frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 29 September 2017
doi: 10.3389/fevo.2017.00115

Comparing the Expression of Olfaction-Related Genes in Gypsy Moth (*Lymantria dispar*) Adult Females and Larvae from One Flightless and Two Flight-Capable Populations

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Specialty section:
This article was submitted to Chemical Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 09 June 2017
Accepted: 12 September 2017
Published: 29 September 2017

Citation:
Clavijo McCormick A, Grosse-Wilde E, Wheeler D, Mescher MC, Hansson BS and De Moraes CM (2017) Comparing the Expression of Olfaction-Related Genes in Gypsy Moth (Lymantria dispar) Adult Females and Larvae from One Flightless and Two Flight-Capable Populations. Front. Ecol. Evol. 5:115. doi: 10.3389/fevo.2017.00115

In insects, flight and sophisticated olfactory systems go hand in hand and are essential to survival and evolutionary success. Females of many Lepidopteran species have secondarily lost their flight ability, which may lead to changes in the olfactory capabilities of both larval and adult stages. The gypsy moth, *Lymantria dispar*, an important forest pest worldwide, is currently undergoing a diversification process with three recognized subspecies: the Asian gypsy moth (AGM), *Lymantria dispar asiatica*; the Japanese gypsy moth (JGM), *Lymantria dispar japonica*; and the European gypsy moth (EGM), *Lymantria dispar dispar*. Females of EGM populations from North America have lost their flight capacity whereas the JGM and AGM females are flight capable, making this an ideal system to investigate the relationship between flight and olfaction. We used next-generation sequencing to obtain female antennal and larval head capsule transcriptomes in order to (i) investigate the differences in expression of olfaction-related genes among populations; (ii) identify the most similar protein sequences reported for other organisms through a BLAST search, and (iii) establish the phylogenetic relationships of these sequences with respect to other insect species. Using this approach, we identified 115 putative chemosensory genes belonging to five families of olfaction-related genes. A principal component analysis (PCA) revealed that the gene-expression patterns of female antennal transcriptomes from different subspecies were more similar to one another than to the larval head capsules of their respective subspecies supporting strong chemosensory differences between the two developmental stages. An analysis of the shared and exclusively expressed genes for three populations shows no evidence that loss of flight affects the number or type of genes being expressed. These results indicate either (a) that loss of flight does not impact the olfactory gene repertoire or (b) that the secondary loss of flight in American EGM populations may be too recent to have caused major changes in the genes being expressed. However, we found higher expression values for

most olfaction-related genes in EGM females, suggesting that differences in transcription rates could be an adaptation of flightless females to their chemical environment. Differences in olfactory genes and their expression in the larvae appear to be unrelated to the flight ability of adult females and are likely adaptations to different ecological pressures.

Keywords: *Lymantria dispar*, transcriptome, odorant receptor, ionotropic receptor, gustatory receptor, odorant binding protein, chemosensory protein

INTRODUCTION

Flight is a leading factor contributing to the evolutionary success of insect species, enabling them to locate food and shelter, avoid predation and competition, and search for optimal oviposition sites for their offspring (Barbosa et al., 1989; Sattler, 1991; Hunter, 1995). Since host–plant location and oviposition in herbivorous insects are largely mediated by chemical cues (Bruce et al., 2005; Bruce and Pickett, 2011; Mescher and De Moraes, 2015), one would expect the evolution of flight to be accompanied by the development of sophisticated olfactory systems. New evidence even suggests that the odorant receptor family (OR), central to the olfactory systems of highly derived insects, emerged around the same time as flight (Missbach et al., 2014; Ioannidis et al., 2017). Furthermore, manipulation of OR-based odor detection in *Drosophila* also indicates that ORs play an important role in flight orientation (Getahun et al., 2016).

The females of many Lepidopteran species have secondarily lost their ability to fly, shifting the responsibility of host selection partly or entirely to the larvae (Barbosa et al., 1989; Sattler, 1991; Hunter, 1995). In this context, it is interesting to investigate whether the loss of flight has an impact on the olfaction of adults and larvae. The gypsy moth *Lymantria dispar* is one of the most important forest pest species worldwide, currently undergoing a diversification process involving the loss of flight by females of some populations (Schweitzer, 2004; Pogue and Schaeffer, 2007). These features make *L. dispar* an ideal model to explore changes in expression of olfaction-related genes that are associated with flight ability.

The first chemosensory proteins (CSPs) from adult *L. dispar* were identified as early as 1989 (Vogt et al., 1989, 1991). Thereafter, Plettner and coworkers have made great contributions to our understanding of olfaction in this species, in particular concerning the structure and function of its pheromone binding proteins (Kowcun et al., 2001; Honson et al., 2003; Honson and Plettner, 2006; Plettner and Gries, 2010; Gong and Plettner, 2011; Yu and Plettner, 2013). Recently, the *L. dispar* olfactory co-receptor (ORCO), a crucial component of olfactory receptor complexes, has been identified (Vosshall and Hansson, 2011; Lin et al., 2015). However, knowledge about olfaction-related proteins and the genes encoding them remains fragmentary for this species.

The gypsy moth is a highly polyphagous herbivore, capable of causing severe and widespread outbreaks in temperate Holarctic regions. At present, there are three recognized subspecies: the Asian Gypsy moth (AGM) *Lymantria dispar asiatica*, the Japanese Gypsy moth (JGM) *Lymantria dispar japonica,* and the European Gypsy moth (EGM) *Lymantria dispar dispar* (which encompasses both European and North American Gypsy moth populations). European Gypsy moth females from North American populations are flightless, possibly due to a founder effect associated with their introduction from Europe in the mid nineteenth century. In contrast, the Asian and Japanese females can fly and disperse over extended distances (Barlow, 2004; NBII, 2011; APHIS, 2013).

The loss of flight in the EGM females restricts their ability to make host-plant choices, transferring the responsibility to the larvae, which disperse either passively through ballooning in the early instars or actively by crawling in the late instars (Capinera and Barbosa, 1976; Lance and Barbosa, 1981, 1982). The extent to which flight capable females are involved in host-plant choices is not yet fully understood, but evidence suggests that both AGM and JGM actively disperse and display oviposition preferences under field conditions (Baranchikov, 1989; Sasaki et al., 2016).

Several efforts have been made to better understand the taxonomic and biogeographic distribution of female flight ability, as well as its heritability and phenotypic plasticity (Keena et al., 2001, 2007, 2008, 2010). However, no studies have yet documented variation in the odor perception systems of *L. dispar* subspecies, despite the likelihood that such differences may accompany the loss of female flight. Therefore, the aims of this study were to (i) Investigate the differences in expression of olfaction-related genes among populations, (ii) identify the most similar protein sequences reported for other organisms through a BLAST search, and (iii) establish the phylogenetic relationships of these sequences with respect to other model insect species, most of which have fully sequenced genomes.

To fulfill these aims we focused on five groups of chemosensory gene families: odorant receptors (ORs), odorant binding proteins (OBPs), CSPs, gustatory receptors (GRs), and ionotropic receptors (IRs). ORs are expressed in the cell membranes of olfactory sensory neurons (OSNs) and are responsible for the detection of odor molecules (Sanchez-Gracia et al., 2009). In general, OSNs will express either ORs or IRs, with the latter mostly tuned to compounds of lower molecular weight (Hallem et al., 2004, 2006; Benton et al., 2009; Silbering et al., 2011). All analyzed Lepidoptera species possess more OR than IR types (Croset et al., 2010; Koenig et al., 2015; van Schooten et al., 2016), and these play a role in the detection of plant volatiles as well as pheromones (Nakagawa et al., 2005; Grosse-Wilde et al., 2006, 2007; Tanaka et al., 2009). In insects, OSNs associated with basiconic or trichoid sensilla express one OR gene, along with the co-receptor ORCO, which is highly conserved and broadly expressed (Krieger et al., 2003; Touhara and Vosshall, 2009).

Insect ORs are seven-transmembrane domain receptors with inverted membrane topology and are not phylogenetically related to vertebrate ORs (Benton et al., 2006).

OBPs contribute to the sensitivity of the olfactory system by binding, solubilizing and transporting odorants through the sensillar lymph (Leal, 2013). CSPs are likely to perform similar roles in chemical communication of insects as OBPs, but unlike these are also expressed in non-chemosensory tissues, and for this reason have been hypothesized to serve additional, as yet undiscovered, functions (Pelosi et al., 2005). Recent evidence suggests that OBPs are an adaptation to the detection of hydrophobic volatiles that became available as olfactory cues in the course of insect terrestrialization (Missbach et al., 2015); however, results in Drosophila suggest a different function for some OBPs (Larter et al., 2016). Structurally, insect OBPs and CSPs generally contain α-helical domains, but folded in two different patterns (Sandler et al., 2000; Lartigue et al., 2002; Tegoni et al., 2004).

GRs are typically expressed in gustatory receptor neurons (GRNs) within the taste sensillae in the mouthparts and are known to detect sugars, bitter compounds and non-volatile pheromones (Montell, 2013). However, some GR genes are also expressed in the antennae, suggesting that some members of this gene family may have an olfactory function (Hallem et al., 2006). This is further supported by the discovery of two GRs in Drosophila that act in the detection of CO_2 (Yao and Carlson, 2010). GR proteins are highly divergent in sequence, sharing as little as 8% amino acid identity across insect species, and it has been hypothesized that the GR gene family is an ancient chemoreceptor family from which insect OR genes subsequently evolved (Robertson et al., 2003; Hallem et al., 2006; Benton, 2015).

IRs are also involved in chemoreception and comprise a large and highly diverse gene family closely related to ionotropic glutamate receptors (iGluR), typically present in the OSNs associated with the coeloconic sensillae in the antennae (Rytz et al., 2013). Recent reports suggest there are multiple variant IRs with different ligand-binding domains that lack the characteristic glutamate-interacting residues (Benton et al., 2009). Unlike ORs, which are exclusively found in pterygote insects, IRs are present in all protostome species studied so far and may have evolved as long as 550–850 million years ago (Croset et al., 2010; Missbach et al., 2014). Similar iGluR-like genes are also present in plants, animals and prokaryotes, indicating that this is an important and ancient group of chemoreceptors (Benton et al., 2009; Rytz et al., 2013).

MATERIALS AND METHODS

Animals

Insects were provided as egg masses by Hannah Nadel, Supervisory Entomologist of the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA). All egg masses came from laboratory cultures that had been maintained using carefully designed mating protocols to avoid the deleterious effects of inbreeding depression, details on the rearing system utilized for these colonies can be found in (Bell et al., 1981). Upon hatching larvae were fed *ad libitum* on artificial wheat germ diet prepared according to manufacturer's instructions (MP Biomedicals LLC, Illkirch, France) and food was replaced twice per week. Caterpillars, pupae, and adult moths were maintained in a climate chamber at 20°C, 60% relative humidity and 16/8 h photoperiod.

The European gypsy moth (EGM) culture (*Lymantria dispar dispar*) originated from flightless *L. dispar* populations collected in New Jersey (US). The Japanese gypsy moth (JGM) culture (*Lymantria dispar japonica*), originated from flight-capable populations coming from the Northern Iwate district and Takizawa, Morika, Nishine (Japan). The AGM culture (*Lymantria dispar asiatica*) originated from flight-capable populations coming from the Primorskiy Krai ports (Vostochnyy, Slavyanka, Vladivostok, Nadhodka) in Russia.

RNA Extraction

RNA extraction was performed following the same procedure as in Koenig et al. (2015), with minor changes as outlined below. Antennae of 50 adult female moths (1–2 days old) from each population were excised from the base of the antennal sclerite. Head capsules from 50 fifth instar larvae from each population were cut at the division point with the prothorax. Tissues were transferred to an Eppendorf tube, cooled with liquid nitrogen and stored at −86°C until extraction. For extraction, tissues were transferred into RL buffer (innuPREP RNA Mini Kit, Analytik Jena, Jena, Germany) and homogenized using a TissueLyser (Qiagen, Hilden, Germany). The resultant homogenate was used with the innuPREP RNA Mini Kit (Analytik Jena, Jena, Germany) following the manufacturers protocol.

Sequencing, Assembly, and Annotation

Total RNA was sent to the Max Planck Genome Centre Cologne (Germany) for construction of TruSeq libraries and subsequent sequencing on an Illumina HiSeq3000. Read data was trimmed and cleaned by the Genome Centre using standard protocols. The resulting Illumina reads were assembled with CLC Genomics Workbench 8 (CLCbio), using the *de novo* algorithm and default parameters. Annotation was performed using Blast2GO 3 (Conesa et al., 2005; Götz et al., 2008). Additionally, assembled transcripts belonging to target chemosensory families (OR, OBP, IR, GR, and CSP) were identified by comparison against custom, manually curated databases created using the available literature on other Lepidopteran species (Wanner and Robertson, 2008; Grosse-Wilde et al., 2011; Heliconius-Genome-Consortium, 2012; Briscoe et al., 2013; Koenig et al., 2015).

Each of the predicted protein sequences was further compared to available sequences using the blastp algorithm and the nr database (NCBI)[1] to identify the most similar sequence, the organism expressing it and its putative function. We only report sequences yielding significant ($E < 0.05$) similarity values.

Alignments and Phylogenetic Trees

Protein sequences conceptually translated from the assembled transcripts were aligned with homologs from *Bombyx mori*,

[1]NCBI https://www.ncbi.nlm.nih.gov/blast/ Accessed 02.02.2017.

TABLE 1 | Normalized expression values (RPKM) for different families of chemosensory genes in the female antennae and larval head capsules of three populations of the gypsy moth *Lymantria dispar*.

		Female antennae			Larval head capsule		
		Normalized gene expression (RPKM)			Normalized gene expression (RPKM)		
Contig	**ID**	**JGM**	**EGM**	**AGM**	**JGM**	**EGM**	**AGM**
CSPs							
58	CSP1	–	–	–	10.801	5.843	3.019
282	CSP2	–	–	0.230	133.508	70.815	97.542
316	CSP3	–	–	–	3.900	1.602	10.032
529	CSP4	–	–	–	362.721	159.136	149.850
546	CSP5	–	–	0.363	757.447	586.861	273.112
2133	CSP6	–	–	–	44.782	14.685	29.899
3155	CSP7	11.326	6.897	16.259	97.599	221.746	319.770
4694	CSP8	–	–	–	46.171	–	0.352
4803	CSP9	3.140	1.954	0.903	6.654	8.187	3.721
4927	CSP10	33.255	**29.942**	13.669	495.729	630.862	168.117
5687	CSP11	1.279	0.239	1.545	16.541	12.390	3.782
6311	CSP12	1.216	6.056	8.222	165.268	23.116	68.945
7764	CSP13	245.022	89.839	343.446	39.865	17.604	81.919
9858	CSP14	–	–	–	7.684	3.130	2.068
10611	CSP15	11.247	0.778	3.056	13.126	12.613	2.014
11424	CSP16	0.084	0.314	0.509	15.545	105.409	66.088
14171	CSP17	299.658	62.941	191.357	17.135	17.746	9.409
20492	CSP18	–	**1.237**	0.572	14.770	2.786	9.797
21710	CSP19	–	–	0.935	2.995	–	3.698
21764	CSP20	–	–	–	212.291	152.589	78.593
24844	CSP21	–	–	–	0.597	0.545	0.768
28449	CSP22	–	**0.438**	0.202	0.519	0.592	2.001
OBPs							
15931	PBP_A*	36.828	**212.317**	54.305	0.674		–
32039	PBP_B*	34.345	**294.223**	95.189	0.098	–	–
32635	PBP_C*	0.605	**38.621**	4.354	–	–	–
33887	PBP_D*	2.019	**14.140**	3.050	–	–	–
34051	PBP_E*	–	**0.831**	–	–	–	–
17785	GOBP_1*	491.190	**3,382.465**	934.072	1.553	7.208	3.330
20294	GOBP_2*	–	–	–	0.842	–	–
80	OBP1	1.705	0.995	1.656	495.207	73.372	228.343
81	OBP2	–	–	–	28.649	4.238	2.320
1985	OBP3	19.360	**30.768**	11.141	182.022	39.913	135.044
2548	OBP4	51.592	**695.792**	184.640	117.613	114.827	150.618
4026	OBP5	0.766	**5.295**	1.455	–	–	–
4999	OBP6	78.013	**233.009**	99.360	67.738	33.244	29.031
5449	OBP7	41.716	–	–	–	–	–
5666	OBP8	–	–	–	15.716	1.984	5.763
11687	OBP9	0.265	–	–	1.756	0.935	–
12090	OBP10	2.555	**6.362**	1.634	9.103	9.695	8.236
18226	OBP11	–	–	–	20.883	7.723	22.590
19950	OBP12	–	–	–	1.877	2.203	–
19951	OBP13	3.772	**10.886**	–	1.517	–	1.170
24041	OBP14	–	–	–	–	7.162	–
24520	OBP15	0.167	**1.089**	0.144	1.474	1.177	0.190
25516	OBP16	1.781	0.416	–	–	–	–

(Continued)

TABLE 1 | Continued

		Female antennae			Larval head capsule		
		Normalized gene expression (RPKM)			Normalized gene expression (RPKM)		
Contig	**ID**	**JGM**	**EGM**	**AGM**	**JGM**	**EGM**	**AGM**
26082	OBP17	–	–	–	1.317	–	–
26834	OBP18	–	–	–	0.554	–	0.570
26945	OBP19	–	–	–	–	3.192	–
31544	OBP20	–	–	–	0.978	2.344	6.919
33379	OBP21	48.640	**587.915**	112.383	0.074	–	–
33405	OBP22	21.469	**132.303**	42.160	0.593	–	–
33456	OBP23	39.212	**171.314**	94.649	0.669	–	–
34786	OBP24	18.142	**65.449**	31.083	–	–	–
34788	OBP25	–	–	3.579	–	–	–
GRs							
587	GR1	0.605	**5.087**	3.135	140.325	98.163	64.391
23948	GR2	0.418	0.130	0.601	0.308	1.757	0.396
32417	GR3	0.374	0.349	0.323	–	–	–
32835	GR4	–	0.471	0.436	–	–	–
33472	GR5	–	–	–	0.596	–	3.065
34141	GR6	0.372	**1.505**	1.070	–	–	–
34172	GR7	0.760	**2.130**	1.313	–	–	–
34277	GR8	–	0.337	0.623	–	–	–
34291	GR9	–	**1.216**	0.321	–	–	–
34451	GR10	–	–	0.347	–	–	–
34464	GR11	–	**0.408**	–	–	–	–
ORs							
20670	ORCO*	0.114	–	–	0.818	3.045	0.712
6380	OR1	0.197	**2.116**	0.766	–	–	–
8989	OR2	3.058	**12.081**	5.635	22.206	18.520	13.248
15892	OR3	0.206	**1.349**	0.713	–	–	–
17788	OR4	–	**1.779**	0.705	–	–	–
27443	OR5	–	–	0.239	–	0.698	0.472
31928	OR6	0.457	**2.218**	0.631	1.617	–	–
32971	OR7	0.099	**1.473**	1.107	0.164	–	–
33087	OR8	–	0.464	0.429	–	–	–
33560	OR9	1.447	**3.860**	1.606	0.171	–	–
33844	OR10	0.577	0.359	1.494	–	–	–
33855	OR11	0.383	**3.357**	1.915	–	–	–
33861	OR12	–	0.409	0.756	–	–	–
33879	OR13	0.707	**1.541**	0.814	–	–	–
33888	OR14	0.367	0.343	–	–	–	–
33903	OR15	0.223	**2.294**	0.578	–	–	–
33963	OR16	0.661	0.925	0.285	–	–	–
33998	OR17	0.759	**1.417**	0.655	–	–	–
34011	OR18	0.761	**1.776**	1.313	–	–	–
34012	OR19	0.378	**2.469**	1.304	–	–	–
34122	OR20	–	**1.178**	–	–	–	–
34175	OR21	0.376	–	0.325	–	–	–
34202	OR22	0.334	**0.779**	1.440	–	–	–
34209	OR23	–	**0.953**	0.881	–	–	–
34210	OR24	–	–	0.800	–	–	–
34217	OR25	0.229	**1.283**	0.198	–	–	–

(Continued)

TABLE 1 | Continued

		Female antennae			Larval head capsule		
		Normalized gene expression (RPKM)			Normalized gene expression (RPKM)		
Contig	**ID**	**JGM**	**EGM**	**AGM**	**JGM**	**EGM**	**AGM**
34226	OR26	0.307	**0.859**	0.265	–	–	–
34270	OR27	0.150	**1.120**	0.518	–	–	–
34280	OR28	–	0.291	0.808	–	–	–
34293	OR29	0.302	**1.412**	0.914	–	–	–
34376	OR30	–	**1.006**	0.558	–	–	–
34421	OR31	–	0.693	1.068	–	–	–
34819	OR32	–	–	0.884	–	–	–
34881	OR33	–	**1.346**	–	–	–	–
IRs							
2720	IR1	–	–	–	6.302	–	16.915
5445	IR2	0.116	**0.731**	0.025	10.520	15.035	13.301
6481	IR3	–	–	–	–	6.003	–
8501	IR4	0.986	**6.627**	7.011	0.022	0.318	0.022
20445	IR5	1.915	**5.653**	1.493	0.034	0.156	–
26528	IR6	1.028	**6.673**	2.796	0.114	0.078	–
30320	IR7	0.323	**2.411**	1.393	–	–	–
32336	IR8	0.423	**1.500**	0.803	0.280	–	–
32470	IR9	–	**0.483**	–	1.859	–	–
32618	IR10	–	**0.720**	0.333	0.213	–	–
33239	IR11	0.391	**2.067**	0.899	0.144	–	–
33881	IR12	1.419	**4.142**	1.021	–	–	–
33900	IR13	1.143	**11.203**	3.233	–	–	–
34101	IR14	0.280	**2.789**	1.611	–	–	–
34111	IR15	0.571	**1.777**	1.314	–	–	–
34374	IR16	–	0.591	0.546	–	–	–
Glu–Rs							
1873	Glu–RX.1*	–	**3.176**	0.117	25.199	28.600	61.456
10442	Glu-RX.2*	0.045	**0.547**	–	8.466	11.092	8.378
10616	Glu-RX.3*	–	**0.676**	–	8.777	7.374	7.925
17885	Glu-RX.4*	0.278	–	–	0.614	–	4.107
26117	Nmdar1*	–	**0.247**	–	3.369	1.070	1.658
3548	Nmdar2*	0.634	**2.748**	0.664	2.228	1.577	1.674

Transcripts have been tentatively labeled following the naming code of closely related sequences (Figures 3–6**).*

CSP, Chemosensory protein; OBP, Odorant binding protein; GR, Gustatory receptor; OR, Odorant receptor; IR, Ionotropic receptor; Glu-R, Glutamate receptor; ORCO, Odorant receptor co-receptor; PBP, Pheromone binding protein; GOBP, General odorant binding protein; Nmdar, N-methyl-D-aspartate receptor; JGM, Japanese gypsy moth; EGM, European gypsy moth and AGM; Asian gypsy moth. Values in bold represent higher expression values for the EGM females in comparison to JGM and AGM populations.

Danaus plexippus, *Heliconius melpomene,* and *Manduca sexta* (Wanner and Robertson, 2008; Grosse-Wilde et al., 2011; Heliconius-Genome-Consortium, 2012; Briscoe et al., 2013; Koenig et al., 2015). In the case of GRs and ORs, sequences from the waterflea *Daphnia pulex* were also included as an outgroup (Peñalva-Arana et al., 2009). For the CSPs and OBPs we included sequences from the Jumping Bristletail *Lepismachilis y-signata* and the Firebrat *Thermobia domestica* (Missbach et al., 2015). In the case of IRs (and Glu-Rs) sequences from *Drosophila melanogaster* have been included (Rytz et al., 2013).

For this purpose, we used MAFFT version 7 (Katoh et al., 2002; Katoh and Standley, 2013) with the "-auto" option. Phylogenetic trees were derived using the program FastTree-2, which uses the maximum likelihood method with a Shimodaira-Hasegawa test to estimate branch support values (Price et al., 2010). Figures were prepared for publication using the FigTree software 1.4.1 (Rambaut, 2007, 2012).

Some transcripts, corresponding to pheromone binding proteins (PBPs), general odorant binding proteins (GOBPs), glutamate receptors (Glu-Rs and Nmdars = N-methyl-D-aspartate receptors) and the ORCO were tentatively labeled following the naming code of closely related sequences.

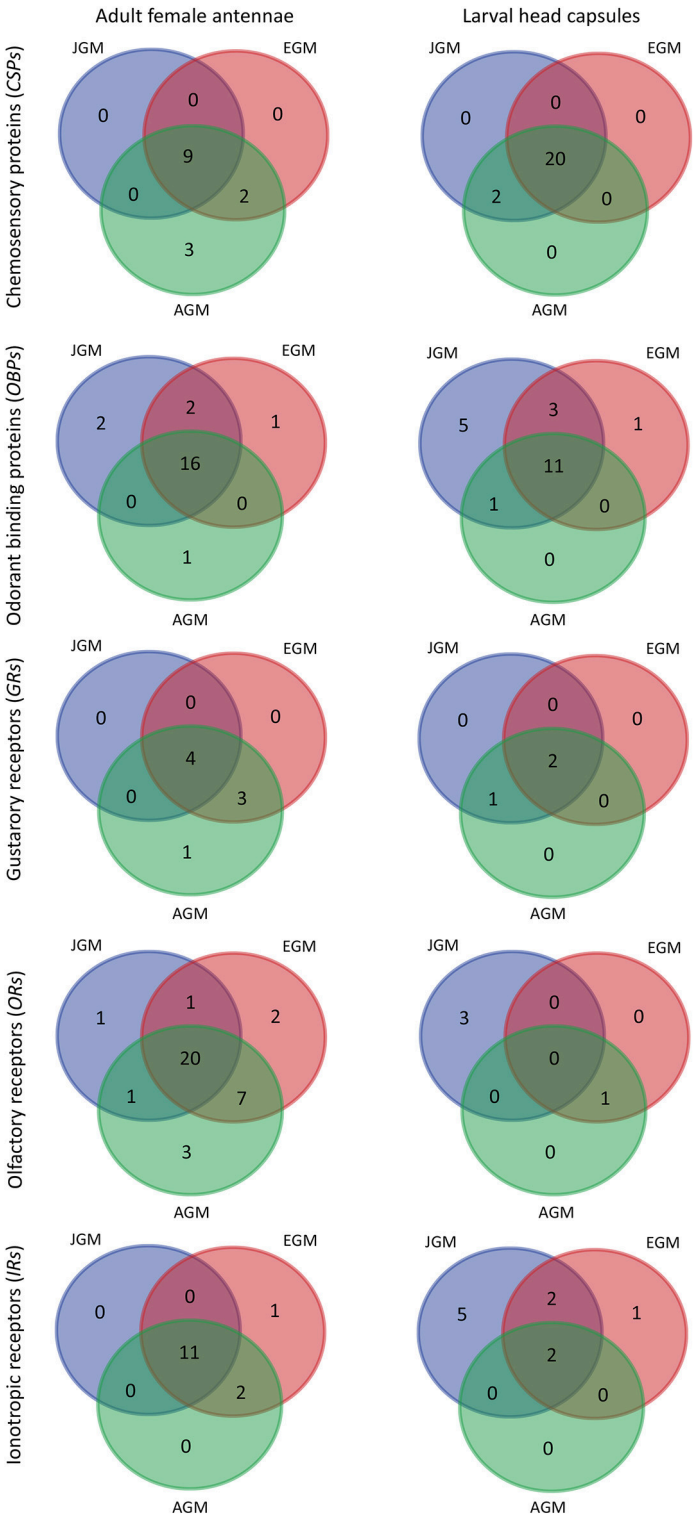

FIGURE 1 | Shared and exclusively expressed genes for three populations of the Gypsy moth for different classes of olfaction-related gene families in both female antennae and larval head capsules.

Quantification of Gene Expression

For the quantification of gene expression levels in the respective tissues/subspecies, the annotated assemblies were used as a template, mapping the raw reads and performing RPKM analysis in CLC Genomics Workbench 8 using default settings. PCA plots were based on normalized count data that was transformed using the regularized log function implemented by the R package DESeq2 (doi: 10.1186/s13059-014-0550-8).

RESULTS

Gene Identification and Expression Patterns for the Three *L. dispar* Populations

We used next generation sequencing to obtain transcriptome assemblies of adult female antennae and larval head capsules from EGM, AGM, and JGM populations of *L. dispar*. The assemblies contained 28,004, 33,208, and 30,820 unique transcripts for EGM, AGM, and JGM populations, respectively. Blastx of the assembled transcripts to the NCBI refseq protein database revealed that that 46.3% (EGM), 52.6% (AGM), and 49.3% (JGM) had high homology ($E < 1e\text{-}5$) to previously characterized proteins at NCBI. To ascertain the transcript coverage of each assembly, we used Blastx to find the proportion of *B. mori* proteins that aligned in a high scoring alignment. We chose *B. mori* because it has one of the best characterized genomes of the Lepidoptera. This analysis showed that an average *L. dispar* transcript encodes just over half the expected protein sequence based on the best blastx hit to *B. mori,* possibly due to a high proportion of partial sequences (**Supplementary Figure 1**).

From the assembled transcripts we were able to identify 115 putative chemosensory transcripts belonging to the five families, 22 CSPs, 32 OBPs (including 2 GOBPs, and four pheromone binding proteins), 11 GRs, 33 ORs, and 16 IRs (**Table 1**). In addition we report 6 glutamate receptors (which are not chemosensory receptors) (**Table 1**). Our results show that 42 olfaction-related genes are found in at least one population in both female antennae and larval head capsules, 52 are exclusive to the female antennae, and 20 to the larval head capsules. A large contribution to the transcripts that are exclusive to the female antennae comes from the ORs (**Table 1**). **Figure 1** depicts the differences and commonalities in gene expression (presence/absence) among the three populations for each chemosensory gene family.

A Principal Component Analysis (PCA) comparing the gene expression patterns (Normalized gene expression values—RPKM) for the antennal and head capsule transcriptomes revealed that female antennal transcriptomes were clustered, being more similar to one another than to the larval transcriptomes of the same population. In contrast, larval transcriptomes were not clustered, but separated along the second component axis (**Figure 2**).

Best Match with Other Protein Sequences

After performing Blast searches with the individual protein sequences, we found that most putative *L. dispar* CSPs have a high sequence homology with those already published for a number of Lepidopteran species, the majority of which are Noctuid moths belonging to the genera *Helicoverpa* or *Spodoptera* (**Table 2**).

Phylogenetic Positioning of Putative Protein Sequences

We constructed phylogenetic trees from alignments of the *L. dispar* CSPs with other published sequences from model insect species (*B. mori, H. melpomene, M. sexta,* and *D. plexippus, D. pulex, L. y-signata*, *T. domestica,* and *D. melanogaster*). Fasta sequences used to construct the trees can be found in Supplementary Files 1–4.

The phylogenetic trees showed that CSPs aligned well within the published sequences, but in a few cases formed clusters containing only *L. dispar* sequences (e.g., CSPs 3, 16, 13, 21; CSPs 2, 8, 14, and 10; CSPs 5, 4, 1) (**Figure 3**). For OBPs most sequences were closely related to those reported for the model species, except OBPs 10, 3, 1, 2, 6, 8, 13, and 14 forming a branch unique to *L. dispar* and a few others forming single nodes (e.g., OBP11) (**Figure 4**).

In the case of the GRs and ORs, sequences are remarkably well nested within those of model species. Of particular interest is GR2 making a single node, and branch containing GR1 and ORs 2 and 4 unique to *L. dispar* (**Figure 5**). For most IRs, we found that the candidate gene sequences were partially aligned with those of the model species, with a few sequences (e.g., IR2) forming single nodes. Most Glu-Rs formed a branch unique to *L. dispar* (**Figure 6**).

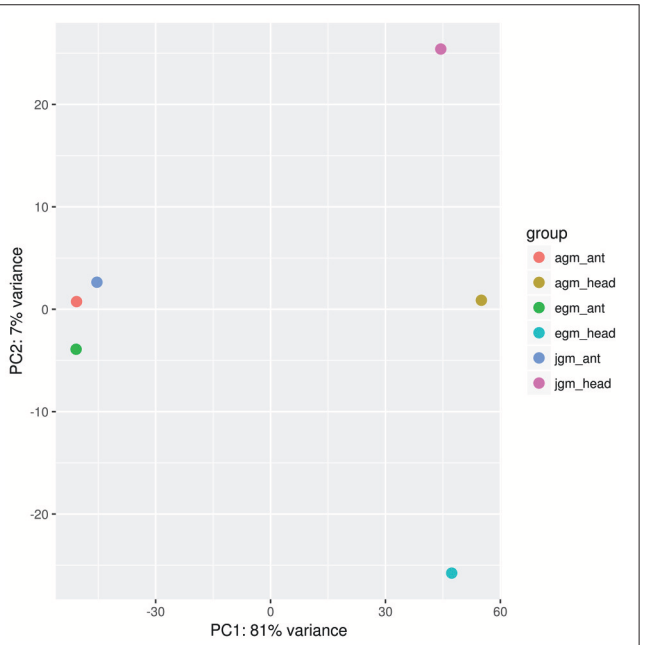

FIGURE 2 | Principal component analysis (PCA), comparing the normalized gene expression patterns for the female antennae (ant) and the larval head capsule (head) transcriptomes of three subspecies of the Gypsy moth (*L. dispar*). The flight capable AGM (Asian gypsy moth) and JGM (Japanese gypsy moth), and the flightless EGM (European gypsy moth).

TABLE 2 | List of *L. dispar* transcripts putatively involved in chemoreception, and characterization for the best hit after comparison with available protein sequences using the BlastP algorithm.

ID	Best match/name	Species	Score	E-value	Acc. Nr
CSPs					
CSP1	Chemosensory protein 1	*Athetis dissimilis (Lep:Noc)*	156	1E-45	ALJ93810
CSP2	Chemosensory protein 12	*Spodoptera exigua (Lep:Noc)*	159	1E-46	AKT26488
CSP3	–	–	–	–	–
CSP4	Chemosensory protein 7	*Spodoptera exigua (Lep:Noc)*	71.2	8E-14	AKT26484
CSP5	Chemosensory protein 24	*Cnaphalocrocis medinalis (Lep:Cra)*	131	2E-36	ALT31606
CSP6	Chemosensory protein	*Helicoverpa assulta (Lep:Noc)*	150	1E-43	ABB91378
CSP7	chemosensory protein 10	*Helicoverpa armigera (Lep:Noc)*	214	2E-68	AFR92094
CSP8	Chemosensory protein 12	*Spodoptera exigua (Lep:Noc)*	132	2E-36	AKT26488
CSP9	Chemosensory protein 16	*Spodoptera exigua (Lep:Noc)*	195	3E-61	AKT26491
CSP10	Chemosensory protein 12	*Spodoptera exigua (Lep:Noc)*	144	4E-41	AKT26488
CSP11	Chemosensory protein 5	*Spodoptera exigua (Lep:Noc)*	182	1E-55	AKT26482
CSP12	Chemosensory protein	*Helicoverpa armigera (Lep:Noc)*	182	3E-56	AIW65100
CSP13	–	–	–	–	–
CSP14	Chemosensory protein 12	*Spodoptera exigua (Lep:Noc)*	161	1E-47	AKT26488
CSP15	Chemosensory protein 25	*Cnaphalocrocis medinalis (Lep:Cra)*	189	2E-58	ALT31607
CSP16	–	–	–	–	–
CSP17	Chemosensory protein 27, partial	*Cnaphalocrocis medinalis (Lep:Cra)*	182	7E-56	ALT31609
CSP18	Putative chemosensory protein	*Sesamia inferens (Lep:Noc)*	97.4	5E-23	AGY49263
CSP19	Hypothetical protein KGM_11196	*Cnaphalocrocis medinalis (Lep:Cra)*	152	1E-43	EHJ76400
CSP20	Bulb-specific protein 3-like	*Papilio machaon (Lep:Pap)*	82.8	2E-17	XP_014365701
CSP21	–	–	–	–	–
CSP22	Chemosensory protein	*Papilio xuthus (Lep:Pap)*	101	2E-24	BAF91714
OBPs					
PBP_A*	Pheromone binding protein 1 precursor	*Sesamia nonagrioides (Lep:Noc)*	165	3E-48	AAS49922
PBP_B*	Pheromone binding protein	*Heliothis virescens (Lep:Noc)*	189	1E-57	CAA65604
PBP_C*	Pheromone-binding protein 3	*Spodoptera litura (Lep:Noc)*	208	4E-65	AIS72934
PBP_D*	Pheromone binding protein 2	*Epiphyas postvittana (Lep:Tot)*	88.6	5E-20	AAL05868
PBP_E*	Pheromone binding protein 3	*Sesamia inferens (Lep:Noc)*	49.3	–006	AEQ30020
GOBP1*	General odorant-binding protein 2	*Heliothis virescens (Lep:Noc)*	424	3E-134	Q27288
GOBP2*	–	–	–	–	–
OBP1	Odorant binding protein	*Spodoptera frugiperda (Lep:Noc)*	139	2E-38	AAR28762
OBP2	Odorant binding protein	*Spodoptera frugiperda (Lep:Noc)*	133	4E-36	AAR28762
OBP3	Odorant binding protein	*Spodoptera frugiperda (Lep:Noc)*	141	3E-39	AAR28762
OBP4	Sericotropin	*Galleria mellonella (Lep:Pyr)*	248	7E-81	AAA85090
OBP5	–	–	–	–	–
OBP6	Odorant binding protein 26	*Spodoptera exigua (Lep:Noc)*	190	3E-58	AKT26503
OBP7	Sericotropin	*Galleria mellonella (Lep:Pyr)*	144	7E-41	AAA85090
OBP8	Odorant binding protein 26	*Spodoptera exigua (Lep:Noc)*	125	5E-33	AKT26503
OBP9	Odorant binding protein	*Spodoptera exigua (Lep:Noc)*	65.1	5E-11	ADY17886
OBP10	Odorant binding protein	*Dendrolimus houi (Lep:Las)*	160	2E-46	AII00969
OBP11	Odorant binding protein 26	*Spodoptera exigua (Lep:Noc)*	141	5E-39	AKT26503
OBP12	–	–	–	–	–
OBP13	Odorant binding protein 26, partial	*Spodoptera litura (Lep:Noc)*	119	8E-33	ALD65900
OBP14	Odorant binding protein 26	*Spodoptera exigua (Lep:Noc)*	89.4	2E-19	AKT26503
OBP15	–	–	–	–	–
OBP16	–	–	–	–	–

(Continued)

TABLE 2 | Continued

ID	Best match/name	Species	Score	E-value	Acc. Nr
OBP17	–	–	–	–	AGH70102
OBP18	Odorant binding protein 9	*Spodoptera exigua (Lep:Noc)*	75.1	8E-15	AGP03455
OBP19	–	–	–	–	–
OBP20	Odorant binding protein 44a, isoform A	*Drosophila melanogaster (Dip:Dro)*	456	3E-148	NP_610358
OBP21	Odorant binding protein 3	*Spodoptera litura (Lep:Noc)*	275	1E-80	AKI87964
OBP22	General odorant binding protein 72-like	*Papilio machaon (Lep:Pap)*	176	4E-53	XP_014369849
OBP23	Odorant binding protein 4	*Spodoptera litura (Lep:Noc)*	184	7E-56	AKI87965
OBP24	Odorant binding protein 13	*Helicoverpa armigera (Lep:Noc)*	253	4E-72	AEB54588
OBP25	Odorant binding protein 1	*Cnaphalocrocis medinalis (Lep:Cra)*	126	2E-34	AFG72998
GRs					
GR1	Uncharacterized protein LOC106133470 (pred)	*Amyelois transitella (Lep:Pyr)*	161	5E-48	XP_013188656
GR2	Ecdysis triggering hormone receptor subtype-A	*Manduca sexta (Lep:Sph)*	535	0	AAX19163
GR3	Gustatory receptor 3, partial	*Athetis dissimilis (Lep:Noc)*	195	5E-60	ALM26253
GR4	Olfactory receptor 1	*Diaphania indica (Lep:Cra)*	84.3	8E-17	BAG71417
GR5	Olfactory receptor 4, partial	*Helicoverpa armigera (Lep:Noc)*	160	6E-45	ACF32962
GR6	Odorant receptor 47, partial	*Athetis dissimilis (Lep:Noc)*	317	8E-102	ALM26237
GR7	Odorant receptor	*Dendrolimus kikuchii (Lep:Las)*	490	3E-169	AII01083
GR8	Gustatory and odorant receptor 24-like (pred)	*Plutella xylostella (Lep:Plu)*	192	2E-58	XP_011558384
GR9	Odorant receptor	*Dendrolimus kikuchii (Lep:Las)*	310	5E-98	AII01083
GR10	Odorant receptor	*Dendrolimus kikuchii (Lep:Las)*	136	9E-36	AII01090
GR11	Odorant receptor, partial	*Helicoverpa armigera (Lep:Noc)*	116	2E-28	AIG51896
ORs					
ORCO*	Protein trapped in endoderm-1 isoform X2 (pred)	*Amyelois transitella (Lep:Pyr)*	349	8E-117	XP_013188595
OR1	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	426	4E-143	AIG51860
OR2	Ecdysis triggering hormone receptor subtype-A	*Manduca sexta (Lep:Sph)*	535	0	AAX19163
OR3	Putative odorant receptor	*Sesamia inferens (Lep:Noc)*	509	6E-175	AGY14579
OR4	Odorant receptor, partial	*Helicoverpa armigera (Lep:Noc)*	59.7	6E-6	AIG51896
OR5	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	86.7	2E-17	AIG51875
OR6	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	335	2E-109	AIG51898
OR7	Putative odorant-binding protein	*Helicoverpa armigera (Lep:Noc)*	169	3E-51	AEJ90553
OR8	Putative odorant receptor, partial	*Sesamia inferens (Lep:Noc)*	286	2E-92	AGY14577
OR9	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	620	0	AIG51879
OR10	Putative olfactory receptor 12	*Spodoptera litura (Lep:Noc)*	274	9E-86	AGG08878
OR11	Olfactory receptor 10	*Helicoverpa armigera (Lep:Noc)*	468	3E-160	AJG42376
OR12	Putative odorant receptor, partial	*Sesamia inferens (Lep:Noc)*	135	5E-39	AGY14575
OR13	Odorant receptor 28	*Athetis dissimilis (Lep:Noc)*	254	9E-80	ALM26217
OR14	Putative olfactory receptor 21, partial	*Ostrinia furnacalis (Lep:Cra)*	147	8E-40	BAR43463
OR15	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	188	9E-55	AIG51873
OR16	Odorant receptor 21	*Athetis dissimilis (Lep:Noc)*	149	6E-41	ALM26210
OR17	Odorant receptor 30a-like (predicted)	*Papilio machaon (Lep:Pap)*	98.6	6E-22	XP_014367947
OR18	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	345	2E-114	AIG51887
OR19	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	268	9E-85	AIG51887
OR20	Olfactory receptor 12, partial	*Helicoverpa assulta (Lep:Noc)*	106	6E-27	AJD81550
OR21	Putative odorant receptor, partial	*Sesamia inferens (Lep:Noc)*	104	4E-26	AGY14570
OR22	Odorant receptor 35	*Athetis dissimilis (Lep:Noc)*	377	1E-127	ALM26225
OR23	Uncharacterized protein LOC106129649 (predicted)	*Amyelois transitella (Lep:Pyr)*	185	1E-53	XP_013183708
OR24	Olfactory receptor 29	*Manduca sexta (Lep:Sph)*	140	3E-37	CUQ99410

(Continued)

TABLE 2 | Continued

ID	Best match/name	Species	Score	E-value	Acc. Nr
OR25	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	130	5E-33	AIG51892
OR26	Odorant receptor, partial	*Helicoverpa armigera (Lep:Noc)*	221	2E-69	AIG51901
OR27	Odorant receptor 41	*Athetis dissimilis (Lep:Noc)*	374	5E-125	ALM26231
OR28	Odorant receptor, partial	*Helicoverpa armigera (Lep:Noc)*	193	3E-57	AIG51872
OR29	Olfactory receptor 56	*Bombyx mori (Lep:Bom)*	169	3E-47	NP_001166617
OR30	Odorant receptor 8	*Athetis dissimilis (Lep:Noc)*	263	8E-83	ALM26196
OR31	Odorant receptor	*Helicoverpa armigera (Lep:Noc)*	273	4E-87	AIG51887
OR32	Odorant receptor 54, partial	*Manduca sexta (Lep:Sph)*	147	9E-41	AFL70817
OR33	Putative olfactory receptor 44	*Spodoptera litura (Lep:Noc)*	328	4E-108	AGG08877
IRs					
IR1	–	–	–	–	–
IR2	Uncharacterized protein LOC106140681, partial (pred)	*Amyelois transitella (Lep:Pyr)*	60.1	1E-5	XP_013197760
IR3	–	–	–	–	–
IR4	Ionotropic receptor 8a.1	*Athetis dissimilis (Lep:Noc)*	1538	0	ALM24945
IR5	Ionotropic receptor 76b, partial	*Helicoverpa assulta (Lep:Noc)*	802	0	AJD81640
IR6	Ionotropic receptor 21a.3	*Athetis dissimilis (Lep:Noc)*	169	1E-45	ALM24946
IR7	Ionotropic receptor 25a, partial	*Helicoverpa assulta (Lep:Noc)*	746	0	AJD81628
IR8	Ionotropic receptor	*Ostrinia furnacalis (Lep:Cram)*	815	0	BAR64811
IR9	–	–	–	–	–
IR10	Ionotropic receptor 75d, partial	*Helicoverpa assulta (Lep:Noc)*	167	5E-49	AJD81642
IR11	Ionotropic receptor 25a, partial	*Helicoverpa assulta (Lep:Noc)*	702	0	AJD81628
IR12	Ionotropic receptor 75q.2	*Athetis dissimilis (Lep:Noc)*	906	0	ALM24940
IR13	Glutamate receptor (pred)	*Bombyx mori (Lep:Bom)*	900	0	XP_012551951
IR14	Ionotropic receptor 31a	*Heliconius melpomene rosina (Lep:Nym)*	498	2E-167	AMM70660
IR15	Ionotropic receptor 75q.1, partial	*Helicoverpa assulta (Lep:Noc)*	290	1E-93	AJD81638
IR16	Putative ionotropic receptor, partial	*Sesamia inferens (Lep:Noc)*	206	6E-65	AGY49252
Glu-R					
Glu-RX.1*	Glutamate receptor ionotropic, kainate 2-like (pred)	*Amyelois transitella (Lep:Pyr)*	494	1E-163	XP_013189500
Glu-RX.2*	Ionotropic glutamate receptor	*Helicoverpa armigera (Lep:Noc)*	1206	0	AIG51930
Glu-RX.3*	Glutamate receptor ionotropic, kainate 2-like (pred)	*Amyelois transitella (Lep:Pyr)*	1095	0	XP_013191608
Glu-RX.4*	Glutamate receptor ionotropic, kainate 2 (pred)	*Plutella xylostella (Lep:Plu)*	331	7E-105	XP_011555112
Nmdar1*	Glutamate [NMDA] receptor subunit 1 (pred)	*Bombyx mori (Lep:Bom)*	546	0	XP_012550364
Nmdar2*	Ionotropic glutamate receptor, partial	*Helicoverpa armigera (Lep:Noc)*	1770	0	AIG51931

Transcripts have been tentatively labeled following the naming code of closely related sequences (see **Figures 3–6). CSP, Chemosensory protein; GR, Gustatory receptor; IR, Ionotropic receptor; OBP, Odorant binding protein; OR, Odorant receptor; ORCO, Odorant receptor co-receptor; PBP, Pheromone binding protein; GOBP, General odorant binding protein; Glu-R, Glutamate receptor; Nmdar, N-methyl-D-aspartate receptor. Only reports yielding significant values (E-value) are shown.*

DISCUSSION

In recent years, considerable progress has been made in our understanding of insect olfaction. Antennal transcriptomes are available for insect species belonging to several orders, including Diptera, Coleoptera, and Lepidoptera (Grosse-Wilde et al., 2011; Andersson et al., 2013; Rinker et al., 2013; Leitch et al., 2015; Zhang et al., 2015). Within the Lepidoptera, the transcriptomes of model species such as *B. mori, D. plexippus, H. melpomene, H. virescens,* and *M. sexta* have been thoroughly investigated (Krieger et al., 2003, 2004; Nakagawa et al., 2005; Wanner et al., 2007; Wanner and Robertson, 2008; Tanaka et al., 2009; Briscoe et al., 2013; Koenig et al., 2015; van Schooten et al., 2016).

This knowledge is rapidly expanding to other economically important species like *Helicoverpa armigera*, *Cydia pomonella,* and *Spodoptera littoralis,* where it could greatly aid in improving already existing and developing new semiochemical-based management strategies (Bengtsson et al., 2012; Jacquin-Joly et al., 2012; Liu et al., 2012). This report represents an expansive characterization of the chemosensory transcripts and their encoded proteins of *L. dispar*, and increases the number of available olfactory-related sequences for Lepidopteran species of

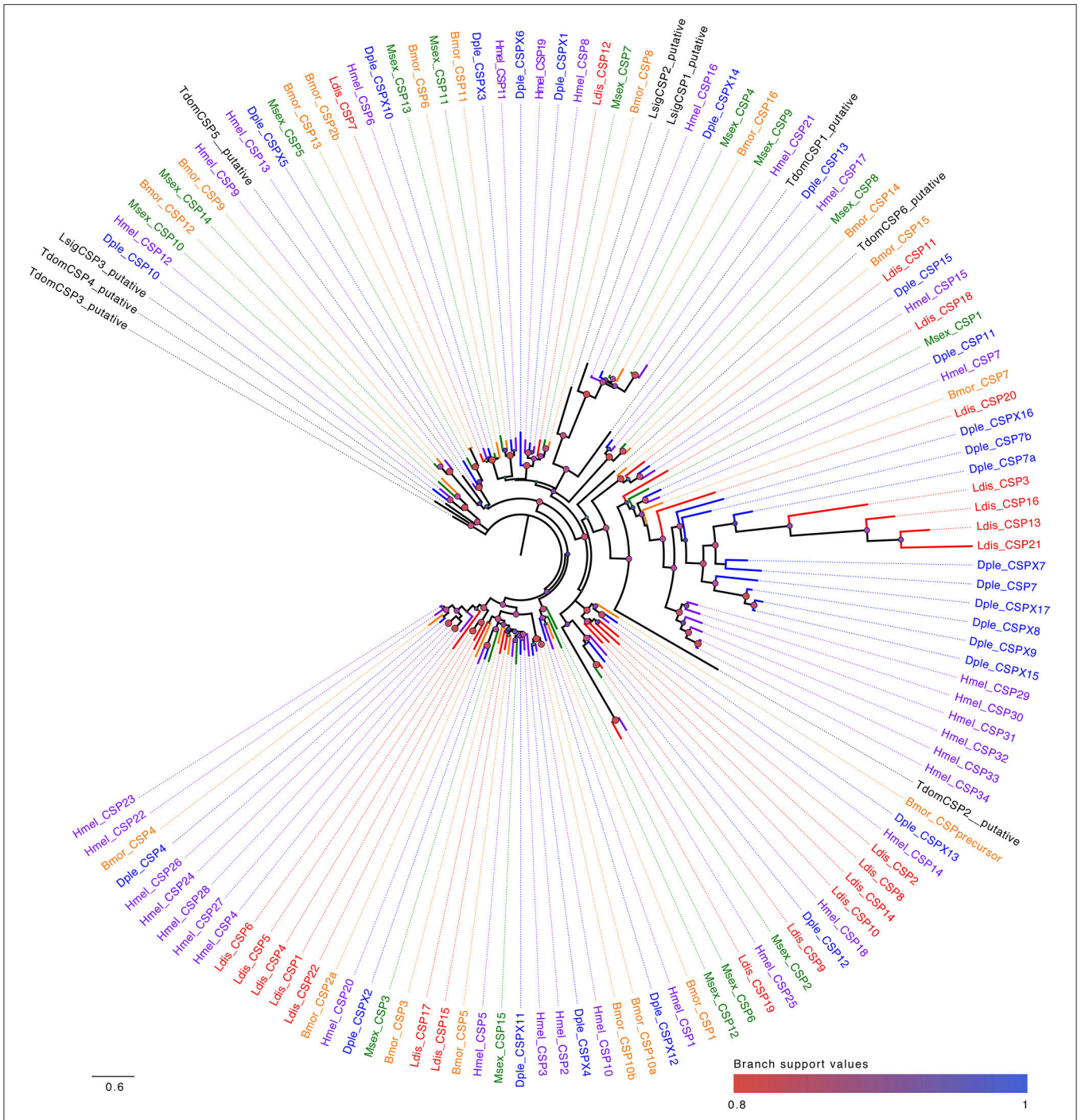

FIGURE 3 | Maximum likelihood dendrogram based on protein sequences of candidate chemosensory proteins (***CSPs***). Included are the putative sequences for *Lymantria dispar* (Ldis) plus those available for model Lepidoptera species *Bombyx mori* (Bmor), *Danaus plexippus* (Dple), *Heliconius melpomene* (Hmel), and *Manduca sexta* (Msex), we also included sequences from the Jumping Bristletail *Lepismachilis y-signata* (Lsig) and the Firebrat *Thermobia domestica* (Tdom).

agricultural relevance. Our results may also help unveil how the expression of chemosensory genes changes throughout insect development and as a result of speciation processes, together with similar reports for other species (Poivet et al., 2013; Zhang et al., 2014, 2015; Walker et al., 2016).

We identified a total of 115 putative olfactory transcripts for *L. dispar*. This number is similar to the one reported on a previous study comparing *S. littoralis* adult antennae and larval head capsules (127) (Poivet et al., 2013), and another study investigating the adult antennal transcriptome of *H. armigera*

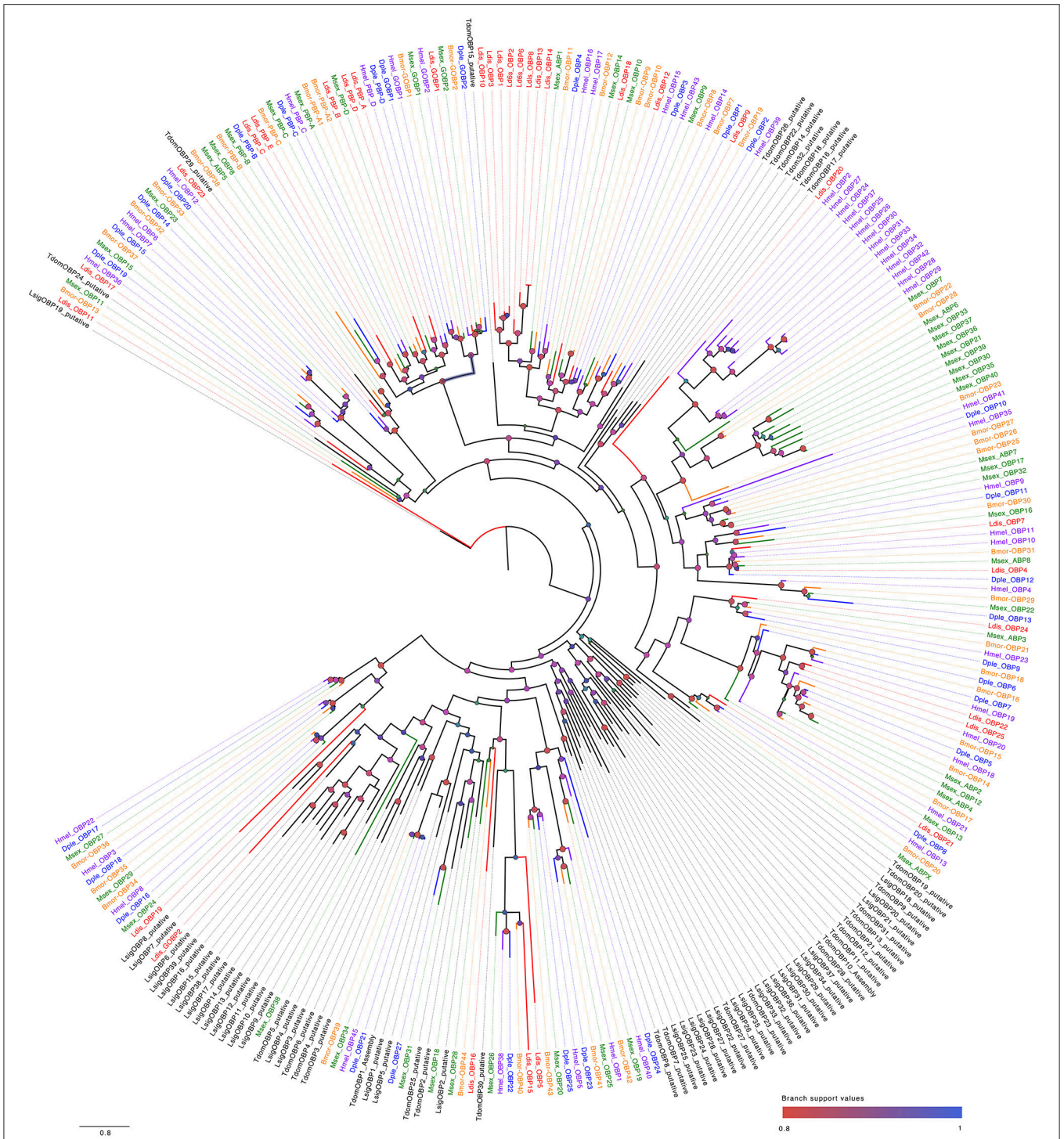

FIGURE 4 | Maximum likelihood dendrogram based on protein sequences of candidate odorant binding proteins ***(OBPs).*** Included are the putative sequences for *Lymantria dispar* (Ldis) plus those available for model Lepidoptera species *Bombyx mori* (Bmor), *Danaus plexippus* (Dple), *Heliconius melpomene* (Hmel), and *Manduca sexta* (Msex), we also included sequences from the Jumping Bristletail *Lepismachilis y-signata* (Lsig) and the Firebrat *Thermobia domestica* (Tdom).

(131), and *H. assulta* (129) (the latter did not include GRs and we excluded sensory neuron membrane proteins from the total count) (Zhang et al., 2014). The conserved number of olfaction-related genes suggests a core group of genes control olfaction in moth species belonging to the superfamily Noctuoidea (Kristensen et al., 2007; Zahiri et al., 2011). Given the similar number of genes across these species, we could speculate that olfactory differences emerge as a product of functional

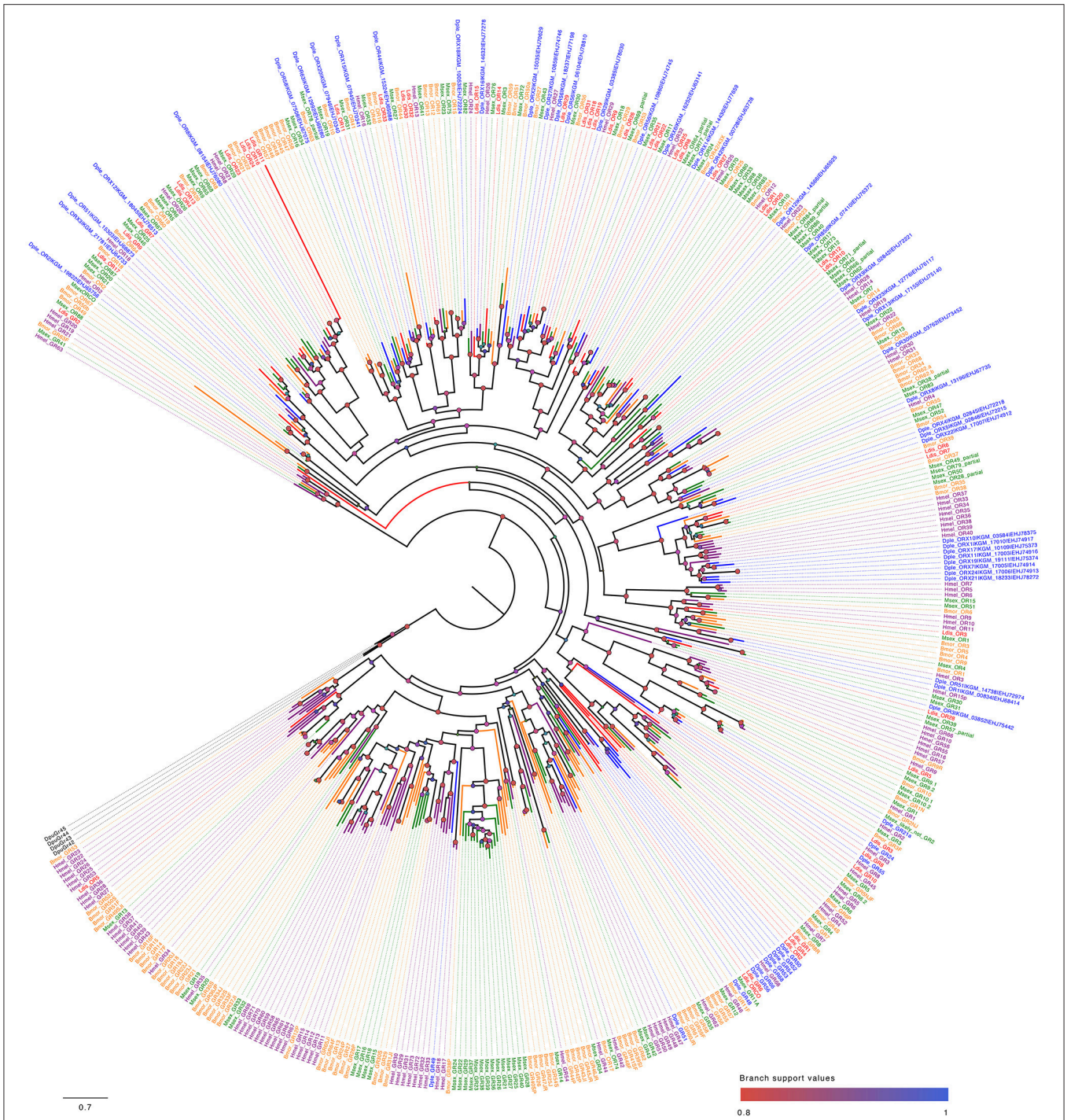

FIGURE 5 | Maximum likelihood dendrogram based on protein sequences of candidate gustatory receptors (***GRs***) and odorant receptors (***ORs***). Included are the putative sequences for *Lymantria dispar* (Ldis) plus those available for model Lepidoptera species *Bombyx mori* (Bmor), *Danaus plexippus* (Dple), *Heliconius melpomene* (Hmel), and *Manduca sexta* (Msex), we also included sequences from the waterflea *Daphnia pulex* (Dpu) as an outgroup.

diversification, while the genes themselves are products of duplication. However, at this stage we can't rule out specific expansions of certain gene clusters balanced by contraction in others, and the evaluation of this possibility must await a more detailed understanding of olfactory differences in the Noctuoidea.

A study investigating expression patterns between adults and larvae of *S. littoralis* found that adults and larvae express similar

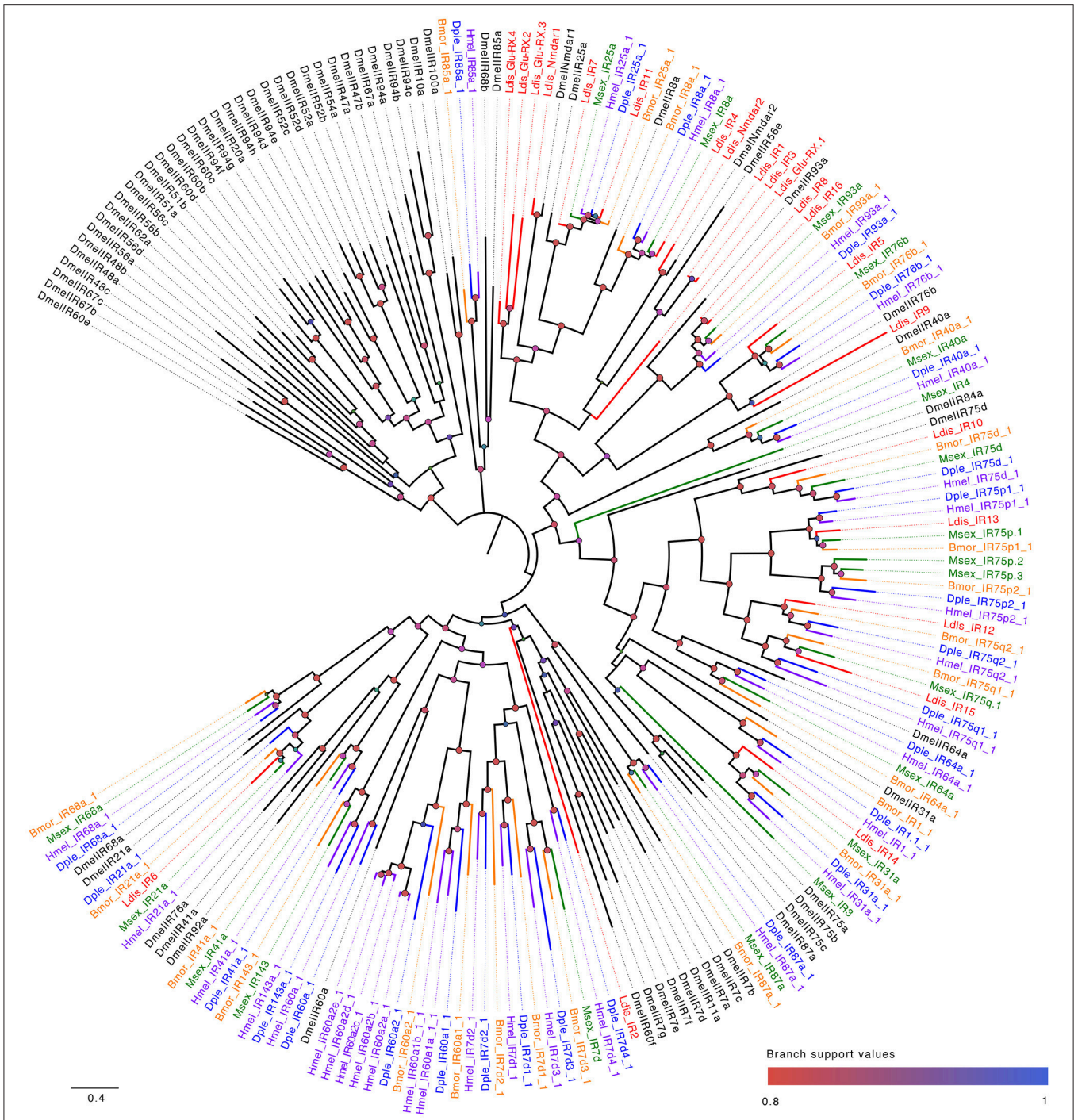

FIGURE 6 | Maximum likelihood dendrogram based on protein sequences of candidate ionotropic receptors (***IRs***). Included are the putative sequences for *Lymantria dispar* (Ldis) plus those available for model Lepidoptera species *Bombyx mori* (Bmor), *Danaus plexippus* (Dple), *Heliconius melpomene* (Hmel) and *Manduca sexta* (Msex), we also included sequences from the fruit fly *Drosophila melanogaster* (Dmel).

numbers of OBPs and CSPs, while the caterpillar OR and IR repertoires were much smaller than the adult ones, and some GRs were found to be adult-specific (Poivet et al., 2013). We also encountered a similar number of CSPs being expressed in both stages and reduced IR, OR, and GR repertoires in the larval stages. However, in contrast to the previous study, we found that larvae had a higher CSP repertoire than adults including eight larval-specific genes. This pattern could reflect species-specific adaptations since in *S. littoralis* host-plant selection is mainly accomplished by adult females, who make suitable choices for the

larvae as eclosion occurs rapidly after egg laying (Anderson and Alborn, 1999; Proffit et al., 2015). In contrast, *L. dispar* eggs of all populations undergo an overwintering process accompanied by changes in the distribution and quality of the resources from oviposition until larval hatching (Barbosa et al., 1989; Sattler, 1991; Hunter, 1995). Therefore, larval stages need to make host-choices to a greater or lesser extent, which may explain the observed differences in the number of CSP genes being expressed in the larval stages.

The strong reduction in the ORs in larvae vs. adults seems to be commonplace in insects and has been reported for a number of species, including *D. melanogaster, Aedes aegypti, M. sexta,* and *B. mori* (Hallem et al., 2004; Kreher et al., 2005; Bohbot et al., 2007; Tanaka et al., 2009; Koenig et al., 2015). In *L. dispar,* this reduction is quite dramatic, with only six ORs being expressed in the larvae vs. 35 in the female antennae. Results from the PCA analysis indicate that gene expression patterns of female antennal transcriptomes from different subspecies are more similar to one another than to the larval head capsules of their respective subspecies, further supporting strong differences in chemosensory perception between adult and larval stages.

After exploring the amount of shared and exclusively expressed genes for three populations (**Figure 1**), we observed that AGM and EGM populations share a high number of commonly expressed genes, whereas the JGM population appears to be more divergent, having a high number (14) of uniquely expressed genes. These results suggest that the observed differences are unrelated to flight capacity, indicating either that (a) loss of flight does not impact the olfactory gene repertoire or (b) the secondary loss of flight in the American EGM populations may be too recent to have caused major changes in the genes being expressed.

Interestingly, females from the flightless EGM population display higher gene expression values (RPKM) when compared with JGM and AGM females for most olfaction-related genes except CSPs (**Table 1**). This could indicate that changes in transcription rates could play an important role in the adaptation of flightless females to their chemical environment. The high variability in olfactory genes and their expression in the larvae suggest that these patterns are unrelated to loss of flight, and we speculate that they are rather adaptations to different ecological pressures.

A detailed comparison of the protein sequences with those reported for other Lepidopteran species through Blast searches and phylogenetic trees supports the common ancestry and high degree of conservation for most olfaction-related gene families within the Lepidoptera, and reveals a high sequence similarity between *L. dispar* and other members of the Noctuidae clade, particularly for ORs and GRs. A recent study investigating the evolution of these chemoreceptors in the Lepidoptera suggests that the common ancestor of this clade harbored only few OR and GR genes, and that while the number of genes increased greatly during the evolution of the clade, it remained relatively low in comparison to other insect groups. This high degree of conservation possibly occurred because olfaction-related gene expression in the Lepidoptera is under strict regulatory control, limiting the establishment of newly emerged genes (Engsontia et al., 2014).

Although most of our candidate sequences had close alignments with those reported for other model species, a few cases remain where *L. dispar* sequences were observed to form clusters or single nodes. Further studies are required to confirm the identity of these sequences and establish whether lineage-specific gene expansion occurs in the Lymantriinae clade (including closely related species such as the douglas-fir tussock moth *Orgyia pseudotsugata* and the nun moth *Lymantria monacha*) or the superfamily Noctuoidea (including more distantly related species such as *Spodoptera* spp. and *Helicoverpa* spp.).

CONCLUSIONS

This work represents the most complete description of chemosensory genes and proteins for *L. dispar* to date. Our results reveal differential gene expression between adult and larval stages characterized by fewer IR, OR, and GR genes being expressed in the larvae, but more CSP genes in comparison to the adults. Comparisons of protein sequences with those from other Lepidopteran species and organisms from different taxa support the common ancestry and high degree of conservation for most olfaction-related gene families. The gene expression patterns in female antennae are more similar to one another than they are to their respective larval stages, whereas larval gene expression patterns are highly divergent across populations. After exploring the number of unique and commonly expressed genes, AGM and EGM populations were found to share a high number of commonly expressed genes, whereas the JGM population appeared to be more divergent. These results indicate that either (a) loss of flight does not impact the olfactory gene repertoire or (b) the secondary loss of flight in American EGM populations may be too recent to cause major changes in the genes being expressed. Nevertheless, higher expression values for GRs, IRs, OBPs, and ORs in EGM females suggest that differences in transcription rates could be an adaptation of flightless females to their chemical environment. Differences in the larval olfactory-related gene expression, on the other hand, are likely responses to unique ecological pressures rather than to female flight ability. Further studies are required to understand the deeper evolutionary and ecological significance of these findings.

AUTHOR CONTRIBUTIONS

AC, EG, CD, MM, and BH designed research; AC and EG collected data; AC, EG, and DW analyzed data; all authors contributed to the writing process. All authors read and approved the submitted version.

FUNDING

This project was possible thanks to the funding provided to AC by the International Postdoctoral Fellowship

Program in Plant Science "Plant Fellows," A Marie Curie initiative managed by the Zurich-Basel Plant Science Center, and funds from ETH Zurich and the Max Planck Society.

ACKNOWLEDGMENTS

We would like to express our gratitude to Hannah Nadel, Supervisory Entomologist of the Animal and Plant Health Inspection Service (APHIS) of the United States Department of Agriculture (USDA) for providing the insects used in this study. We would also like to acknowledge the reviewers for their constructive criticism and valuable suggestions.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2017.00115/full#supplementary-material

Supplementary Figure 1 | Percent of *Bombyx mori* protein recovery in a blast search for three subspecies of the Gypsy moth (*L. dispar*). The flight capable AGM (Asian gypsy moth) and JGM (Japanese gypsy moth), and the flightless EGM (European gypsy moth).

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.