preferences, might then represent additional factors that, in association with the phenological offset described above, could locally reduce the probability of co-occurrence and reinforce pre-zygotic barriers to hybridization between *M. europaea* and *M. fulvipes*. However, when considering the whole distribution ranges of these bees, large-scale partitioning factors such as climatic preferences (see below) are more likely to play essential barriers to hybridization and competition by limiting sympatry between these two bee species.

4.3. New insights on the climatic preferences and spatial distributions of *M. europaea* and *M. fulvipes*

In our study, at least three observations confirm the shift in ecological niches of the two species: (1) despite model predictions forecasting rather similar optimal areas for the two species in a large part of central Europe (Figure 4), we have identified only scarce sympatric populations (15 sites on our whole European sampling, see Figure 1); (2) occurrences in our sampling were spatially more continuous for *M. europaea* than for *M. fulvipes*, the latter being most frequent in mountainous areas; and (3) niches were significantly different as attested by the significant test for the Schoener's metric (i.e., the one of *M. fulvipes* being slightly larger than that of *M. europaea*). When confronting (1) and (2) to the paradigm of ecological valence sensu Dajoz (1978), we can consider *M. fulvipes* as an euryoecic species tolerating a larger ecological range (e.g., potential habitats, flower preference) than *M. europaea*, which is more stenoecic. Indeed, *M. fulvipes* is not only present in more climatically harsh regions (e.g., the Alps; see Figure 4), but it is also able to collect oil from other *Lysimachia* species (Figure 1, also see the *L. nummularia* pollination study in the

Supplementary material), even if *L. vulgaris* remains its more frequent oil-providing plant species in Europe. In contrast, *M. europaea* shows higher probabilities of occurrence in more temperate climatic zones and there is no convincing evidence yet of *M. europaea* feeding on other oil-providing plants (data compiled until here are anecdotal). Consequently, *M. europaea* is also expected to be more competitive than *M. fulvipes* when both species are found in sympatry on *L. vulgaris*. The less competitive *M. fulvipes* would then replace *M. europaea* in habitats with harsher conditions, as shown by the significantly higher mean altitude where *M. fulvipes* was collected (see Figure 3). Sympatric sites are therefore not as frequent as expected by the ecological niche models because they could only be located in transitional areas, where *M. europaea* reaches its ecological limits and becomes less competitive. Indeed, the 15 sympatric sites stand at a mean altitude of 580 m. a.s.l., the almost exactly halfway between mean altitudes of each bee species when found in allopatry. In the sympatric sites where the respective climatic preferences of *M. europaea* and *M. fulvipes* overlap, their considerable genetic distance still acts as a strong post-zygotic barrier, keeping hybridization events as rare as found in the current study.

5. CONCLUSION

Due to the probable combination of both genetic and ecological factors impeding the formation of hybrids between *Macropis* species, the frequency and localization of such events remain difficult to forecast. A more intensive screening of the sympatric populations, especially from the Balkanic range (where populations enclosing all three European *Macropis* species might exist, and with a single hybrid detected from the Bulgarian population TUL) could reveal further hybridization events, which could also involve *M. frivaldszkyi* as another potential parent for hybrid specimens. Despite the fact that the two bees share similar ecological and morphological features, the current

study suggests that European *Macropis* species efficiently avoid hybridization, as was evidenced by (1) a high level of genetic divergence and (2) differences in their climatic niches, locally associated with more subtle factors such as phenological offset and differences in floral choice or habitat preferences.

Chevauchement des niches écologiques chez 2 espèces sœurs: comment les abeilles récolteuses d'huile *Macropis europaea* et *M. fulvipes* (Hymenoptera : Melittidae) évitent-elles l'hybridation et la compétition?

Macropis / mutualisme / *Lysimachia* / niche écologique / hybridation

Die Überlappung ökologischer Nischen bei Geschwisterarten: Wie vermeiden die ölsammelnden Bienen *Macropis europaea* und *M. fulvipes* (Hymenoptera: Melittidae) die Hybridbildung und innerartliche Konkurrenz?

Macropis / Mutualismus / *Lysimachia* / ökologische Nische / Hybridbildung

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