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Anne-Emmanuelle Félix[abc], Paul-André Calatayud[bd], Bruno Le Ru[bd], Claire Capdevielle-Dulac[b], George Ong'amo[e], Jean-François Silvain[b] & Brigitte Frérot[a]

[a] INRA, UMR PISC 1272, Route de St Cyr, 78026 Versailles Cedex, France

[b] IRD, UR072, Laboratoire Evolution, Génomes et Spéciation (LEGS), UPR 9034, CNRS, 91198 Gif-sur-Yvette cedex, France and Université Paris-Sud 11, 91405 Orsay Cedex, France

[c] School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK

[d] IRD, UR072, c/o icipe, PO Box 30772-00100, Nairobi, Kenya

[e] School of Biological Science, College of Physical and Biological Sciences (Chiromo Campus), University of Nairobi, Nairobi, Kenya

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To be or not to be a species: use of reproductive isolation experiments and genetic analysis to clarify the taxonomic status of two *Busseola* (Lepidoptera: Noctuidae) species in Kenya

Anne-Emmanuelle Félix[a,b,c]*, Paul-André Calatayud[b,d], Bruno Le Ru[b,d], Claire Capdevielle-Dulac[b], George Ong'amo[e], Jean-François Silvain[b] & Brigitte Frérot[a]

[a]INRA, UMR PISC 1272, Route de St Cyr, 78026 Versailles Cedex, France; [b]IRD, UR072, Laboratoire Evolution, Génomes et Spéciation (LEGS), UPR 9034, CNRS, 91198 Gif-sur-Yvette cedex, France and Université Paris-Sud 11, 91405 Orsay Cedex, France; [c]School of Biological Sciences, University of Plymouth, Drake Circus, Plymouth PL4 8AA, UK; [d]IRD, UR072, c/o icipe, PO Box 30772-00100, Nairobi, Kenya; [e]School of Biological Science, College of Physical and Biological Sciences (Chiromo Campus), University of Nairobi, Nairobi, Kenya

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Summary. Phylogenetic analysis combined with chemical ecology can contribute to the delimitation of closely related insect species, particularly in Lepidoptera. In this study, the taxonomic status of a species in the genus *Busseola* (Lepidoptera: Noctuidae) was discussed using morphological data, cross-mating experiments, sex pheromone chemistry, field-trapping, and molecular classification. The results of the chemical ecology experiments corroborated those from the phylogeny studies. It was concluded that several reproductive isolation components, namely host plants, geography, pheromone emission time, pheromone blend, and post-zygotic isolation factors, led to the separation of *Busseola* **n. sp.** from its closely related species *B. segeta*. Molecular data showed a strong difference between these two species, regardless of the marker used. The new species named *Busseola nairobica* was morphologically described and a hypothesis about the evolutionary history of the studied species was put forward.

Résumé. Etre ou ne pas être une espèce : utilisation d'expérimentations d'isolation reproductive et d'analyse génétique pour clarifier le statut taxonomique de deux espèces de *Busseola* (Lepidoptera : Noctuidae) du Kenya. L'analyse phylogénétique combinée avec l'écologie chimique peut contribuer à la délimitation des espèces d'insectes très voisines, particulièrement chez les lépidoptères. Dans la présente étude, le statut taxonomique d'une espèce du genre Busseola (Lepidoptera : Noctuidae) a été discuté en utilisant données morphologiques, expériences d'accouplements croisés, chimie des phéromones sexuelles, piégeages et classification moléculaire. Le résultat des expériences d'écologie chimique ont confirmé l'étude phylogénétique. On a ainsi conclu que plusieurs composantes d'isolation spécifique (plantes-hôtes, géographie, période d'émission de phéromones, mélande de phéromone et plusieurs facteurs post-zygotiques) ont mené à la séparation d'un taxon nouveau *Busseola nairobica* **n. sp.** de l'espèce proche *B. segeta.* Les données moléculaires ont montré une forte différence entre ces deux espèces, quelque soient les marqueurs utilisés. La morphologie de *Busseola nairobica* **n. sp.** a été décrite et quelques hypothèses sont présentées au sujet de l'hisoire évolutive de cette espèce.

Keywords: *Busseola fusca*; *Busseola segeta*; *Busseola nairobica*; morphology; pheromone; field-trapping; molecular phylogeny; cytochrome b; L5 ribosomal protein gene

In Lepidoptera, sex pheromone compositions are species specific and the pheromone evolution processes can support systematic positions, even among closely related species (Descoins & Frerot 1979; Roelofs & Brown 1982; Newcomb & Gleeson 1998; Hille et al. 2005; Frolov et al. 2007), and participate in the complex processes of reproduction and gene flow (Mallet et al. 2007) by driving mate localization, recognition and acceptance. Chemical cues play an essential role in specific mate recognition systems (SMRS) (Paterson 1985) and they maintain the reproductive isolation between closely related species. The reproductive isolation is at the origin of the "biological species" concept developed by Mayr in 1942.

Sex pheromones consist of a precise blend of compounds with different chemical structures, and the resulting chemical signature of a species induces in the conspecific partner of the opposite sex a specific behaviour (Karlson & Luscher 1959). Between closely linked species, related or identical molecules are used, and the chemical signature is the result of different ratio of the same compounds. Males are very sensitive both to the ratio and to the total amount of pheromone components (Baker et al. 1981; Linn et al. 1986). Sex pheromone chemistry can be used in addition to morphological and molecular classification to confirm phylogeny and to distinguish very closely related species.

*Corresponding author. Email: annemmanuelle@aol.com

In Africa, geographical distribution, host plant and species status of a noctuid complex in the *Busseola* genus remain unclear. *Busseola fusca* (Fuller 1901) is the best-known species, being one of the major stem borer pests of maize (*Zea mays* L. 1753) and sorghum (*Sorghum bicolor* Moench 1794) in sub-Saharan Africa. Recent surveys in Kenya showed that *Busseola segeta* (Bowden 1956) is increasingly attacking maize in Western Kenya (Le Ru, Ong'amo, Moyal, Muchugu, et al. 2006; Le Ru, Ong'amo, Moyal, Ngala, et al. 2006; Ong'amo et al. 2006). *Busseola segeta* can develop on at least nine host plants, including Napier grass (*Pennisetum purpureum* Schumacher 1827), maize and sorghum. It is absent in eastern Kenya where another *Busseola* species, called "*B.* **n. sp**" (Felix et al. 2011), is mainly found on broadleaf panicum (*Panicum deustum* Thunb 1794) and on one occasion only on *P. purpureum* in Tanzania, but never on maize (Ong'amo et al. 2006; Le Ru, Ong'amo, Moyal, Muchugu, et al. 2006; Ong'amo 2009). From pheromonal and preliminary morphological studies, *B. segeta* and *B.* **n. sp** appeared to be closely related species: Felix et al. (2011) identified the same pheromone components, but in slightly different ratios.

To date, no genetic analysis has been carried out on *B. segeta* and *B.* **n. sp**., whereas phylogenetic analysis using a mitochondrial marker was performed on *B. fusca*, which consists of three major clades corresponding to three geographic units, one originating in the West African region (*W*), one restricted to East Africa (*KI*), and one found in Central to East Africa (*KII*) (Sezonlin et al. 2006). Also, no morphological description of the *B.* **n. sp**. is available.

The purpose of this study was to assess whether *B.* **n. sp**. is a species different from *B. segeta* by morphological description, inter-crossing studies, field trapping with synthetic pheromones, and molecular analyses. In addition, this paper presents a taxonomic description of *B.* **n. sp.** Based on the phylogenetic studies, the systematic position and evolutionary scenarios are discussed.

Materials and methods

Insects

Because the mating success can be modified by the rearing conditions (Calatayud et al. 2008), mating experiments were conducted with adults originating from larvae collected in the field. *Busseola fusca* individuals were obtained from larvae collected on maize in Machakos (45 km from Nairobi, East Kenya, 1°29′ S, 37°16′ E, 1819 m asl) while *B. segeta* individuals were obtained from larvae collected on *P. purpureum* (2005 and 2006) in Kakamega (West Kenya, (0°12′ N, 34°55′ E, 1612 m asl). *Busseola n. sp.* individuals were collected on *P. deustum* in the Ngong forest (Central Kenya, 1° 19′ S, 36°44′ E, 1833 m asl). All larvae were reared at the International Centre of Insect Physiology and Ecology (ICIPE) on an artificial diet (Onyango & Ochieng'-Odero 1994) until pupation. The pupae were sexed and held in individual containers on moist vermiculite at 25°C, 85 ± 10% relative humidity (RH) and under a 12:12 h light:dark reversed photoperiod until adult emergence. Adults were used at ICIPE for mating experiments or sent to Institut de Recherche pour le Développement (IRD) (Gif-sur-Yvette, France) for genetic characterizations: DNA from 30 individuals of *B. segeta* from Kakamega and 50 individuals of *B.* **n. sp.** from Ngong forest was extracted.

Reproductive isolation

Mating experiments

Intra- and inter-specific crossings were tried with virgin adults. Tests were conducted on the first night after emergence. All possible combinations within three species were tested (Table 1). Depending on the availability of insects, eight to 58 pairs were used per treatment. Each pair was introduced into a transparent cylindrical plastic jar (16 × 9 cm), containing a wet piece of cotton wool that maintained relative humidity at around 80%. The mating success was assessed by counting the eggs laid after four nights on three surrogated oviposition supports made from three rectangular pieces of nylon cloth (15 × 5 cm) rolled helicoidally from top to bottom (Juma 2005). Eggs were then incubated at 25°C to check egg hatchability. For each pair, female moths were dissected to assess spermatophore number, which in Lepidoptera indicates the number of successful matings (Lum 1979).

Field trapping

To test the reproductive isolation under natural conditions, field-trappings were carried out at Mahi-Mahiu (Rift Valley, Kenya, 1°2.07′ S, 36°36.08′ E, 2042 m asl), Kakamega and Ngong forest where *B. fusca*, *B. segeta* and *B.* **n. sp.** are the dominant species, respectively. The three pheromone mixture compositions (Table 2) were based on previous specific pheromone identifications (Felix et al. 2009, 2011) and bore the name of the species they represented. The lures were loaded with an appropriate composition and amount of synthetic chemicals: (*Z*)-11-tetradecen-1-yl acetate (Z11-14:Ac), (*E*)-11-tetradecen-1-yl acetate (E11-14:Ac), (*Z*)-9-tetradecen-1-yl acetate (Z9-14:Ac), (*Z*)-11-hexadecen-1-yl acetate (Z11-16:Ac) and (*Z*)-11-tetradecenol (Z11-14:OH), in hexane solution. The pheromone mixture compositions were: (1) *B. fusca* mixture: 620 µg of Z11-14:Ac; 150 µg of E11-14:Ac; 50 µg of Z9-14:Ac; 100 µg of Z11-16:Ac; (2) *B.* **n. sp.** mixture: 620 µg of Z11-14:Ac; 200 µg of E11-14:Ac; 50 µg of Z9-14:Ac; 100 µg of Z11-16:Ac; 50 µg of Z11-14:OH; (3) *B. segeta* mixture: 620 µg of Z11-14:Ac; 150 µg of E11-14:Ac; 50 µg of Z9-14:Ac; 100 µg of Z11-16:Ac; 100 µg of Z11-14:OH. The lures loaded onto rubber septa (Sigma-Aldrich, France, SARL), were placed inside multicoloured Unitrap traps (Biosystèmes France, SARL; green cover, yellow top, and white bottom) at the edge of host plant patches, approximately 1.5 m above the ground and at least 30 m apart.

Genetic characterization

Total genomic DNA was extracted from insect thoraxes and legs, using a DNeasy Blood and Tissue Kit (QIAGEN®). A 965 bp fragment of the mitochondrial gene encoding cytochrome *b* was amplified via PCR using the primers CP1 forward (5′GAT GAT GAA ATT TTG GAT C 3′) and Tser reverse (5′TAT TTC TTT ATT ATG TTT TCA AAA C 3′), on a Biometra® (France, SARL) thermocycler using the following conditions: 94°C initial denaturation for 5 min; 40 cycles of 94°C for 1 min, 46°C for 1 min 30 s, 72°C for 1 min 30 s, and 5 min final extension at 72° C. All sequencing was performed in both directions but some individuals were sequenced in two fragments using the primer

Table 1. Oviposition rates, spermatophore transfer and egg viability of different *Busseola fusca*, *B. segeta* and *B. nairobica* **n. sp.** pairings.

Type of pair[a]	*N*	No. of eggs laid/female after 4 nights (means ± SE)[b]	%females[c] with a spermatophore	%females[d] giving fertile eggs
$F_{B.\ fusca}$ × M $_{B.\ fusca}$	**15**	**200.9 ± 60.2 c**	**53.3 bc**	**100**
$F_{B.\ fusca}$ × M $_{B.\ segeta}$	17	39.5 ± 16.7 a	5.9 a	0
$F_{B.\ fusca}$ × M $_{B.\ nairobica.}$	42	65.3 ± 12.7 a	2.4 a	0
$F_{B.\ segeta}$ × M $_{B.\ fusca}$	21	82.9 ± 22.4 a	4.8 a	0
$F_{B.\ segeta}$ × M $_{B.\ segeta}$	**8**	**114.4 ± 37.0 b**	**37.5 b**	**100**
$F_{B.\ segeta}$ × M $_{B.\ nairobica}$	9	182.0 ± 56.0 bc	33.3 b	67
$F_{B.\ nairobica}$ × M $_{B.\ fusca}$	58	28.0 ± 7.8 a	5.2 a	0
$F_{B.\ nairobica}$ × M $_{B.\ segeta}$	11	96.3 ± 44.7 ab	18.2 ab	100
$F_{B.nairobica}$ × M $_{B.nairobica}$	**31**	**213.1 ± 26.1 c**	**77.4 c**	**100**

[a]F, female; M, male.
[b]Means in a column followed by the same letter(s) are not significantly different at the 5% level (Student–Newman–Keuls test, result of ANOVA: $F_{8,\ 203} = 8.914$, $p < 0.0001$).
[c]Percentages followed by the same letter(s) are not significantly different at $p \leq 0.05$ (Fisher's exact test [2 × 2] for multiple percentage comparisons following chi-square test for the contingency table [2 × 9]) (chi-square test result for the contingency table [2 × 9]: df = 8, $Chi^2 = 92.055$, $p < 0.0001$).
[d]Among the females with a spermatophore, the %females giving fertile eggs were exhibiting more than 50% of eggs hatching. For 0%, no egg hatching at all was observed.

Table 2. Catches of *Busseola* males in traps baited with different pheromone lures in different localities in Kenya (mean[a] ± SE male caught per trap/week).

Locality		*B. fusca* mixture	*B.* **n. sp.** mixture	*B. segeta* mixture
Mai-Mahiu	*B. fusca* catches	22.3 ± 7.2c	4.1 ± 1.2b	0.5 ± 0.3a
	B. segeta catches	0	0	0
	B. nairobica catches	0	0.3 ± 0.1	0
Kakamega	*B. fusca* catches	0.7 ± 0.3	0	0
	B. segeta catches	0	15.4 ± 1.8b	10.9 ± 2.2a
	B. nairobica catches	0	0	0
Ngong forest	*B. fusca* catches	0	0	0
	B. segeta catches	0	0	0
	B. nairobica catches	0	0.9 ± 0.2a	0.7 ± 0.3a

[a]Means within a line followed by the same letter(s) are significantly different from each other according to the Tukey–Kramer test at the 5% level.

pairs CP1/CB3H (5′ AGC AAA TAA AAA ATA TCA TTC 3′) and Tser/CB3RL (5′CAT ATT CAA CCT GAA TGA TAT TT 3′) (Sezonlin et al. 2006).

A 794 bp fragment of nuclear marker encoding L5 ribosomal protein gene (RpL5) was amplified via PCR using primers from Mallarino et al. (2005) on a Biometra® thermocycler using the following conditions: 95°C initial denaturation for 6 min, 35 cycles of 45 s at 95°C, 50 s at 63°C, 60 s at 72°C, and 10 min final extension at 72°C. Some individuals were amplified after cloning with ligation Invitrogen kit (TA Cloning Dual Promoter, France, SARL).

All PCR amplifications were performed in 25 µl reactions containing 10 ng template DNA, 3 mM $MgCl_2$, 0.4 µM of each primer, 0.4 mM dNTPs and Promega Go*Taq* Flexi DNApolymerase (1U, France, SARL). PCR products were cleaned using Gel and PCR Clean-up System (Promega). The products were sequenced on an Applied Biosystems ABI PRISM®3130 automated sequencer (France, SARL).

Morphology study

The specimens were identified by comparison with *Busseola* types housed at the Natural History Museum, London (BMNH). Genitalia were dissected after immersion of the abdomen in a boiling 10% potassium hydroxide bath for a few minutes, then cleaned, immersed in absolute ethanol for a few minutes and mounted on slides in Euparal, after separating the aedeagus from the rest of the male genitalia. Types of the new species were deposited in the Museum National d'Histoire Naturelle (MNHN) in Paris, France and in the National Museums of Kenya (NMK) in Nairobi.

Data analyses

Numbers of eggs laid per female were $\log(x + 1)$ transformed and the means were separated by Student–Newman–Keuls test following analysis of variance (ANOVA). Chi-squared test and Fisher's exact test were used to compare the percentages for contingency tables [2 × 9] and [2 × 2], respectively. For analysis of the field-trapping bioassays, following the Kruskal–Wallis test the ranks were generated using proc Rank of SAS 9.1 (SAS Institute 2003) and then analysed using proc GLM. Means were then separated using the Tukey–Kramer comparison procedure.

Sequence alignments were performed manually with the Bioedit Sequence Alignment Editor software by comparison with previous sequences from *B. fusca* (Sezonlin et al. 2006, Tom Hall, Carlsbad, CA). Bootstrap parsimony tree and divergence degrees (Kimura two-parameter) were obtained with Mega

4.0 software (Tamura et al, Tokyo, Japan). Robustness of the inferred trees was tested by bootstrapping (1000 replicates). The outgroup used was a sequence of *Sesamia nonagrioides* (Lefèbvre) (Lepidoptera: Noctuidae) for the mitochondrial marker and a sequence of *S. calamistis* (Hampson) for the nuclear marker.

Results

Reproductive isolation

Mating experiments

The results indicated that inter-specific mating between *B. segeta*, *B.* **n. sp.** and, in rare cases, with *B. fusca* was possible with resulting transfer of spermatophores. The number of eggs laid by females was significantly higher in intra-specific (in bold in Table 1) than in inter-specific crosses, regardless of the female and male species used (Table 1). For inter-specific crosses involving *B. fusca*, the number of eggs was very low compared to inter-specific crosses involving *B. segeta* and *B.* **n. sp**. Successful mating as shown by the percentage of females with spermatophores was significantly lower for all inter-specific crosses involving *B. fusca* than the two other species. Moreover, for *B. segeta* and *B.* **n. sp.**, all inter-specific crosses produced viable eggs, whereas egg viability was zero with *B. fusca*.

Field trapping

In all localities, the *B. fusca* pheromone mixture only caught *B. fusca* (Table 2). In Mai-Mahiu, where *B. fusca* was the most abundant, traps containing the *B. segeta* and *B.* **n. sp**. mixtures caught significantly fewer *B. fusca* males than the *B. fusca* mixture. In Kakamega, where *B. segeta* was the most abundant, *B. segeta* males were significantly more attracted by the *B.* **n. sp.** than by the *B. segeta* mixture. In Ngong forest, where *B.* **n. sp.** was the most abundant, *B.* **n. sp.** males were equally attracted by *B.* **n. sp.** and *B. segeta* mixtures.

Genetic characterization

The phylogenetic tree constructed with the mitochondrial marker coding for cytochrome *b* yielded a significant homogeneity in the group of studied *Busseola* species (Figure 1). The results indicated a monophyly of *B. fusca*, well separated from the two other species, *B. segeta* and *B.* **n. sp**. The latter species were gathered in a well-supported group, within which two independent sister clades were distinguished, clustering *B. segeta* and *B.* **n. sp**. haplotypes, respectively. This clade separation was strongly supported by high and sufficient bootstrap values (99%). The divergence percentages between the three species averaged 6.9% (data not shown). By comparison, divergences between *S. nonagrioides* outgroup

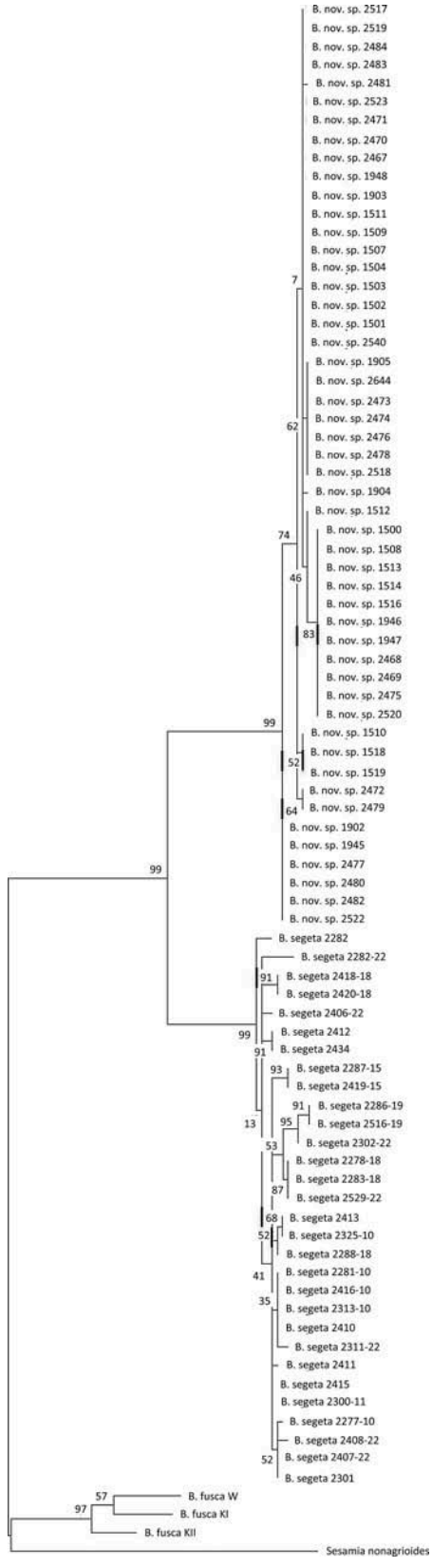

Figure 1. Maximum parsimony analysis phylogenetic tree for the mitochondrial marker cytochrome b. Numbers at nodes indicate the statistical support as obtained from 1000 bootstrap replicates.

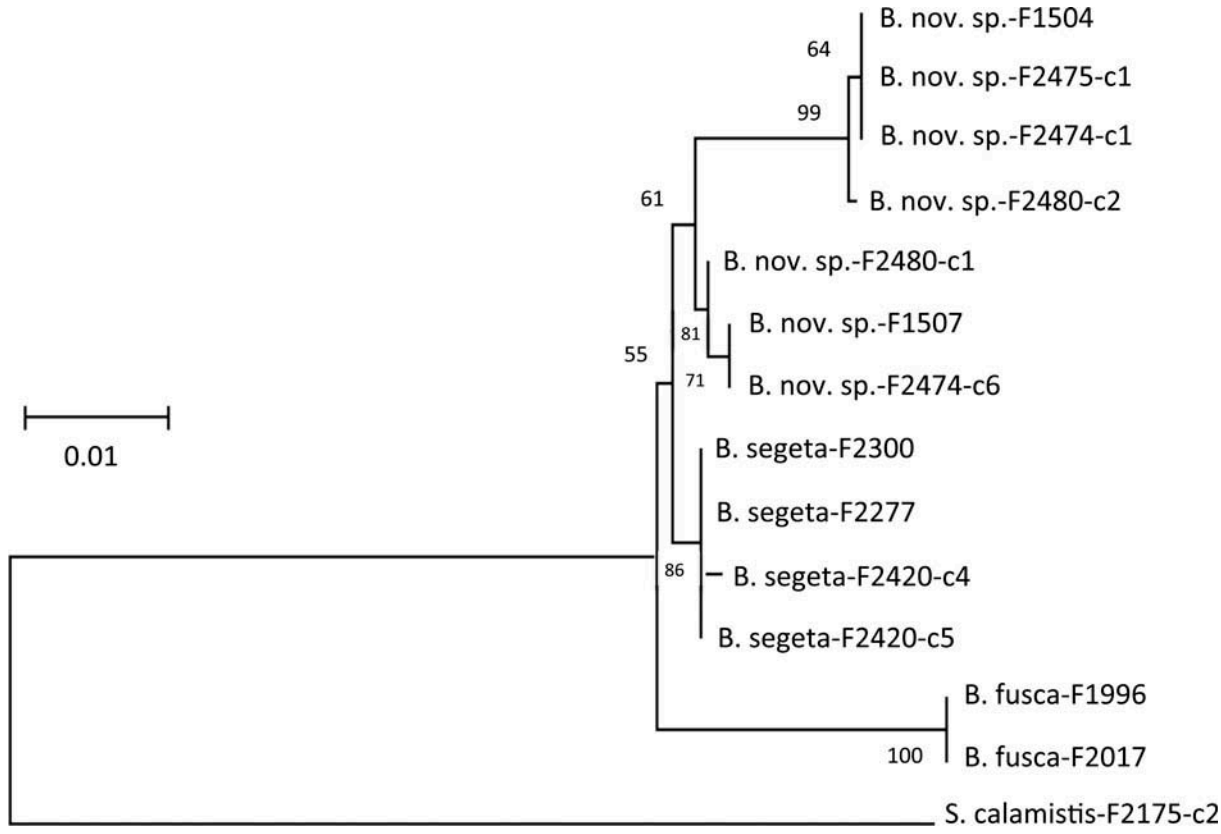

Figure 2. Maximum parsimony analysis phylogenetic tree for the nuclear marker RpL5. Numbers at nodes indicate the statistical support as obtained from 1000 bootstrap replicates.

and *Busseola* species averaged 10.5%, and between *B. n. sp.* and *B. segeta* 4.9%. Divergences within group (data not shown) were very weak, i.e. 0.2% and 0.6% for *B.* **n. sp.** and *B. segeta*, respectively. By comparison, the divergence between *B. fusca* haplotypes was 2.6%.

The phylogenetic tree showing nuclear marker data (Figure 2) allowed separation of homogeneous clades, corresponding to three species. Divergence percentages were as follows: 1.2% between *B. segeta* and *B.* **n. sp.**, an average of 2.8% with *B. fusca* and 11.8% with *S. calamistis* outgroup (data not shown).

Morphology study

Busseola n. sp. was found in mid altitude areas (994 m < *x* < 2042 m) in seven localities East of the Gregory Rift Valley in Kenya and Tanzania, and in one locality only West of Gregory Rift Valley (Masaï Mara) (see online supporting table and map). The main morphological differences between the two species concerned the genitalia (Figures 3, 4).

Male: The valves were more elongated in *B. segeta* (ratio length–width = 2.3, *n* = 5) than *B. n. sp.* (ratio length–width = 1.7, *n* = 6); the valve sclerotized process was usually much more developed in *B. segeta* than in *B. n. sp.*; the costal process was well developed in both species, but rather blunt in *B. segeta* compared to *B.* **n. sp.** in which it was sharply pointed.

Female: in *B. segeta* the anterior lip of the ostium was convex strongly protruded into ostium in the middle part while it was flat in *B.* **n. sp.**

Description of the new species

Busseola nairobica Le Ru n. sp. (Figure 4) (= *Busseola* n. sp.)

Type material examined. Holotype ♂, Kenya, Race Course Forest (00°52.580′ S, 37°24.390′ E, 1754 m asl), XI.2004, ex larva [in stem of *Panicum deustum* Thunb. B. Le Ru, Leg., gen. prep. BLR G276, MNHN, Paris, France. Paratypes: 1 ♀ (gen. prep. BLR G388), same data as holotype, B. Le Ru, Leg., NMK, Nairobi, Kenya; 1 ♂ and 1 ♀, same data as holotype, B. Le Ru, Leg., NMK, Nairobi, Kenya; 1 ♂ and 1 ♀, same data as holotype, B. Le Ru, Leg., MNHN, Paris, France; 1 ♀ from Naro Moru (00°09.278′ S, 37°00.706′ E, 1956 m asl), Kenya, VII.2007, ex larva [in stem of *Panicum deustum* Thunb], 1 ♂ from Keekorok (01°35.308′ S, 35°14.210′ E, 1754 m

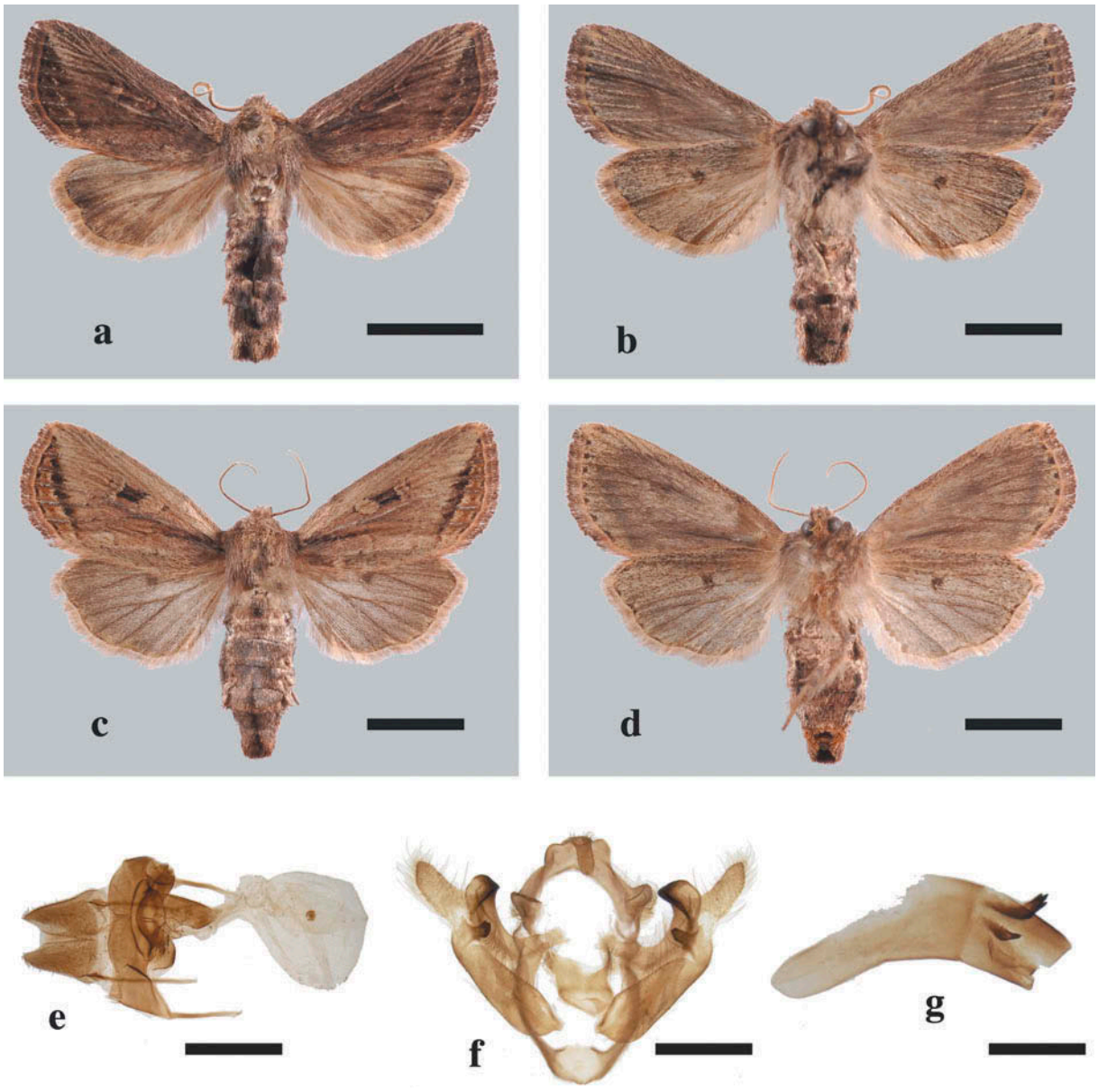

Figure 3. *Busseola segeta*: **a,** male dorsal side; **b,** male ventral side (scale bar: 1 cm); **c,** female dorsal side; **d,** female ventral side (scale bar: 0.8 cm); **e,** female genitalia (scale bar: 1 mm); **f,** male genitalia; **g,** male aedeagus (scale bar: 0.5 mm).

asl), Kenya, VI.2007, ex larva [in stem of *Panicum deustum* Thunb], B. Le Ru, Leg., MNHN, Paris.

Description. Antennae short-bipectinate, serrate at apex in the male, filiform in the female, flagellum adorned dorsally with grey scales, underside pale buff. Palpus grey buff; body colour and wing pattern are similar in both sexes. Head and thorax grey buff especially on tegulae and epaulets covered with long hairs; eyes black. Abdomen grey suffused with buff and black, underside of abdomen adorned with two lateral lines of dark buff scales. Anal tuft grey suffused with buff. Legs dark buff especially forelegs, tibia and each segment of tarsi with a white ring at apex. Forewing grey buff suffused with blackish scales at base and along costa; orbicular and reniform spots rather indistinct and defined by a black area in between; a slightly curved feebly dentate and indistinct anteromedian black fascia; an indistinct post-median fascia black, curved outwards from costa, strongly dentate from vein 8 to inner margin. An oblique subterminal row of 4–5 interneural cream spots defined proximally by black scales extended by 2 cream interneural reniform spots on the apex. Veins outlined with white, fringe infuscate with a narrow basal yellow line. Hind wing pale, densely suffused with black scales, veins outlined with black scales; fringe very pale, flecked with dark grey. Underside of forewing pale,

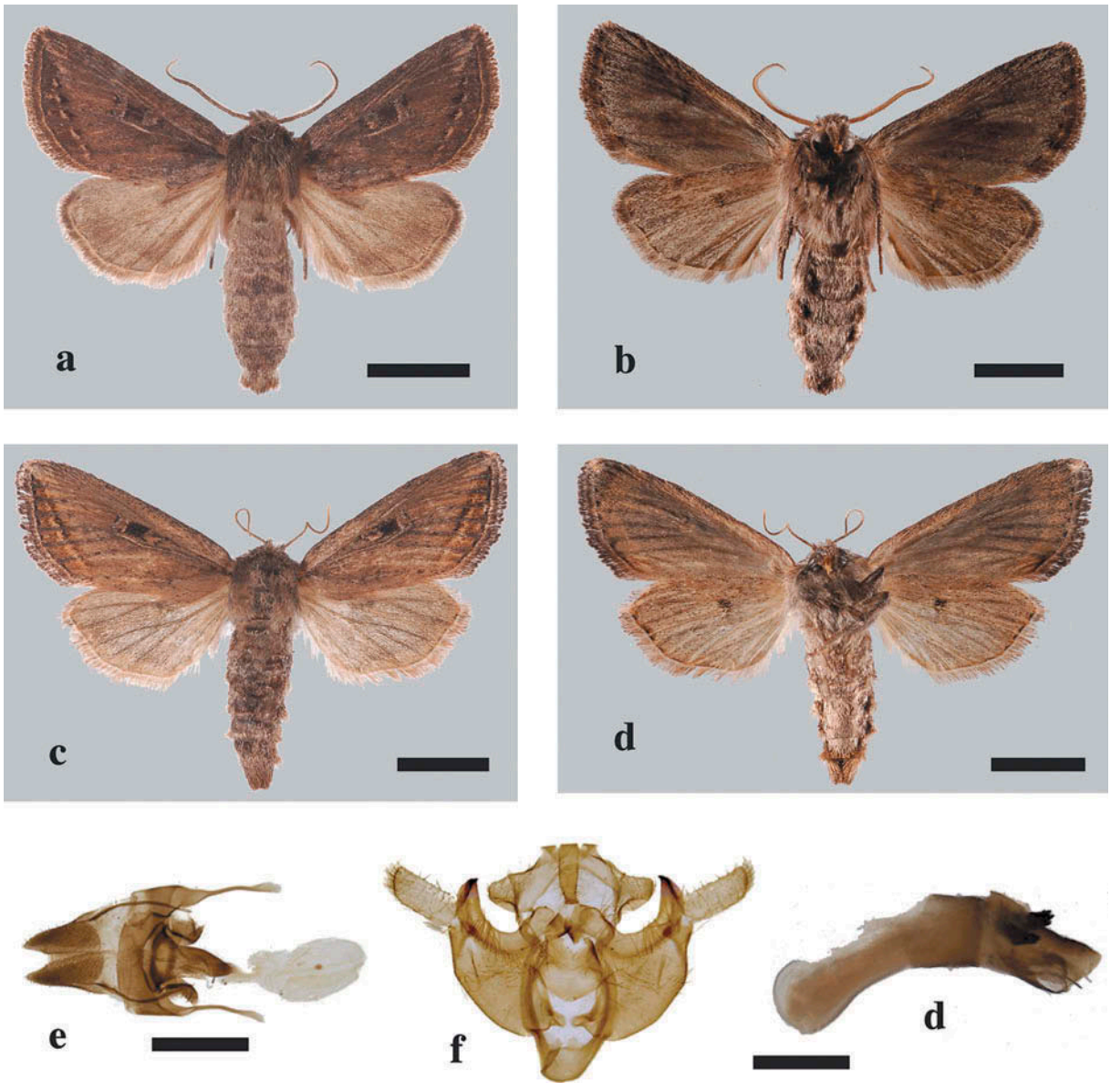

Figure 4. *Busseola nairobica* **n. sp.**: **a,** male dorsal side; **b,** male ventral side (scale bar: 1.3 cm); **c,** female dorsal side; **d,** female ventral side (scale bar: 0.8 cm); **e,** female genitalia (scale bar: 1 mm); **f,** male genitalia; **g,** male aedeagus (scale bar: 0.5 mm).

suffused with black, more densely suffused along the costa; a rather indistinct fuscous discal spot; a terminal series of small interneural black spots; veins outlined with black; fringe infuscate. Underside of hind wing pale, suffused with black, more densely suffused along costa and apex; a fuscous discal spot; fringe pale infuscate at apex.

Wingspan. 25–29 mm (males) (n = 13); 27–32 mm (females) (n = 11).

Male genitalia (Figure 4). Uncus small, broad, blunt apically. Tegumen with large rounded peniculi. Vinculum with a medium sized oval saccus. Valve short and broad, the sclerotized process small and blunt; costal process quite large and sharply pointed. Juxta shield-like, rounded ventrally. Aedeagus short with two small cornuti.

Female genitalia (Figure 4). Corpus bursae short with two small signa; ductus bursae heavily sclerotized from ostium for about two third its length, ending obliquely; ostium crescent-shaped, its anterior lip flat in the middle. Ovipositor lobes 2.9 times longer than wide (n = 5).

Discussion

The crossing experiments showed that interspecific breeding between *B. fusca* and the two other species was not

compatible, even if mating was possible. This post-mating isolation mechanism corroborated the pre-mating isolation mechanism, which prevents cross attraction between *B. fusca* and *B. segeta* because of different periods of female calling as shown by Frerot et al. (2006) and Felix et al. (2011). Besides varying numbers of compounds, the pheromones of the three species also differed in compound ratios (Felix et al. 2009, 2011). In field trapping experiments in Mai-Mahiu, the additional compound present in the *B. segeta* and *Busseola nairobica* **n. sp.** pheromones, was not sufficient to ensure a complete reproductive isolation of *B. fusca*: some males of *B. fusca* were attracted by *Busseola nairobica* **n. sp.** and *B. segeta* mixtures. However, *B. fusca* formulation did not attract the two other species, which probably required the Z11-14:OH to recognize the female. Mayr's "biological species" concept (1942), based on the factors isolating the species, can be employed to describe the isolation mechanisms preventing *B. segeta* and *Busseola nairobica* **n. sp.** from interbreeding with *B. fusca*. This isolation is achieved by the periods of female calling, the pheromone blends and post-zygotic mechanisms (no fertile egg) even in experimental conditions.

Interspecific crosses involving *B. segeta* and *Busseola nairobica* **n. sp.** yielded viable eggs, suggesting that they belong to the same species. However, the time of female calling of *B. segeta* and *Busseola nairobica* **n. sp.** was shown to be different, which indicates a pre-mating isolation mechanisms (Felix et al. 2011). Moreover, only 5–10% of the hybrids could develop to pupation at the first generation and thereafter they could not develop more (Calatayud, unpublished data), corroborating the fact that *B. segeta* and *Busseola nairobica* **n. sp.** are two distinct species. The phylogenetic tree results showed that *B. segeta* and *Busseola nairobica* **n. sp.** belonged to two different clades, corroborating results of the morphological study and reinforcing the separation of *B. segeta* and *Busseola nairobica* **n. sp.** into two different species. The divergence observed between clades indicated two different hypotheses. Divergence between *B. segeta* and *Busseola nairobica* **n. sp.** was 4.9%, a value which was also observed between two close *Sesamiina* species (Moyal, Le Ru, Conlong, et al. 2011). This value was also superior to the one observed for the three mitochondrial *B. fusca* populations (2.6%), and superior to values published for host races in other noctuids (2.3%) (Prowell et al. 2004; Lewter et al. 2006). However, this 4.9% mitochondrial divergence was weaker than the currently observed divergence of 7.8% for the same marker between *B. fusca* and *Busseola nairobica* **n. sp.** This divergence was also weaker than the 8% observed between two distant species in the genus *Spodoptera* (Silvain J.-F., unpublished data). Other studies showed a very weak divergence (2–4%) between "young" species of Lepidoptera (Roelofs et al. 2002; Hebert et al. 2003, 2010; Hajibabaei et al. 2006; Moyal et al. 2010; Moyal, Le Ru, van den Berg, et al. 2011) or a high divergence (9.7–11.2%) between populations of the same species (Salvato et al. 2002). Considering these results, the genetic divergence on its own might not be a suitable parameter to elucidate species differentiation, as discussed by Ferguson (2002). The separation between *B. segeta* and *B.* **n. sp.** is manifest taking into account the mitochondrial and nuclear phylogenies and, in our case, a genetic divergence of 4.9% in noctuids was sufficient to distinguish two different taxa. A combination of these molecular results with pre-mating and post-mating isolation, in the sense of Mayr's "biological species" concept (1942), demonstrated clearly the incompatibility between *B. segeta* and *Busseola nairobica* **n. sp.** and their separation into two different species.

Results of the chemical ecology and phylogeny studies appeared to be congruent. Taking into account all the results of the SMRS in *B. fusca* species and of the reproductive isolation mechanisms between *Busseola* spp., we can draw a hypothesis about their evolutionary history. Referring to Brower (1994), the divergence percentage observed between mitochondrial gene sequences dates the separation between *B. fusca* and the two other species to around three million years ago (6.9% divergence), and between *B. segeta* and *Busseola nairobica* **n. sp.** to around two million years ago (4.9% divergence). Concerning the pheromone, the common ancestors of these three species might have produced a blend of five compounds, including the alcohol. In the *B. fusca* female pheromone, the alcohol is now completely transformed into acetates. Around one and a half million years ago, *B. fusca* was separated in three groups: West, East I and East II haplotypes. The East–West separation was probably due to the Cameroon volcanic line formation and the East separation to the Gregory Rift Valley (Sezonlin et al. 2006). East African haplotypes are nowadays in sympatry and inter-fertile. The West haplotype group is still in allopatry, but pheromone analysis and behaviour experiments showed that no SMRS differentiation occurred between these West and East geographic populations as the pheromone compositions are identical and males and females from the three haplotypes are strongly inter-attractive (Felix et al. 2009). The separation between *B. segeta* and *Busseola nairobica* **n. sp.** could be due either to an allopatric differentiation associated to a geographic barrier, the Gregory Rift Valley, which could have limited gene flow, or to an ecologically driven divergence by host-plant switch.

Conclusion

A multi-disciplinary approach using a combination of ecology, morphology, behaviour, and molecular data allowed us to clarify the species status of *B.* **n. sp**. This new species was described and named as *Busseola nairobica* **n. sp.** The major criteria to separate *B. nairobica* from its sister

species, *B. segeta*, were the ecology (geography and host plants), the morphology of male and female genitalia, the calling periods of females, the non-viability of hybrids, and the molecular data.

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