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Discovery of a disused desaturase gene from the pheromone gland of the moth *Ascotis selenaria*, which secretes an epoxyalkenyl sex pheromone

Takeshi Fujii [a,b,c,*], Masataka G. Suzuki [b,d], Susumu Katsuma [c], Katsuhiko Ito [c], Yu Rong [c], Shogo Matsumoto [b], Tetsu Ando [a], Yukio Ishikawa [c]

[a] Graduate School of Bio-Applications and Systems Engineering (BASE), Tokyo University of Agriculture and Technology, Koganei, Tokyo 184-8588, Japan
[b] Molecular Entomology Laboratory, RIKEN (The Institute of Physical and Chemical Research), Wako, Saitama 351-0198, Japan
[c] Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan
[d] Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Chiba 277-8562, Japan

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ABSTRACT

Female *Ascotis selenaria* (Geometridae) moths use 3,4-epoxy-(*Z,Z*)-6,9-nonadecadiene, which is synthesized from linolenic acid, as the main component of their sex pheromone. While the use of dietary linolenic or linoleic fatty acid derivatives as sex pheromone components has been observed in moth species belonging to a few families including Geometridae, the majority of moths use derivatives of a common saturated fatty acid, palmitic acid, as their sex pheromone components. We attempted to gain insight into the differentiation of pheromone biosynthetic pathways in geometrids by analyzing the desaturase genes expressed in the pheromone gland of *A. selenaria*. We demonstrated that a Δ11-desaturase-like gene (*Asdesat1*) was specifically expressed in the pheromone gland of *A. selenaria* in spite of the absence of a desaturation step in the pheromone biosynthetic pathway in this species. Further analysis revealed that the presumed transmembrane domains were degenerated in *Asdesat1*. Phylogenetic analysis demonstrated that *Asdesat1* anciently diverged from the lineage of Δ11-desaturases, which are currently widely used in the biosynthesis of sex pheromones by moths. These results suggest that an ancestral Δ11-desaturase became dysfunctional in *A. selenaria* after a shift in pheromone biosynthetic pathways.

1. Introduction

Females of many moth species secrete species-specific sex pheromones to attract conspecific males. To date, the sex pheromones of more than 640 lepidopteran species have been reported [1]. The sex pheromones of moths have generally been classified into two types based on the presence (type I) or absence (type II) of a terminal functional group [1,2]. Type-I sex pheromones are composed of C_{10}–C_{18} unsaturated acyclic aliphatic compounds with a functional group such as a formyl, hydroxyl, or acyloxyl group [3]. On the other hand, type-II sex pheromones are predominantly composed of C_{17}–C_{23} hydrocarbons with two or three (*Z*) double bonds at the 3, 6, or 9 positions, and their corresponding epoxy derivatives [4].

Although the final step in the biosynthesis of both types of pheromones occurs in the pheromone gland, which is commonly located at the abdominal terminus of female moths, the two types of pheromones largely differ in their origins and biosynthetic pathways (Fig. S1). Most type-I pheromones are synthesized from a common saturated fatty acid, i.e., palmitic acid, via enzymatic steps such as desaturation, reduction, and acetylation. In contrast, type-II pheromones are synthesized from dietary linoleic or linolenic acid typically via chain elongation, decarboxylation, and subsequent epoxidation [2,5,6]. Thus, the double bonds characteristic to type-II compounds originate from dietary polyunsaturated fatty acids.

The biosynthesis of female sex pheromones has been mainly studied using moth species that secrete type-I pheromones, in particular, the silkworm *Bombyx mori*. Therefore, more information is available on the biosynthesis of type-I pheromones. Among the enzymes involved in the biosynthesis of type-I pheromones, fatty acyl-CoA desaturase (hereafter, 'desaturase') has been extensively studied following pioneering work on the Δ11-desaturase used in sex pheromone biosynthesis in the cabbage looper moth

Abbreviations: bp, base pair; ORF, open reading frame; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RAT, rod-like abdominal tip; RT-PCR, reverse transcription PCR; hpi, hours post infection.

* Corresponding author at: Graduate School of Agricultural and Life Sciences, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan. Fax: +81 3 5841 5061.
E-mail address: pippi.boo@gmail.com (T. Fujii).

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Table 1
Sequences of primers used in this study.

	F/R[a]	Primer sequence from 5′ to 3′
DP	F	GGNATHACNGCNGGNGCNCA
	R	RTGRTGRTARTTRTGRAANCCYTC
Tubulin	F	ATCTGCTCGTCCACCTCCTTCATGG
	R	CCTACTGTATCGACAACGAGGCCC
Asdesat1 GSP	F	GGGTCAAAGATCACAGACTG
	R	TAGAATGAATCCCATGA
Asdesat2 GSP	F	GCGCTGGACTGGGCGCGC
	R	AGGCGAGCGGCATCAGTA
Asdesat3 GSP	F	GGAAACATCCTCAAATCAAGGC
	R	CCGAGAACGACCAGAGATACGGG
sal-Asdesat1 GSP	F	GTCGACCATGGCGCCATACCTCACCG
Asdesat1 GSP-His-sph	R	GCATGCTTAGTGGTGATGATGGTGGTGATCAGCTGATTCCTC
Asdesat1 GSP-sph	R	GCATGCTTAATCAGCTGATTCCTC

[a] F and R indicate forward and reverse primers, respectively.

Table 2
Profiles of desaturase fragments isolated from the pheromone gland of *A. selenaria*.

	No. of clones[†]	Signature motif[‡]	Top hit by a tBLAST X search of the database[a]		
			Species	Description	E value
Asdesat1	3	APSQ	*Manduca sexta*	Acyl-CoA desaturase (d3APTQ gene)	2.00E − 83
Asdesat2	15	KPSE	*Helicverpa assulta*	Acyl-CoA delta-9 desaturase	1.00E − 128
Asdesat3	2	NPVE	*Spodoptera littoralis*	delta-9 desaturase	1.00E − 118

[†] Sequences of 46 clones obtained by degenerate PCR were analyzed. Most of the remaining 26 clones were classified as housekeeping genes, unknown, or read-error by the tBLAST X search.
[‡] Signature motif is the sequence of four amino acid residues in insect desaturases located between the second and third His-boxes (see Knipple et al. [11]).
[a] DNA data bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/Welcome-j.html).

Trichoplusia ni [7]. This membrane-bound enzyme typically introduces a single double bond at a specific position in a saturated long chain fatty acyl-CoA [8–11]. Insect desaturases are characterized by transmembrane domains and three histidine-rich clusters (histidine-boxes), which are presumed to coordinate two iron atoms at the active center [11].

Insect desaturases have been classified into 5 groups, i.e., Δ9 (16 < 18), Δ9 (18 < 16), Δ9 (14–26), Δ10; 11, and Δ14 desaturases based on the position of the double bond formed and the preference of the enzyme for the carbon-chain length of the substrate [10]. Desaturases have a 'signature motif', a sequence of four amino acid residues located between the second and third His-boxes, which was shown to be useful in predicting the function of a newly sequenced desaturase [11]. For example, the signature motifs KPSE and NPVE are characteristic of Δ9-desaturase, while QPVE is characteristic of Δ14-desaturase [11]. Similarly, XXXQ is characteristic of Δ11-desaturase; however, the signature motif in Δ11-desaturase is not conserved as well as that in Δ9-desaturase.

Female moths of the Japanese giant looper *Ascotis selenaria* (Geometridae) use 3,4-epoxy-(*Z,Z*)-6,9-nonadecadiene as the main sex pheromone component, which is presumed to be biosynthesized from dietary linolenic acid. The use of compounds derived from dietary polyunsaturated fatty acids as sex pheromone components has only been observed in moth species belonging to Geometridae, Arctiidae, Noctuidae, and Lymantriidae [1] (Fig. S2). How dietary polyunsaturated fatty acids have become adopted as the precursor of sex pheromone components in these more recently evolved groups of moths is of interest (Fig. S2). In the present study, we attempted to gain insight into the differentiation of sex pheromone biosynthetic pathways in geometrids by analyzing desaturase genes expressed in the pheromone gland of *A. selenaria*. Although no desaturase is currently involved in the biosynthesis of sex pheromones in this species, we considered that we may be able to obtain information on the desaturase that had been working in their ancestors.

2. Materials and methods

2.1. Insects and a cell line

Larvae of *A. selenaria* were collected in a tea garden at Shizuoka, Japan (34.97°N, 138.43°E) and reared on an artificial diet (Insecta LF, Nosan Corp., Yokohama, Japan) at 25 °C under a photoperiod of 16 h light and 8 h dark. The insect cell culture (Sf9) was maintained in TC-100 medium (Life Technologies) with 10% fetal bovine serum as described previously [12].

2.2. Tissue collection and mRNA isolation

To obtain spatiotemporal information on the expression of desaturase-like genes (*Asdesat1–3*), the antennae (Ant), epidermis (Ep), flight muscle (FM), fat bodies (FB), eggs (Eg), midgut (MG), and rod-like abdominal tip (RAT) were carefully dissected from 1- to 3-day-old adult moths of *A. selenaria*. In one experiment, the RAT was divided into the pheromone gland, which is a modified intersegmental membrane between the 8th and 9th abdominal segments [13], and the remaining part. In another experiment, the RAT was excised from female pupae 3 days and 1 day before eclosion, and from newly emerged and 1-day-old female moths. Poly A^+ RNA (mRNA) was isolated from each tissue using the Micro-FastTrack™ 2.0 kit (Life Technologies) according to the manufacturer's instructions.

2.3. Cloning of acyl-CoA desaturase cDNA

One hundred nanograms of mRNA prepared from the pheromone gland of *A. selenaria* was reverse-transcribed using the RNA PCR Kit, Ver. 3.0 (Takara Bio, Ohtsu, Japan) with an oligo-dT adaptor primer. The resultant cDNA was used as the template for cloning. Degenerate primers (DP in Table 1) were designed based on

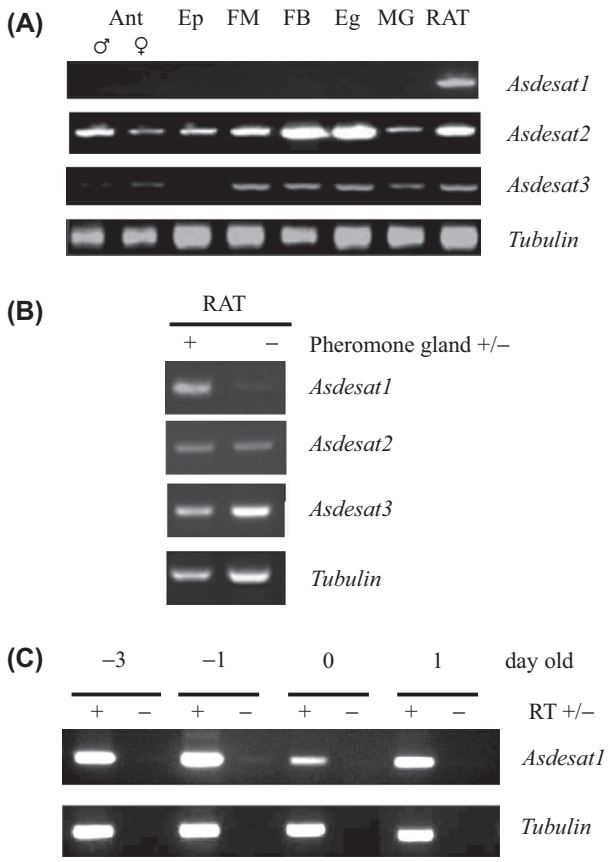

Fig. 1. Tempo-spatial expression patterns of *Asdesat* genes. (A) The distributions of *Asdesat1–3* transcripts in various tissues of *A. selenaria*. Ant: Antennae, Ep: Epidermis, FM: Flight muscle, FB: Fat body, Eg: Egg, MG: Midgut, and RAT: Rod-like-abdominal tip. (B) Expression of *Asdesat1–3* in the pheromone gland (PG+) and remaining part (PG) of the RAT of *A. selenaria* [13]. (C) *Asdesat1* transcripts in the RAT at different developmental stages. Day 0 indicates a newly emerged adult.

the sequence of the region spanning the histidine clusters (His-boxes), which are characteristic of non-heme desaturases. PCR was performed with Ex Taq DNA polymerase (Takara Bio) under the following conditions: 94 °C for 1 min, 5 cycles of 94 °C for 30 s, 37 °C for 30 s, and 68 °C for 40 s, and 30 cycles of 94 °C for 30 s, 42 °C for 30 s, and 68 °C for 40 s. PCR bands produced by the contamination of exogenous sources were distinguished by a parallel PCR performed with the same components, except for the cDNA template (Fig. S3). The full-length sequences of *Asdesat1* and *Asdesat2* were determined by the 3′-and 5′-RACE method using the GeneRacer™ Kit (Life Technologies). To verify the connection between the central and 3′- and 5′- end sequences, PCR was performed with a pair of gene-specific primers designed within the two end sequences.

2.4. Tissue distribution analysis of acyl-CoA desaturase

mRNA (100 ng) prepared from the tissues of adult *A. selenaria* was reverse-transcribed with the SuperScript III First-Strand Synthesis System (Life Technologies). RT-PCR was performed with gene-specific primer pairs for three acyl-CoA desaturases (Asdesat1–3 GSPs in Table 1) and Ex-Taq DNA polymerase (Takara Bio) under the following conditions; 94 °C for 1 min and 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 68 °C for 25 s. In an experiment to determine the timing of *Asdesat1* expression in female pupae, non-transcribed RNA (RT) was used in place of cDNA to monitor the carryover of genomic DNA.

2.5. Secondary structure and phylogenetic analyses

The secondary structures of ASDESAT1 and ASDESAT2 were predicted by the SOSUI program (http://bp.nuap.nagoya-u.ac.jp/sosui/). The phylogenetic tree of desaturases including ASDESAT1 and ASDESAT2 was reconstructed using an online service provided by the DNA Data Bank of Japan (http://clustalw.ddbj.nig.ac.jp/top-e.html). Amino acid sequences were aligned using Clustal W, and the phylogenetic tree was constructed by the neighbor-joining method with the DISTANCE option set to "Kimura." We evaluated the reliability of the inferred tree by bootstrap analysis with 1,000 resamplings.

2.6. Construction of recombinant baculoviruses

Recombinant *Autographa californica* nuclear polyhedrosis viruses (AcNPVs) were constructed using the Bac-to-Bac® Baculovirus Expression System (Invitrogen), as described previously [14]. The coding region of *Asdesat1* with or without the His-tag at the C-terminus was PCR-amplified with a pair of gene-specific primers, which contained restriction enzyme sites (sal-Asdesat1, Asdesat1-His-sph, and Asdesat1-sph) as listed in Table 1. The *Asdesat1* fragments were subsequently cloned into the vector, pFastBac1 (Invitrogen), and bacmids containing the *Asdesat1*s were isolated. Recombinant AcNPVs were generated by transfection with bacmid DNAs as described previously [12]. The resultant AcNPVs carrying *Asdesat1* with and without the His-tag were named Asdesat1-His-AcNPV and Asdesat1-AcNPV, respectively.

2.7. Expression and intracellular localization of recombinant ASDESAT1

Western blotting was performed as described previously [12]. The samples were separated by SDS/PAGE and transferred to Immobilon-P membrane (Millipore). The membrane was incubated with mouse anti-His antibody (Qiagen, 1:3000 dilution) and subsequently with goat anti-mouse IgG-HRP conjugate (1:3000). Immobilon Western Chemiluminescent HRP Substrate (Millipore) was used according to the instruction of the manufacturer, and chemiluminescence was detected using Luminescent Image Analyzer LAS-3000 (Fuji Film).

Sf9 cells were infected with Asdesat1-His-AcNPV, Asdesat1-AcNPV, or LATPG1-His-AcNPV, which carries *latpg1*, a desaturase gene from the moth *Ostrinia latipennis* [15]. We used LATPG1-His-AcNPV as a positive control of the baculovirus expression system producing recombinant desaturases [15]. The supernatant was removed at 72 hpi, and the cells were washed twice in PBS and fixed in 4% formaldehyde in PBST (1% Triton X-100 in PBS) for 10 min. The fixed cells were subsequently washed three times with TBST (0.2% Tween in TBS), permeabilized in 0.5% Tween 20 in TBS for 10 min, and blocked with 4% Block ACE (DS Pharma Biomedical, Japan) overnight at 4 °C. The cells were then incubated with an anti-His antibody (Qiagen, 1:500 dilution) for 1 h, washed three times in TBST, and incubated with an Alexa 488-conjugated goat anti-mouse antibody (Molecular Probes, 1:500 dilution) for 1 h. The cells were subsequently stained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (DOJINDO, 1:3000), and examined by FLoid™ Cell Imaging Station (Life technologies).

2.8. Data deposition

The nucleotide sequences of *Asdesat1* and *Asdesat2* have been submitted to DDBJ (http://www.ddbj.nig.ac.jp/) with Accession Nos. AB373030 and AB373031, respectively.

Fig. 2. Comparison of the deduced amino acid sequence of *Asdesat1* with those of Δ11- and Δ14-desaturases retrieved from public databases. Amino acid residues identical to those of Asdesat1 are represented by hyphens(-), and gaps introduced to maximize alignment are indicated by dots (•). Three histidine-boxes are shaded and the signature motif [11] is double underlined. The transmembrane domains of desaturases, except for ASDESAT1, which were predicted using the SOSUI program, are underlined. ASDESAT1 was predicted to be a soluble protein by SOSUI.

3. Results

3.1. Cloning Asdesat genes

To investigate the acyl-CoA desaturase-like genes expressed in the pheromone gland of *A. selenaria*, we performed RT-PCR analysis with cDNA prepared from the RAT and degenerate primers (DP) shown in Table 1. Cloning and sequencing of the single band of approximately 570 bp (Fig. S3) showed that this band contained many different sequences, three of which appeared to be the fragments of acyl-CoA desaturases (*Asdesat1–3*). The amino acid sequences deduced from the 3 fragments included the following

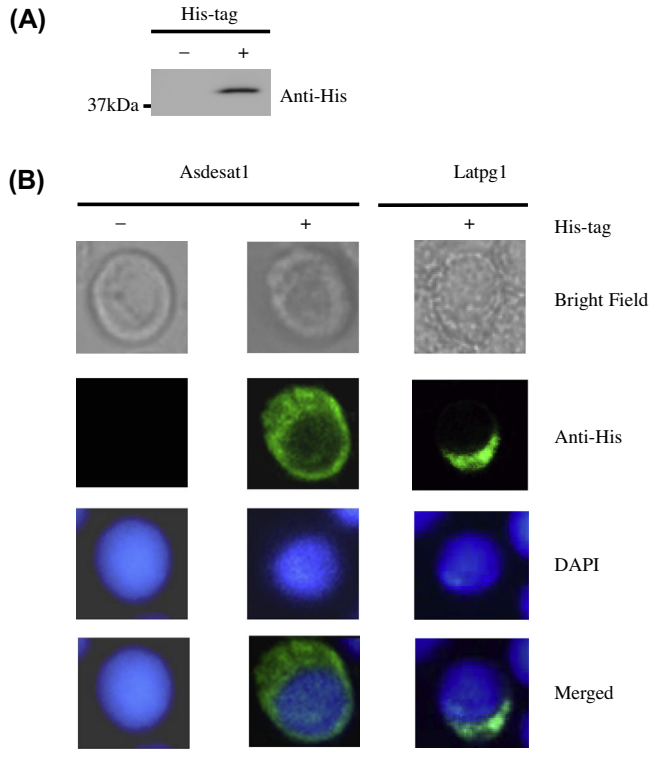

Fig. 3. Expression and localization of ASDESAT1 in Sf9 cells infected with AcNPV. (A) Detection of ASDESAT1-His (+/) expressed in Sf9 cells. The molecular weight of recombinant ASDESAT1 was estimated as 38.9 kDa based on the deduced amino acid sequence. (B) Localization of recombinant ASDESAT1 and LATPG1 (control) in Sf9 cells. The recombinant protein and nucleus are shown in green and blue, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

signature motifs; APSQ in *Asdesat1*, KPSE in *Asdesat2*, and NPVE in *Asdesat3* (Table 2). Signature motifs predicted that *Asdesat1* belonged to Δ11, whereas *Asdesat2* and *Asdesat3* belonged to the Δ9 desaturase family [11]. Among the clones sequenced, *Asdesat2* outnumbered *Asdesat1* and *Asdesat3* (Table 2).

3.2. Tissue distribution of transcripts of three Asdesat genes

The expression of *Asdesat1–3* in the tissues of *A. selenaria* adult moths was examined by RT-PCR with the gene-specific primer sets listed in Table 1 (Fig. 1A and B). The transcription of *Asdesat1* was specific to the RAT of *A. selenaria* (Fig. 1A). Furthermore, the expression of *Asdesat1* was detected specifically in the pheromone gland (Fig. 1B). In contrast, *Asdesat2* and *Asdesat3* transcripts were detected in all or in several tissues examined (Fig. 1A). *Asdesat1* transcripts in the abdominal terminus of pupae were detectable as early as 3 days prior to eclosion (Fig. 1C).

3.3. Sequence analysis of Asdesat1 and Asdesat2 cDNAs

We obtained the complete open reading frame (ORF) of *Asdesat1* because this gene was specifically expressed in the pheromone gland. In addition, we also obtained the ORF of *Asdesat2* because this gene was abundantly expressed in many tissues. The *Asdesat1* gene contained an ORF of 1,011 bp, encoding 337 amino acid residues, with a predicted molecular mass of 38.9 kDa. Although the deduced amino acid sequences around each of the three His-boxes were conserved (Fig. 2), analysis with the SOSUI program predicted that the four presumed transmembrane domains of ASDESAT1 lost their original properties as the transmembrane domain. ASDESAT1 was predicted to be a soluble protein.

The *Asdesat2* gene contained an ORF of 1,056 bp, encoding 352 amino acid residues, with a predicted molecular mass of 40.5 kDa. The deduced amino acid sequence of *Asdesat2* showed a high degree of similarity to Δ9 desaturase from the pheromone gland of *Helicoverpa assulta* (Oriental tobacco budworm) (Table 2). The degeneration of transmembrane domains was not detected in this gene.

We subsequently compared the translated region of *Asdesat1* with other desaturases (Fig. 2). Alignment of the amino acid sequences that were retrieved from public databases with high scores in the Blast-P search indicated that the degree of homology was uncommonly low in the transmembrane domains (Fig. 2).

3.4. Expression and intracellular localization of recombinant ASDESAT1

To obtain information on the intracellular localization of ASDESAT1, recombinant ASDESAT1 with and without the His-tag was expressed by AcNPV (Asdesat1-His-AcNPV and Asdesat1-AcNPV, respectively). Sf9 cells were independently infected with the two AcNPVs and collected at 72 hpi. Western blotting with the anti-His antibody showed that the His-tagged form was expressed with a molecular mass of approximately 39 kDa (Fig. 3A). We next investigated the intracellular localization of the recombinant ASDESAT1 protein in Sf9 cells infected with AcNPVs. The expression of recombinant ASDESAT1 was observed in the entire cytosol, whereas that of LATPG1 was polarized (Fig. 3B).

3.5. Phylogenetic analyses

Consistent with their biochemical functions, the known acyl-CoA desaturases of lepidopteran and dipteran species were largely classified into five clusters, i.e., Δ9 (14–26), Δ9 (16 < 18), Δ9 (16 > 18), Δ10;11, and Δ14 (Fig. 4). *Asdesat2* was classified into the Δ9 (16 > 18) desaturase cluster as predicted by its signature motif KPSE. On the other hand, *Asdesat1* fell between the Δ10;11 and Δ14 clusters, which suggested that *Asdesat1* anciently diverged from the lineage of Δ10;11 desaturases, which are currently widely used in the biosynthesis of sex pheromones by moths. Non-functional desaturases found in *Spodoptera littoralis* (AY362880) and *Choristoneura rosaceana* (AF518018) as well as *Asdesat1* had no common phylogenetic origin (Fig. 4).

4. Discussion

Asdesat1 is suggested to have anciently diverged from the lineage of Δ11-desaturases, which had specifically evolved early in the lepidopteran clade and currently function in the biosynthesis of sex pheromones by the majority of moth species. The degeneration of transmembrane domains in ASDESAT1 suggests that this protein, even if it is produced in the cells, is not likely to take the conformation essential for the formation of an active center; thus, this protein is dysfunctional as a desaturase. Species utilizing type-II pheromones have been identified in a few relatively recently evolved moth families (Geometridae, Arctiidae, Noctuidae, and Lymantridae), and account for approximately 15% of moth species whose sex pheromones have been identified. The distribution of moth families utilizing type-II pheromones in the lepidopteran phylogenetic tree suggests that this trait is derivative. The discovery of a disused desaturase gene in *A. selenaria* is consistent with the assumption that an ancestral Δ11-desaturase, which had been functioning in the ancestors of *A. selenaria,* was disused after the shift in pheromones to type-II. Since not all families in the super-

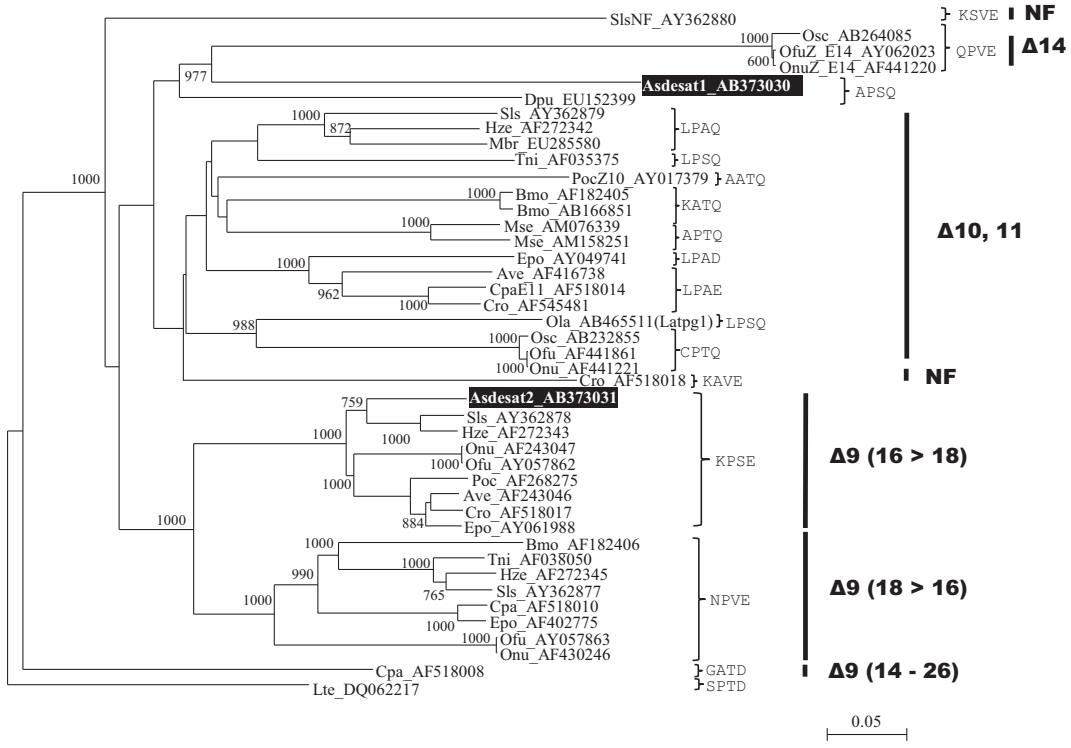

Fig. 4. Phylogenetic analysis of ASDESAT1, ASDESAT2, and other insect desaturases. The phylogenetic tree was reconstructed by the neighbor-joining (NJ) method. A desaturase from the wasp *Lysiphlebus testaceipes* (DQ062217) was used as an outgroup. NF: Non-functional. The accession numbers of the sequences are shown next to the abbreviated species names of insects. Bootstrap values >750 after 1000 re-samplings are shown near the branch. The branch length is drawn to the genetic distance.

family Noctuoidea contain species producing type-II pheromones, the adoption of type-II pheromones appears to have occurred more than once during the course of divergence in the Noctuoidea lineage (Fig. S2).

Asdesat1 transcripts were specifically found in the pheromone gland of *A. selenaria* (Fig. 1A). Moreover, *Asdesat1* transcripts started to appear in the abdominal tip of the female pupae two days ahead of the emergence (Fig. 1C). These aspects are typical of the genes encoding enzymes actually engaged in the biosynthesis of type-I pheromones (for example, see [16]). Whether *Asdesat1* is translated into protein in *A. selenaria* has yet to be examined; however, it is interesting that the promoter controlling the pheromone-gland-specific transcription of *Asdesat1* appears to stay intact. In this regard, it should be noted that the adoption of dietary polyunsaturated fatty acids as the source of sex pheromones is not always associated with the disuse of desaturases. For example, the winter moth *Operophtera brumata* (Geometridae) uses a single hydrocarbon, 1,Z3,Z6,Z9-nonadecatetraene, as its female sex pheromone. The Z3, Z6, and Z9 double bonds in this compound originate from dietary linolenic acid as in ordinary type-II pheromones; however, the terminal double bond was shown to be introduced by a fatty acyl-CoA desaturase [17]. This desaturase was classified into Δ11-desaturase, but was relatively distantly related to a subgroup of Δ11-desaturases currently used for the production of type-I pheromones. Extensive studies on the desaturases expressed in the pheromone gland of moths that produce type-II pheromones may shed light on the transition of type-I to type-II pheromones in moths.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.10.143.

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