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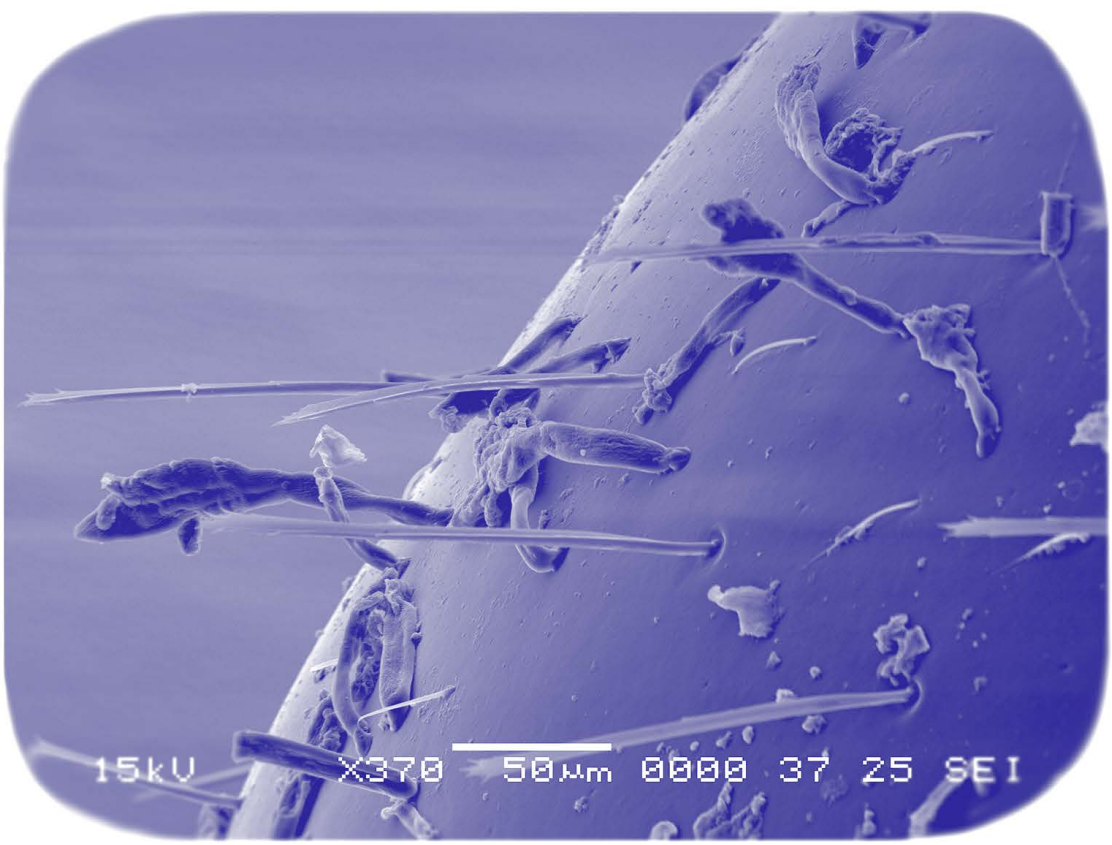

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1

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Entomological diversity associated with tomato cultivation under organic shelter in the El-Outaya Region, Biskra (Algeria)

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Abstract. The aim of the present work is to determine harmful and beneficial insects associated with tomato greenhouse cultivation in the arid region of El-Outaya (Biskra, Algeria). Insect trapping was conducted using yellow water plates and yellow glue traps. Faunistic data have revealed the presence of 2754 individuals distributed on seven orders, 33 families and 38 species of insects. From specific point of view, the majority of these insects were represented by potential insect pests with sixteen phytophagous species amongst them three species attacking tomatoes. Whereas nine species are predators and two pollinators. Our results can be used to plan strategies for the management of harmful pests and beneficial insects associated with the tomato greenhouse crops in this locality.

Keywords: Entomofauna, inventory, tomato, greenhouse, arid.

Introduction

Greenhouse tomato production offers an opportunity for growers to produce a marketable product at times when supplies are low. Greenhouse tomatoes are not an easy crop to grow profitably. Besides growing time, temperatures, pollination, irrigation, fertilization, weeds in a soil system, diseases

and pests require different management techniques than outdoor crops (Rutledge, 2015). Although growing tomato is labour intensive, greenhouse production of tomato is getting increasingly popular. Even though greenhouse can control the growing environment of tomato, problem such as pests and diseases build-up, can occur (Sainju and Dris, 2014). In Algeria greenhouse tomato production reaches 129 0829.7tonnes during 2014-2015a gricultural campaign (MADRP, 2016). Although Biskra province (South east Algeria) is famous for date cultivation that represents the principal agricol product in Algeria, in the mid of the 1980's, greenhouses have appeared on Biskra's oases marges, opening the way to new agricol dynamics (Amichi *et al*., 2015). This region is the largest producer of greenhouse tomato at the national level with a production reaching more than 91300 tonnes (MADR, 2009), which represents more than 50% of the total national production. Despite this importance, few studies tackled the total load greenhouse plants fauna in Biskra region. While in Algeria, previous studies have often targeted a-well-defined species in order to estimate its damage and / or to know its life cycle (Houamel, 2013; Yahoui, 2015; Badaoui, 2018; Ourchene, 2019). Meanwhile numerous studies worldwide (*e.g.* Tonessia *et al*., 2018; Son *et al*., 2018; Patouma *et al*., 2020) focused on this subject. Hence, our article aims to identify the insect diversity and abundance by listing different orders, families, genus and species of tomato greenhouse cultivation under Bio-conditions in El-Outaya (Biskra, Algeria).

Material and Methods

Study area

This study was conducted at CRSTRA's Bio-Ressources Experimental Station in El-Outaya (Plaine, north-east of Biskra; 34 ° 55'41 73 "N, 5 ° 38'59 86" E, 263 m). The arid climate of this region is characterized by low rainfall and high evapo-transpiration.

Tomato (*Solanum lycopersicum* L., 1753) seedlings aged of 48 days of the Tofanea standard, large and vigorous hybrid variety (Chenafi *et al*., 2020; Assassi *et al*., 2017) were sown on 12/09/2017, irrigated and monitored daily in the nursery of the station, they were transplanted on 29/10/2017 in two different semi-closed greenhouses of an area of at least 200 m2 (20 × 10), on 8 lines (at the rate of 384 and 372 plants in the two half-greenhouses). Irrigation is done by a drip system at frequencies depending on the soil situation which is of the loam-clay type. The farming technics used are plowing, mulching and weeding. No chemical fertilisers neither phytosanitary treatment were used during the whole period of the test.

Sampling methods

The inventory of the entomological fauna took place in the two half-greenhouses during the period between September 2017 and April 2018. At ground level a yellow water plate is used and a yellow glue trap is installed at a height of 30 cm above of culture in each half-greenhouse. Insect trapping was done from the transplanting stage to fruiting and maturity of the first fruits while the sampling frequency is one week. Identification of the harvested specimens is made possible by specialized books and keys (Chinery, 1988; D'Aguilar and Fraval, 2004; Tolman and Lewington, 1999; Wolfgang and Werner, 2009).

Temperature and humidity were recorded during the experiment in both half greenhouses to check their effect on insect's diversity.

Diversity evaluation

Family diversity was evaluated by Shannon-Weaver index. This index permits to evaluate taxa diversity (in our case the family) of each half greenhouse is calculated (Magurran, 2004), of which formula is as follows:

$$H' = \sum p_i \ln p_i.$$

With ***H'***: Shannon biodiversity index; ***i***: a species from the study environment; ***Pi***: the relative frequency of the species.

The ratio of the number of species common to two districts to the total number of species collected in the two districts together reported by Jaccard (1912) as coefficient of community (Number of species common to the two districts / Total number of species in the two districts * 100); is used to evaluate similarity between the two half greenhouse with the formula:

$$\text{Jaccard index} = Nc/(N_1+N_2-Nc)$$

With Nc: Common number of taxons (in our case the family) between two half greenhouses, N1 and N2 taxon numbers present respectively in the half greenhouse G1 and G2.

Results

Diversity of insect species associated to tomato growing under greenhouse

The identification revealed sequence presences in 33 families and 38 genera and species of insects with significant species richness in the order of Diptera (Tab. 1).

Table 1. Different families, genera and species of insects inventoriedin Biskra, Algeria.

Order	Families	Species
Coleoptera	Meloidae	*Lytta vesicatoria*
	Chrysomelidae	*Phyllotreta* sp.
	Coccinellidae	*Coccinella septempunctata*
	Buprestidae	*Acmaeodera* sp.
	Cryptophagidae	*Cryptophagus* sp.
	Staphylinidae	*Anotylus tetracarinatus*
Diptera	Syrphidae	*Sphaerophoria philanthus*
		Episyrphus sp.
		Lapposyrphus lapponicus
	Agromyzidae	*Liriomyza bryoniae*
	Calliphoridae	*Lucilia* sp.
	Muscidae	*Musca domestica*
	Chironomidae	*Chironomus* sp.
	Culicidae	*Culex* sp.
	Sciaridae	*Neociara* sp.
	Tephritidae	*Rhagoletis* sp.
	Simuliidae	*Simulium* sp.
Hemiptera	Aphididae	*Macrosiphum euphorbiae*
		Aphis sp.
		Myzuspercicae
	Cicadellidae	*Cicadella* sp.
	Delphacidae	*Laodelphax* sp.
	Psyllidae	*Cacopsylla* sp.
Hymenoptera	Microgastrinae	*Cotesia glomerata*
	Opiinae	*Opius* sp.
	Pemphredonidae	*Diodontus* sp.
	Cryptidae	*Dichrogaster aestivalis*
	Bauchinae	*Exetastes syriacus*
	Andrenidae	*Andrena* sp.
	Apidae	*Apis mellifera*
Lepidoptera	Guelechiidae	*Tuta absoluta*
	Nymphalidae	*Cynthia (=Vanessa) cardui*
Neuroptera	Chrysopidae	*Chrysoperla carnea*
	Hemerobiidae	*Hemerobius* sp.
Thysanoptera	Aeolothripidae	*Aeolothrips intermeduis*
	Melanthripidae	*Melanthrips fuscus*
	Thripidae	*Frankliniella occidentalis*
		Odontothrips confusus

The Diptera was richest order (nine families), followed by Hymenoptera (seven families) and Coleoptera (six families). The richest suborder Brachycerea with five families, followed by the suborder Nematocerea with four families; however, the richest families were determined to be Syrphidae and Aphididae, both with three genera and species. Thus, 16 species or genera are phytophagous. Consisting of *Lytta vesicatoria* (Linnaeus, 1758) (Coleoptera: Meloidae), *Phyllotreta* sp., *Acmaeodera* sp., *Liriomyza bryoniae* (Kaltenbach, 1858) (Diptera: Agromyzidae), *Rhagoletis* sp., *Macrosiphum euphorbia* (Thomas, 1878) (Hemiptera: Aphididae), *Aphis* sp., *Myzus percicae* (Sulzer, 1776) (Hemiptera: Aphididae), *Cicadella* sp., *Laodelphax* sp., *Cacopsylla* sp., *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Gelechiidae), *Cynthia cardui* (Linnaeus, 1758) (Lepidoptera: Nymphalidae), *Melanthrips fuscus* (Sulzer, 1776) (Thysanoptera: Melanthripidae), *Frankliniella occidentalis* (Pergnade, 1895) (Thysanoptera: Thripidae) and *Odontothrips confusus* (Priesner, 1926)(Thysanoptera: Thripidae).

Abundance of individuals, families and species

Samples taken during our experiment captured a total of 2745 insects including 1697 insects in the yellow plates and 1048 in the glue traps their distribution and Shannon Index among orders are shown in (Tab. 2).

Table 2. Number of individuals, families and Shannon Index per orders.

Orders	**Greenhouse1**		**Greenhouse 2**		**Total**		
	Ind* (Water traps)	Ind (Glue traps)	Ind. (Water traps)	Ind. (Glue traps)	Ind	Fam*	Spc*
Coleoptera	14	3	2	2	21	6	6
Diptera	602	322	699	534	2157	9	11
Hemiptera	205	6	46	10	267	4	6
Hymenoptera	13	6	26	22	67	7	7
Lepidoptera	3	2	11	1	17	2	2
Neuroptera	0	1	0	2	3	2	2
Thysanoptera	58	53	18	84	213	3	4
Total	895	393	802	655	2754	33	38
Shannon index	**0.927**	**0.640**	**0.553**	**0.652**	**0.781**		

Legend: Ind: number of individuals, Fam: number of families, spc: number of species.

The captured insects belong to the orders Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera, Neuroptera and Thysanoptera. The Diptera is the largest order with 2157 captured specimens. It is followed respectively by

Hemiptera (267 individuals), Thysanoptera (213 individuals), Hymenoptera (67 individuals), Coleoptera (21 individuals), and Lepidoptera (17 individuals), whereas, the order Neuroptera was determined as the least captured insects (3 individuals).

The Shannon index is similar in the two half-greenhouses G1 and G2 (0.64 and 0.65 respectively) for the glue traps while it varies slightly in the two half-greenhouses G1 and G2 (0.92 and 0.55 respectively) for the water traps. The Jaccard similarity index showed that the tow half-greenhouses are strongly similar for the glue traps (1) while it is dissimilar for the water traps (0.81).

The results showed that the population of Diptera captured by yellow water plates was dominant in both half-greenhouses G1 and G2 (67.26% and 87.16% respectively), followed by Hemiptera in both half-greenhouses G1 and G2 (22.91% and 5.74% respectively), Thysanoptera in the half-greenhouse G1 (6.48%), Hymenoptera in the half-greenhouse G2 (3.24%), Coleoptera in the half-greenhouse G1 (1.56%) and Thysanoptera in the half-greenhouse G2 (2.24%). Then Hymenoptera in the half-greenhouse G1 (1.45%) and Lepidoptera in the half-greenhouse G2 (1. 37%). However, Lepidoptera and Coleoptera did not reach 1% in both half-greenhouses G1 and G2 correspondingly (0.34% and 0.25% respectively). What is reported is the absence of the order of the Neuroptera in both half greenhouses G1 and G2 in the yellow plates (Fig.1).

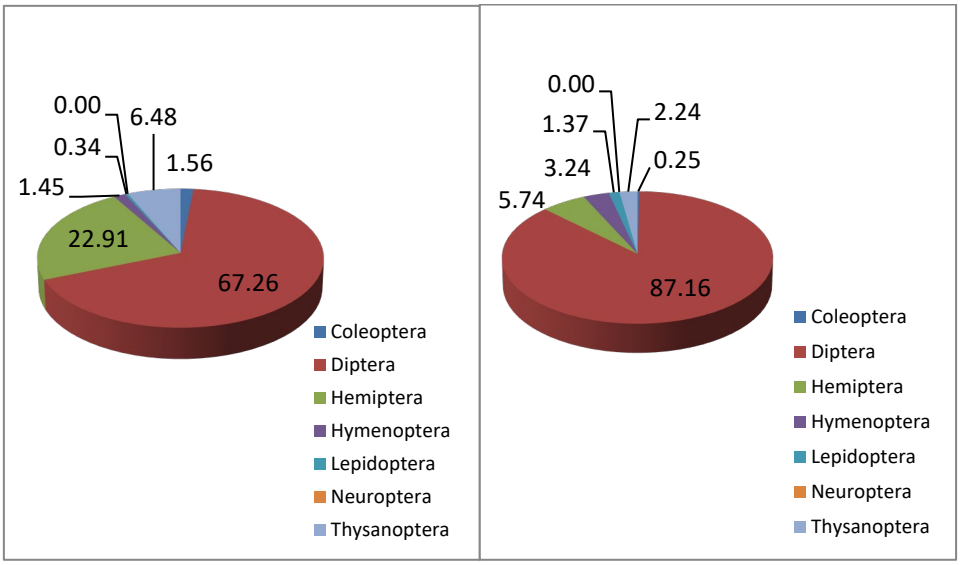

Figure 1. Relative abundance of different orders in water traps (G1 on the left and G2 on the right).

Diptera also dominate in glue traps (Fig.2) in both half-greenhouses G1 and G2 (81.93% and 81.53% respectively) followed by Thysanoptera (13.49% and 12,82% respectively), Hymenoptera and Hemiptera in the half-greenhouse G1 (1.53% for each order) while in thehalf-greenhouse G2 Hymenoptera followed by Hemiptera (3.36% and 1.53% respectively). Meanwhile, Coleoptera, Lepidoptera and Neuroptera did not reach 1% in both half-greenhouses G1 and G2 (0.76%; 0.51%; 0.25% and 0.31%; 0.15%; 0.31% respectively).

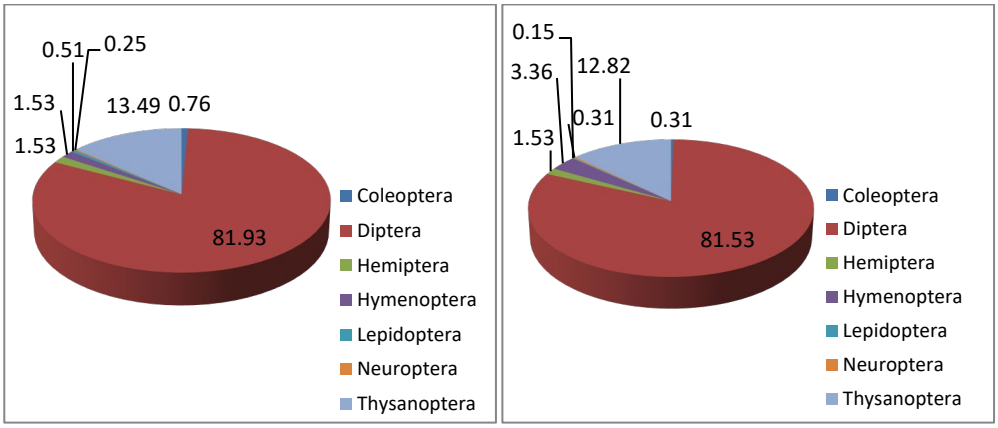

Figure 2. Relative abundance of different orders in glue traps (G1 on the left and G2 on the right).

Beneficial insects inventoried

The insect inventory has identified beneficial species that belong to five orders and are divided into seven families including four predatory species, three parasitoids and two pollinators (Tab. 3).

Table 3. Beneficial insects encountered in the two half-greenhouses of the El-Outaya bio-resources station.

Orders	Families	Genera and species	Prey or host
Coleoptera	Coccinellidae	*Coccinella septempunctata*	White flies and aphids
Diptera	Syrphidae	*Sphaerophoria philanthus* *Lapposyrphus lapponicus*	Aphid and mite eggs.
Hymenoptera	Andrenidae Braconidae	*Apis mellifera* *Andrena* sp. *Cotesia glomerata*	Pollinator. Pollinator. Cabbage mothlarvae.

Orders	Families	Genera and species	Prey or host
		Opius sp. *Diodontus* sp.	Diptera. Thysanoptera and Collembola.
Neuroptera	Chrysopidae Hemerobiidae	*Chrysoperla carnea* *Hemerobius* sp.	Mites, aphids and white flies.
Thysanoptera	Aeolothripidae	*Aeolothrips intermedius*	Thrips.

Temperature and humidity records

During the experiment the G1 recorded higher means of temperature than G2 with a highest value of 46°C. Meanwhile G2 recorded higher levels of humidity than G1with a highest value of 65% (Fig. 3).

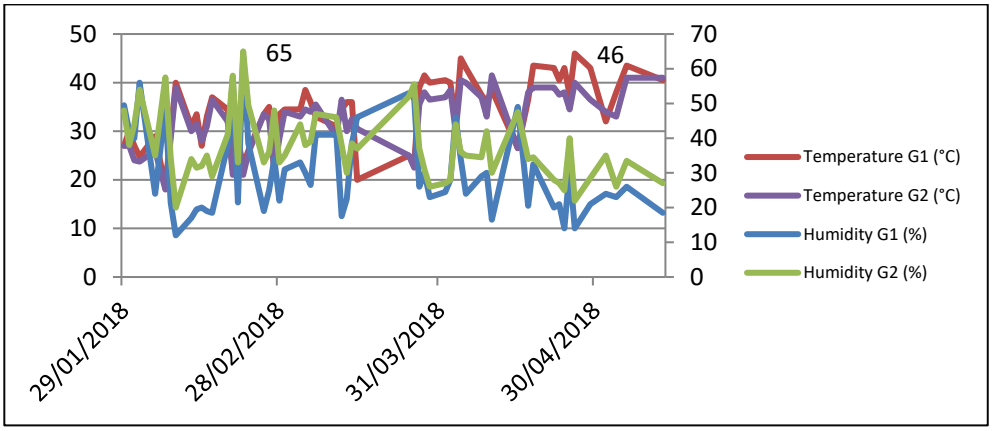

Figure 3. Temperature and humidity means recorded in the two half greenhouses

Discussion

Sixteen species are phytophagous, thus *Lytta vesicatoria* adults are reported as phytophagous (Binon*et al*., 2015), *Acmaeodera* sp. being a jewel beetles (Coleoptera: Buprestidae) thus, mostly are xylophagous species, although a few buprestids mine leaves (Evans *et al*., 2007). *Rhagoletis* sp. is also reported as Phytophagous (Bush,1992), as well as *Cacopsylla*sp. (Hemiptera: Psyllidae) are phloem feeding insects (Sagar andBalikai, 2013). Three aphid species that found in this study have been already reported in the region of Biskra by Laamari *et al.* (2010). Though, past studies have identified 18 species of aphids attacking

tomato in open-field agriculture and greenhouses. However an in-depth review of the literature reveals only two species, *Macrosiphum euphorbiae* and *Myzuspersicae*, as frequent and common aphid pests of tomato throughout the world (Perring, 2018). The leafhoppers of the genus *Cicadella* (Hemiptera: Cicadellidae) usually feed on different plants sap and are considered as agricultural pests while some leafhoppers are the vector of many plant viruses (SHAH*et al.* 2019). The planthoppers of the genus *Laodelphax* (Hemiptera: Delphacidae)was reported as vectors of plant pathogens (O'Brien and Wilson, 1985). *Cynthia cardui*is a polyphagous species, with a clear host plant preference (Stefanescu, 1994). *Melanthrips fuscus*, *Odontothrips confusus* and *Frankliniella occidentalis* are phytophagous thrips already identified on faba beans in this site (Bengouga, 2018).

Thus, the most interesting species attacking tomatoes are *Phyllotreta* sp. which serves as minor insect pests causing damage to tomatoes (Brust *et al*., 2018). The tomato leaf miner, *Liriomyza bryoniae* (Kaltenbach, 1858)(Diptera: Agromyzidae) can cause severe infestations in protected tomato production (Srinivasan and Manickam, 2018). Meanwhile, the tomato pinworm, *Tuta absoluta* (Meyrick, 1917) (Lepidoptera: Guelechiidae) is among the most harmful pests encountered on greenhouse tomato cultivation, whose larva can cause serious damage at all stages of the culture by digging galleries in the leaves, stems, buds, flower buds as well as in the fruits during formation or maturity (Aprel *et al.,* 2017). This pest has been observed in several Mediterranean countries, which was reported from Spain in 2006 and from Tunisia in 2008 (Lebdi *et al*., 2011). Similarly in Algeria this pest was reported in 2008 by Guenaoui (2008) in Mostaganem located north / west of Algeria. The precise identification of this micro-Lepidoptera has been confirmed by the study of its genitalia. (Badaoui and Berkani, 2011). Similarly, Belhadi (2008) reported that some greenhouse gardeners in Tolga a commune of Biskra reported the presence of an insect attacking their tomato plants; this insect has been identified as *T. absoluta* by a team from the CRSTRA El-Outaya Bio-Ressources station who reported that this pest first appeared, at Zab Elgharbi (West of Biskra), then at Zab Echargui (East of Biskra) with a lag of one and a half to two months between these two major ecological entities of the Ziban. The recrudescences of this pest at the Bio-Ressources station of El-Outaya have been studied by Berrjouh *et al*. (2016), who reported that the evolution of adult catches of tomato leafminer increases from one month to another to reach the peak during the month of April. This increase is influenced by the rise in temperatures in the greenhouse.

Similarly, *Frankliniella occidentalis* is a significant and the most destructive thrips of greenhouse crops. It causes direct damage on foliage and flowers, and indirect damage as vector viruses (Cloyd, 2010).

Meanwhile, the presence of auxiliaries; *Coccinellasepemt punctata* (Linnaeus) (Coleoptera: Coccinellidae), *Sphaerophoria philanthus* (Meigen) (Diptera: Syrphidae), *Lapposyrphus lapponicus* (Zetterstedt) (Diptera: Syrphidae), *Opius* sp., *Diodontus* sp., *Chrysoperla carne* (Stephens) (Neuroptera: Chrysopidae), *Hemerobius* sp. and *Aeolothrips intermedui*s (Bagnall) (Thysanopera: Aeolothripidae) is useful for suppressing harmful pests this ascertainment is consolidated by Naika *et al*. (2005) and the presence of pollinators; *Apis mellifera* (Linnaeus) (Hymenoptera: Apidae) and *Andrena* sp. (Hymenoptera: Andrenidae) also ensures better fruiting.

From the quantitative point of view,our results are similar to those of Choudourou *et al*. (2012) even they performed their work on two open fields, of three tomato varieties in Benin and identified 37 species of insects belonging to nine orders and 26 families from May to July. Similarly, Lahmar (2008) identified 44 genera and species of insects distributed in eight orders and 23 families on tomato cultivation under greenhouse in Ouarglaby the Barber pots collection method. From the qualitative point of view, Sid-Rouhou (2014) also reported that the most represented order in a tomato greenhouse over eight months from November to June is Diptera followed by Hymenoptera, Lepidoptera, Homoptera and Coleoptera. Similarly, Imine (2011) recorded six insect orders on greenhouse tomato cultivation with 21 families and 22 species with Dipterian order dominance. The dominance of Diptera can be attributed of being ubiquitous and widely distributed insects most families of Diptera are nearly worldwide in distribution (Vockeroth, 1993). Indeed, our inventory of insects took place during the period between September 2017 and April 2018, where climate factor covers humidity, rainfall and temperature are optimal for dipteran larval development. Emantis (2017) reported that factors of humidity and rainfall have positive correlation with the abundance of dipteran larvae. Meanwhile, temperature factor has negative correlation.

Using the same methods adopted in our work (yellow plates with water and glue traps); in Burkina-Faso; on a total trial area of 691.2 m2 (Kere, 2016); reported the capture of 13525 insects in tomato field that belong to 10 orders divided into 83 families. Where the Hemiptera was more abundant followed respectively by the orders of the Diptera, Hymenoptera, Heteroptera, Coleoptera, Orthoptera, Thysanoptera, Lepidoptera, Trichoptera, and Neuroptera. In comparison with our results the difference in the number of specimens as well as the number of orders and families can be justified by the combination of certain factors including, capture on open field, the area being larger as well as climatic conditions of the experimentation site which took place in Bobo-dioulassour (Burkina-Faso) characterized by a tropical savanna climate

compared to our experiment conditions, capture under greenhouse, small area and arid climate.

In addition, with the method of Barber pot in a farm of which 5 ha reserved for tomato cultivation of the variety Tavira in open field during 4 months of study (June-September) and which was treated by several phytosanitary products; 23 species of insects represented by 245 individuals were recorded (Bissaad*et al*., 2016).

By comparing the two trapping methods; similar observations have been made by Sid-Rouhou (2014) about the dominance of Diptera, however the abundance of other orders is different. While Lahmar (2008) using the Barber pots on greenhouse tomato cultivation in the region of Hassi Ben Abdallah in Ouargla (Algeria); found that Hymenoptera was the most numerous order followed by Coleoptera and Diptera. These differences can be explained by pedo-climatic differences and differences in cultural techniquesand in type of trap "Barber pots" that capture crawling insects by the underground traps.

While Sid-Amar (2011) using the yellow plates recorded 8 orders of insects under greenhouse sheltering different market gardening including the tomato in the station Mouley Nadjem and 7 orders in the station Sbaihi. It is noted in our experiment that Thysanoptera pre-date Hemiptera and that Neuroptera are present in glue traps; this can be explained by the difference in the size and shape of the wings between the different orders.

The similarity in Shannon and Jaccard Indices indicate that the two half-green houses are colonized by approximately the same number of insect families. Thus, the slight differences can be attributed to climatic conditions and position of each green house that the halhf-greenhouse2 is more exposed to wind so it recorded low temperatures than the half-greenhouse1, at the same time more insects are captured in the glue traps. The difference of the Shannon index between the two-half greenhouse can be attributed to temperature and humidity differences recorded in the two half greenhouses (Figure3) according to Bale et al. (2002) temperature is identified as the dominant abiotic factor directly affecting insect abundance.

Conclusion

The inventory showed that the insects that are subservient to tomato cultivation under greenhouse bio conditions in the El Outaya Bio-resource Station vary in abundance and diversity. A total of 38 genera and species of insects were registered. These insects belong to seven orders and 33 families. Although the majority of these insects are pests in tomato cultivation, there are

some predators, parasitoids and pollinators. The collected data provide a basis for preliminary knowledge of tomato crop entomofauna in the El-Outayaplain and can thus be used to design pest management strategies in this region.

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Antibacterial activity of *Garcinia kola* and *Hunteria umbellata* extracts on bacterial isolates from consumed sachet water in Edo State, Nigeria

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Abstract. Water is an essential resource and its purity should not be negotiated when it comes to human consumption. This study investigated the antibacterial activity of *Garcinia kola* and *Hunteria umbellata* seed and epicarp on some bacterial isolates from sachet water. Duplicate samples of ten brands of sachet water were purchased from sales outlets around Ugbowo community, Benin City. Plate count techniques, minimum inhibitory and minimum bactericidal concentrations of the extracts were adopted in this investigation. Heterotrophic bacterial counts revealed highest range of $3.72\pm0.50x10^2$ cfu/mL in EJ water and lowest in IB water ($0.00\pm0.0x10^2$ cfu/mL) while total coliform counts revealed its highest value in EJ water ($3.62\pm0.30x10^2$ cfu/mL) and lowest value (0.00 ± 0.0 $x10^2$ cfu/mL), in OL, IB, NOS and UNI water. *Aeromonas* sp., *Bacillus* sp., *Pseudomonas* sp., *Staphylococcus aereus* and *Enterobacter* sp. were isolated. The antimicrobial susceptibility profile revealed varying zones of inhibition of 4 mm for *Aeromonas* sp., for *Garcinia Kola* and 20 mm recorded against *Staphylococcus aureus* and *Bacillus* sp. for *Hunteria umbellata* epicarp extract. The Minimum Inhibitory Concentration of susceptible bacteria to *Hunteria umbellata* and *Garcinia kola* extract were 6.25mg/mL and 12.5mg/mL,

respectively, except *Aeromonas* sp. which had 50 mg/mL. *Hunteria umbellata* epicarp had a greater bactericidal effect of 6.25mg/mL against *Staphylococcus aureus,* while *Garcinia kola* had its greatest bactericidal effect on *Enterobacte*r sp. with a minimum bactericidal concentration of 12.5mg/mL. This study has revealed the potentials of *Hunteria umbellata* epicarp and *Garcinia kola* as effective natural therapeutic agents against some harmful bacteria, preventing their pathogenic effect.

Keywords: Herbal extracts, Hygiene education, Minimum Bactericidal Concentration, Minimum Inhibitory Concentration, Sachet water.

Introduction

Water is indispensable to life so that it has a substantial effect on public health, living standard and has an uneven distribution the world over (Kılıç, 2020). It's of immense importance and an essential substance needed to maintain vital actions of humans such as respiration, nutrition, circulation, excretion and reproduction (Kılıç, 2020). The adult human body consists of 70 percent water, while 95 to 98 percent of the bodies of lower animals/aquatic animals are made up of water (Gordalla *et al.,* 2007). Most times water ascertained as clean is often non available to countless human populations globally, which remains relatively distressing with the harmful aspects linked with contaminated water frequently used (Khalifa and Bidaisee, 2018). Generally, people prefer to drink and use unhygienic water than using nothing at all. The amount of people that consume unhygienic water across the globe are about two billion people, which is alarming (Khalifa and Bidaisee, 2018).

Water is prone to contamination by microorganisms and organic matter, among other contaminants, irrespective of source (Gangil *et al.,* 2013; Anyamene and Ojiagu, 2014; Oludairo and Aiyedun, 2016). Potable water encourages economic growth as well as helps to improve public health. Contaminated water is often the reason for economic and social costs via water-related ailments like dysentery, typhoid fever, hepatitis A, poliomyelitis, Vibrio illness, *E. coli* infection and increases in therapeutic treatment expenses (Rossi *et al.,* 2012; Mohsin *et al.,* 2013). The presence of *Escherichia coli*, *Klebsiella* and *Enterobacter* spp. in water possibly connotes the existence of infective organisms such as *Clostridium pafringens*, *Salmonella* and Protozoa (Anyamene and Ojiagu 2014).

Antimicrobials refer to a constituent that kills or impedes the development of microorganisms like bacteria, fungi and viruses (Ajayi and Ojelere, 2014). Antimicrobial drugs either kill microorganisms (micro-biocidal) or impede the development of microorganisms (micro-biostatic) (Ajayi and Ojelere, 2014). Antibiotics either occur naturally or are synthetic organic compounds known to impede or terminate choosy bacteria, normally at low concentrations (Brooks *et al.,* 2007).

Hunteria umbellata (K. Schum) is a tropical rainforest plant which belongs to the Apocynaceae family. It is referred to locally as 'abeere' amongst the Yoruba (South-West Nigeria), 'nkpokiri' by Ibos and 'osu' by Edos (Adeneye and Adeyemi, 2009b). It is a therapeutic plant having a history of usage in treatment of infections, illnesses and diseases in Nigeria and Ghana (Adeneye and Adeyemi, 2009b). It has been reported that numerous extracts prepared from its various parts have been used for the treatment of several human diseases like sexually transmitted infections, yaws, stomach ulcers, pains and swellings, diabetes mellitus, dysmenorrhea and to induce or augment birth labor by African folklore medicine (Falodun *et al.,* 2006; Adeneye and Adeyemi 2009). Studies have shown that various medicinal plant extracts have several biological properties such as antimicrobial, antioxidant, anti-inflammatory, anticancer and anti-diabetic activities (Wang *et al.,* 2018; Ahmed *et al.,* 2019; Cai *et al.,* 2019; Tuama and Mohammed 2019; Olaokun *et al.,* 2020). Also, the analgesic and antipyretic effects the aqueous extract of its fruit pulp has been investigated and proven to be effective in the regulation of pain and fever and these effects were independent of its antibacterial activities (Igbe *et al.,* 2009).

Garcinia kola commonly known as bitter kola, belongs to the family Guittiferal, it is valued in Nigeria for its medicinal nut within many Nigerian communities. Bitter kola is chewed extensively by Nigerian locals as a masticator to enhance nervous alertness and has been proven to exhibit pharmacological uses in the treatment of coughs and throat infections (Farombi *et al*., 2005). *Garcinia kola* stem bark has been shown to contain a complex mixture of phenolic compounds such as tannins, guttiferin, biflavonoids, xanthenes, benzophenone, kola flavanone and garcinia flavanone Adamu *et al.,* 2020 and Niemenak *et al*., 2008, all of which are reported as having antimicrobial activity. Besides, *G. kola* has been reported as exhibiting purgative, anti-parasitic, anti-inflammatory, anti-bacterial and antiviral properties (Akoachere *et al*., 2002).

It is against this background that this study attempts to assess the antimicrobial effect of *Garcinia kola* (bitter kola) and *Hunteria umbellata* on bacterial isolates from selected sachet water brands sold around Ugbowo community in Benin Metropolis to provide data for future reference purposes.

Materials and methods

Study Area

The study was carried out around Ugbowo community in Egor Local Government area, Benin City, the capital of Edo state, in the southern part of Nigeria. Its geographic location is at latitude 6°11′ and 6°29'N, and longitude 5°33′ and 5°47'E.

Sample collection

Ten different brands of sachet water were purchased from a total of five shops within Ugbowo environs in Benin City. Duplicate samples were purchased for each brand. The sachet water samples were purchased at the following locations: Faculty of Life Sciences Shopping Complex, University of Benin (6°23'46" N, 5°37'8" E); Faculty of Physical Sciences Shopping Complex, University of Benin (6°23'48" N, 5°36'59" E); Ekosodin (6°24'19" N, 5°37'35" E); and two locations in BDPA area (6°23'44" N, 5°36'13" E and 623'41" N, 5°36'9" E).

The purchased sachet water samples were stored under ice and transported to the laboratory for analysis (Figs. 1, 2).

Figure 1. Sachet water displayed for sales (Photo credit: Blessing Offeh)

Figure 2. Sampling locations

Sterilization and preparation of media

The glare wares used (conical flask, round bottom flask) were washed, drained and dried. They were wrapped with aluminum foil and sterilized using autoclave at 121°C for 15 minutes. An aseptic working environment was achieved with the use of Bunsen burner flame and disinfection of work surfaces with alcohol. The media were prepared according to manufacturers' manufacturer's instructions. Pour plate technique was employed (Holt *et al*., 1994).

Enumeration and isolation of bacterial species

The isolation, enumeration and characterization of the bacterial species were carried out using standard procedures and characterization of the isolates were done using Bergey's manual (Holt *et al*., 1994).

Preparation of plant organic extracts

The *Garcinia kola* (bitter kola) and *Hunteria umbellata* (osu) were grinded to fine powder to increase the surface area. 100 g of each extract was soaked in 250 mL of solvents (distilled water) in conical flasks plugged and with cotton plugs, respectively. It was observed on a shaker for 48 and 72 hrs. The

stock concentration was 400 mg/mL. The extracts were filtered through a Whatman No.1 filter paper and Muslin cloth severally and concentrated to dryness with the aid of a rotary evaporator. The stocks were kept at 4°C in a refrigerator until further use. The concentrated extracts of osu seed, osu epicarp and bitter kola were varied into different concentrations; the quantities used were 100 mg and 200 mg, according to the methods of Adeogun *et al.* (2016).

Figure 3. *Hunteria umbellata* (left) and *Garcina kola* (right)

Antimicrobial screening

Agar well diffusion method was used to screen the antibacterial activities of different solvent extracts (Daoud *et al.,* 2015). One mL of fresh bacterial culture was pipetted in the center of sterile Petri dish. Molten cooled (MHA) was then poured into the Petri dish containing the inoculum and mixed well. Upon solidification, wells were made using a sterile cork borer (10 mm in diameter) into agar plates containing inoculums. Then, 100 µl of each extract 20 % (w/v) was added to respective wells. The concentration of extracts 20 % (w/v) has been selected based on our pre-experiments, and previous literature. The plates were placed in the refrigerator for 30 mins to let the extracts diffusion well into the agar. Then, the plates were incubated at 37OC for 18 hours. Antimicrobial activity was detected by measuring the zone of inhibition (including the wells diameter) appeared after the incubation period.

Determination of minimum inhibitory concentration

The tested extracts which exhibited antimicrobial activity at a concentration of 20 % (w/v) were used to determine their minimum inhibitory concentrations (MIC) using agar well diffusion method, and to evaluate their effectiveness in

pathogens. Different concentrations, 100, 75, 50, 25, 12.5, 6.25, 3.12 and 1.56 mg/mL, were prepared by two-fold serial dilution. One ml of each prepared inoculum was pipetted into sterile Petri dishes followed by the addition of molten agar and mixed well. One mL of the inoculum was also transferred into tubes containing the extracts. Then, wells were made on each plate, and 100 µl of 100, 75, 50, 25, 12.5 6.25, 3.12 and 1.56 mg/mL concentrations were transferred to the respective wells. Plates were kept in the refrigerator for 30 mins and then incubated at 37°C for 18 hours. The MIC was considered as the lowest concentration which inhibited the growth of the respective microorganisms.

Determination of minimum bactericidal concentration

A modification of the dilution method for the determination of MBC was used. Various concentrations of 200, 100, 50, 25, 12.5 and 6.25 mg were placed in sterile nutrient broth in test tubes. Using standard wire loop (Merck), a loopful (10 µl) of isolates, 0.5 McFarland standards was inoculated into test tubes containing 1 mL of the various concentrations. The tubes were incubated at 37°C for 18 to 24 hours and thereafter observed for growth or turbidity. Subsequently, a loopful of broth from each test tube not showing growth was inoculated into nutrient agar plate. Thereafter, equal volumes of sterile nutrient broth were added into the test tube cultures and incubated further for 24 hours at 37°C. Then, the tubes and agar plates were examined for turbidity or growth (Daoud *et al.,* 2015).

Results

The results obtained from the bacteriological analyses of the water samples are shown in Figures 4 and 5. The total heterotrophic bacterial counts were highest in EJ water at (3.72±0.50) $x10^2$ cfu/mL and least in IB water at (0.00±0.0) $x10^2$cfu/mL. For the total coliform count, the highest count was recorded as (3.62±0.30) $x10^2$cfu/mL for EJ water, while the least was (0.00±0.0) $x10^2$cfu /mL in OL, IB, NOS and UNI waters.

Five bacterial species were isolated and identified in the water samples, namely: *Aeromonas* sp. (EJ, OL and FM), *Bacillus* sp. (SB, FL and NOS), *Pseudomonas* sp. (SB and UNI), *Staphylococcus aureus*. (OJ) and *Enterobacter* sp. (NL).

The percentage frequency of occurrence of the various bacterial isolates identified in the samples is shown in Fig. 6. The highest value (30%) was recorded for *Aeromonas* sp. and *Bacillus* sp. each while the lowest value (10%) was recorded for *Staphylococcus aureus* and *Enterobacter* sp.

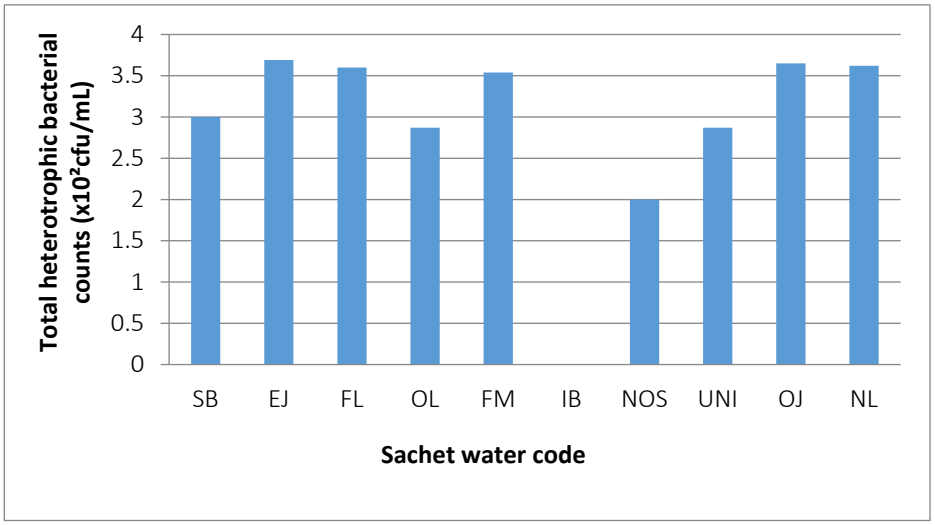

Figure 4. Total heterotrophic bacterial counts

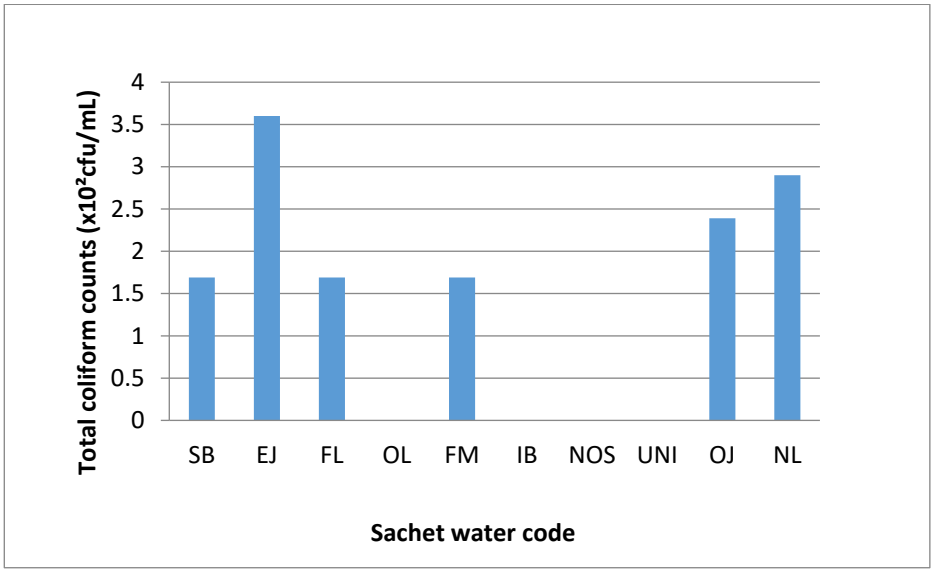

Figure 5. Total coliform counts

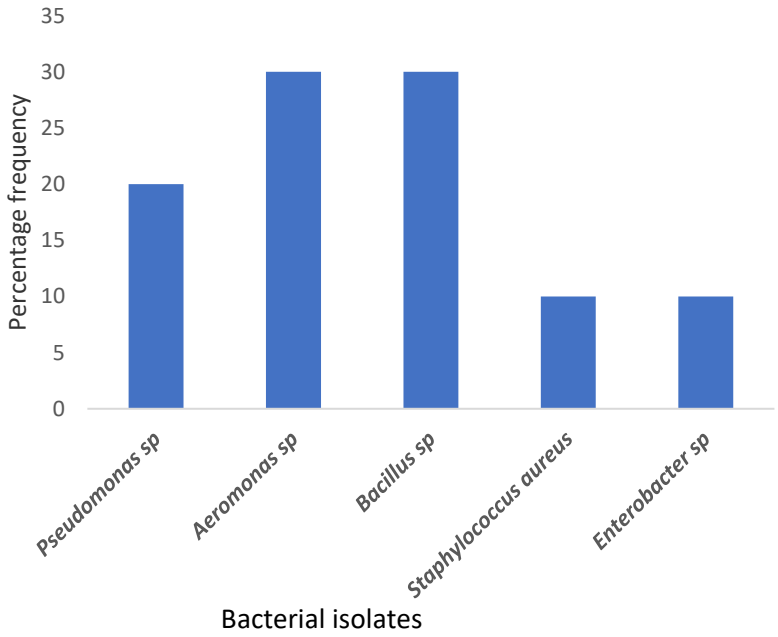

Figure 6. Percentage frequency of occurrence of isolates

Antibacterial activity of *Garcinia Kola* (bitter kola) and *Hunteria umbellata* (osu) extracts was also investigated (Tabs. 1-5). Table 1 shows the zone of inhibition of the various extracts, (*Hunteria umbellata)* (osu) seed, epicarp and bitter kola extracts. There was no zone of inhibition for *Hunteria umbellata* (osu) seed extract effective against the various isolates. The highest zones of inhibition for (*Hunteria umbellata* (osu) epicarp extract, 20mm, was effective against *Staphylococcus aureus, Bacillus* sp. and the lowest zone of inhibition, 14mm, was recorded against *Aeromonas* sp. *Garcinia Kola* had its highest and lowest zones of inhibition recorded against *Staphylococcus aureus* and *Aeromonas* sp., 14 and 4mm, respectively.

Tables 2 and 3 reveal the minimum inhibitory concentrations of *Hunteria umbellata* (osu) epicarp and *Garcinia kola* extracts. The minimum inhibitory concentration of *Hunteria umbellata* (osu epicarp) of the various isolates (*Staphylococcus aureus, Enterobacte*r sp. and *Bacillus* sp.) was 6.25mg/mL except for *Aeromonas sp.* which had 50mg/mL. For bitter kola extract, the MIC for *Staphylococcus aureus, Enterobacte*r sp. and *Bacillus* sp. was 12.5mg/mL while *Aeromonas* sp. had 50mg/mL. The tubes containing the extract inoculated with *Pseudomonas sp.* were turbid, indicating that the extract

was not effective against the isolates. The minimum bactericidal concentration results obtained for the various extracts are shown in Tables 4-5. Bitter kola (*Garcinia kola*) result from the non-turbid tubes (clear tubes) plated on nutrient agar showed growth after 24 hours. *Staphylococcus aureus* and *Bacillus sp.,* both had MBCs of 100 mg/mL, while *Enterobacte*r sp. had a MBC of 12.5 mg/mL. The results showed growths on agar plates and tubes from those of *Pseudomonas* sp. and *Aeromonas* sp., indicating that the extract did not have any bactericidal effect on the organisms (Tab. 4). Minimum bactericidal concentration results obtained for *Hunteria umbellata* (osu epicarp) extract were 6.25, 100, 100, 50 mg/mL from tubes of extract inoculated with *Staphylococcus aureus, Enterobacte*r sp., *Aeromonas* sp. and *Bacillus* sp., indicating that the extract had bactericidal effects against all isolates except *Pseudomonas* sp. (Tab. 5).

Table 1. Zones of Inhibition of *Hunteria umbellata* (osu seed, epicarp) and *Garcinia kola* (bitter kola) extracts

Isolates	**Osu seed (100mg)**	**Osu epicarp (100mg)**	**Bitter kola (200mg)**
Staphylococcus aureus	0mm	20mm	14mm
Bacillus sp.	0mm	20mm	12mm
Aeromonas sp.	0mm	14mm	4mm
Enterobacter sp.	0mm	18mm	10mm

Table 2. Minimum Inhibitory Concentration (MIC) for *Hunteria umbellata* (osu epicarp) extract on the various isolates from sachet water (mg/mL)

Isolates	**100**	**75**	**50**	**25**	**12.5**	**6.25**	**3.125**	**1.56**
Bacillus sp.	-	-	-	-	-	-	+	+
Staphylococcus aureus	-	-	-	-	-	-	+	+
Aeromonas sp.	-	-	-	+	+	+	+	+
Enterobacter sp.	-	-	-	-	-	-	+	+
Pseudomonas sp.	+	+	+	+	+	+	+	+

KEY: - = no growth; + = growth

Table 3. Minimum inhibitory concentration for *Garcinia kola* extract on the various isolates from sachet water (mg/mL)

Isolates	**100**	**75**	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.56**
Bacillus sp.	-	-	-	-	-	+	+	+
Staphylococcus aureus	-	-	-	-	-	+	+	+
Aeromonas sp.	-	-	+	+	+	+	+	+
Enterobacter sp.	-	-	-	-	-	+	+	+
Pseudomonas sp.	+	+	+	+	+	+	+	+

KEY: - = no growth; + =growth

Table 4. Minimum bactericidal concentration for *Garcinia kola* extract on the various isolates from sachet water (mg/mL)

Isolates	200	100	50	25	12.5
Bacillus sp	-	-	+	+	+
Aeromonas sp.	+	+	+	+	+
Enterobacter sp.	-	-	-	-	+
Staphylococcus aureus.	-	-	+	+	+
Pseudomonas sp.	+	+	+	+	+

KEY: - no growth, + = growth

Table 5. Minimum bactericidal concentration for *Hunteria umbellata* (osu epicarp) extract on the various isolates from sachet water (mg/mL)

Isolates	200	100	50	25	12.5	6.25
Bacillus sp.	-	-	-	+	+	+
Aeromonas sp.	-	-	+	+	+	+
Enterobacter sp.	-	-	+	+	+	+
Staphylococcus aureus.	-	-	-	+	+	+
Pseudomonas sp.	+	+	+	+	+	+

KEY: - = no growth, + = growth

Discussion

The health risk implication that drinking of contaminated water could cause to man cannot be overemphasized, hence this study was conducted to investigate the microbial contamination of sachet water, otherwise called 'pure' water, and to see how potent using natural remedies (osu and bitter kola) could be.

The result obtained from the bacteriological analysis of sachet water revealed the presence of heterotrophic and total coliform counts in some of the sampled water which were otherwise branded as 'pure water'. EJ water had the highest counts in both the heterotrophic and total coliform count. This could be due to unhygienic practices leading to the contamination of the environment and production process corroborating with the report of Idu *et al.* (2011) and Ademoroti (1996), who indicated that contamination by microbes may be the result of the environment in which they were produced, improper handling or storage.

The bacteriological investigation revealed the presence of five isolates: *Bacillus* sp. *Pseudomonas* sp.*, Staphylococcus aureus, Enterobacter* sp *and Aeromonas* sp. This agrees with the work of Funmilayo *et al.* (2021), who revealed the presence of microorganisms in packaged sachet water as a result of post contamination as some of the organisms are commensals in groundwater and are present in the environment; it also conforms to the work done by Daniel and Daodu (2016),

who isolated eight bacterial species (*Staphylococcus aureus, Pseudomonas* sp.*, Aeromonas* sp.*, Corynebacterium* sp.*, Bacillus* sp.*, Bacillus badius, Proteus vulgaris* and *Escherichia coli*) from sachet water.

The antimicrobial investigation of *Hunteria umbellata* (osu) seed extract showed little or no zones of inhibition against the various isolates indicating its non-effectiveness against these isolates. This result, although, does not correlate with work done by Udinyiwe *et al.* (2017), who reported an MIC/MBC of 15mg/mL and 50mg/mL for *Hunteria umbellata* seed extract bacteriostatic and bactericidal effect against *Staphylococcus aureus*, *Micrococcus* sp., *Bacillus* sp., *Proteus* sp. and *Streptococcus pneumoniae*. The variations in the diameter for zone of inhibition for plant extracts might be due to the alterations in the chemical composition of their extracts (Issah *et al.,* 2020).

Both *Hunteria umbellata* (osu) epicarp and bitter kola (*Garcinia kola*) revealed that the extract was effective against the various bacterial species isolated, especially *Staphylococcus aureus, Bacillus* sp. and *Aeromonas* sp. There was no zone of inhibition seen for *Pseudomonas sp.* This agrees with the findings of Ogu *et al.* (2017), who recorded high resistance of *Pseudomonas aeruginosa* to various antibiotics such as Norfloxacin (NF), Amoxicillin (AX), Cafuroxime (CF) and Ampicillin (AM). Gram-negative bacteria are known to be more resistant to regular antibiotics, especially some nosocomial strains such as *Acinetobacter baumannii, P. aeruginosa* and *Klebsiella pneumonia*, due to the presence of a peptidoglycan layer (Li *et al.,* 2006; Abdallah *et al.,* 2015).

The MIC/MBC of bitter kola (*Garcinia kola)* extract revealed a value of 12.5-50 mg/mL and 12.5-100 mg/mL, respectively, and that of *Hunteria umbellata* (osu) epicarp extract recorded for the various extract was between 6.25-50 mg/mL and 6.25-100mg/mL, which correlates with the work done by Anibijuwon *et al.* (2011) who reported a range of 20 to 100 mg (MIC/MBC) at treatment with *Hunteria umbellata* fruit extract on bacterial isolates.

Conclusion

The antimicrobial potential of *Hunteria umbellata* (osu epicarp) and *Garcinia kola* (bitter kola) extract has been demonstrated in this study. Both extracts have the potential as natural therapeutic agent against four out of the five isolates investigated with greatest impact on *Bacillus* sp., *S. aureus and Enterobacter* sp. The effect of *H. umbellata* epicarp extract against both Gram Positive and Gram Negative bacteria indicates it will be a useful tool for drug synthesis with a broad spectrum of activity with the possibility of developing therapeutic substances which will be active against multi-drug resistant organisms.

The health risk of drinking contaminated water cannot be overemphasized, hence regular water quality monitoring, combined with community-led intervention with a focus on sanitation, hygiene education, better source water protection strategies and source water treatment is recommended to avoid contamination of pathogenic bacterial isolates in consumed sachet water.

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A laboratory experimental assessment of the sensibility of *Rana temporaria* tadpoles to the effects of car traffic-associated seismic disturbances

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Abstract. Vibrations generated by road traffic are considered to be a form of physical environmental pollution. Nonetheless, the effect of this disturbance in natural habitats, and in particular on the aquatic stage of amphibians, received very little attention from the scientific community.

This study aims to assess the direct effect of mechanical waves and the consequent water turbidity on the aquatic larval stage of *Rana temporaria* by exposing an experimental group to laboratory-induced vibrations and comparison with a non-exposed control group.

Our results show that this kind of pollution had no significant effect on the development rate, the length of the larval period, and the mortality of the larvae. However, we identified a significant effect on the size of animals, both during the larval period, and at metamorphosis. This result raises concern about the long-term risks to the amphibian population exposed to this type of low-profile pollution.

Key words: development, growth, metamorphosis, amphibian performance, traffic-related pollution

Introduction

Commercial and recreational vehicle traffic in the peri-urban and rural areas is on a constant rise, increasing the pressure on the already deteriorated amphibian populations by supplementing the existing chemical contamination and rising road crossing injuries and mortality (Adlassnig *et al*., 2013; Hamer *et al*., 2015). However, studies on vehicle traffic-related consequences are scarce and focus mostly on nuptial migration-related road-crossing mortality (Elzanowski *et al*., 2009). Referring strictly to Europe (van Gelder, 1973; Hels and Buchwald, 2001; Cooke and Sparks, 2004), this type of disturbance seems to affect the Common toad (*Bufo bufo*) in suburban areas and the Common frog (*Rana temporaria*) in the countryside (Elzanowski *et al*., 2009; Puki, 2006). Furthermore, all mentioned research refers to adult frogs of reproductive age. Studies regarding the impact of car traffic on amphibians in the larval stage are few and refer to indirect effects (noise - Castaneda *et al*., 2022, de-icing chemical contamination - Sanzo and Hecnar, 2006).

When a vehicle strikes an irregularity in a road surface, an impact load is generated, which in turn, gives rise to long-wavelength mechanical waves in the form of seismic vibrations that travel through the soil (Toplak *et al*., 2016). In addition, these waves are exacerbated by obstacles such as potholes and bumps and are considered a form of environmental pollution (Chilton *et al*., 1975; Hunaidi, 2000; Ducarne *et al.,* 2018; Niazmand-Aghdam *et al.,* 2021).

As far as we know, the effect of car tire impact on the soil and water surface on the larval-stage of amphibians hasn't been researched yet, even though the presence of the lateral line indicates their high sensitivity to this type of stimuli (Quinzio and Fabresi, 2014).

Amphibian life history theory predicts that in suboptimal habitats, larvae will accelerate their development and growth-rate coupled with a shorter larval period, to minimize mortality (Newman, 1992; Stearns and Coella, 1986; Wilbur and Collins, 1973). The faster development rate may have repercussions, such as smaller size at metamorphosis and reduced survival rate during adulthood (Arendt, 1997). Therefore, the value of this adaptive strategy depends on the cost-benefit ratio after the metamorphic transition.

This study aimed to evaluate the effects of seismic disturbances and water turbidity produced by car traffic on a *Rana temporaria* population from the Făgetul Clujului - Valea Morii protected area. The area is a Site of Community Importance (SCI,46°42′53″N 23°34′18″E), covering 1.7 ha that includes forest habitats of community-interest and 33 protected plant and animal species (M.M.D.D., 2008).

Although access by motor vehicles in natural protected areas is strictly prohibited and constitutes contravention according to Romanian Law (O.U.G., 2007), the above-mentioned protected area is constantly, and intensively subjected to this type of traffic. During the week, the vehicle traffic is utilitarian in nature (heavy vehicles and tractors), and on weekends, the traffic is recreational (4x4 cars, ATVs, and dirt bikes - personal observations). For the studied species, the Common frog, this traffic can represent a permanent disturbance of the breeding ponds through the indiscriminate and sometimes intentional use of the more rugged off-road tracks, which also happen to create puddles that function as amphibian breeding areas in the spring. In our experimental design, we formulated two hypotheses:

1. Seismic disturbances and increased water turbidity will affect larval development exposed to this type of treatment.

2. The larvae exposed to disturbances will metamorphose faster and have a smaller average size than the control group.

Materials and methods

1 Experimental design

The study was carried out in the laboratory of the University Babeș-Bolyai Vivarium, Cluj-Napoca, Romania, between April and June 2022.

To test our hypotheses, we designed an experiment based on two groups (control and experimental), each consisting of 24 individuals of Rana temporaria larvae.

Simulating the traffic-related vibrations in the laboratory is a rather complicated endeavour, considering the wide range of frequencies in the resulting ground-borne mechanical waves. From the low frequencies produced by the so-called wheel hop (8-16 Hz; Watts, 1992) to the higher frequencies produced by the tire impact forces (800-1500 Hz; Hajek et al., 2006), all these vibrations travel through the soil and water and may influence the natural habitats. A perfect reproduction of the whole range of the mentioned frequencies is, therefore, impossible at the moment. However, we simulated vibrations similar to those produced by the impact of wheels on the soil and water surface of the breeding ponds by placing the experimental rearing containers on a high-frequency vibrating reptile feeder (Exoterra Vivicator) for 15 minutes twice a day (30 min total time/day), three days a week (90 minutes/week). We also simulated the turbidity resulting from these disturbances by agitating the water twice a day, each day, for 1 min, by vigorously moving a paddle along the length of the growth containers.

The animals were collected as eggs (three clutches) on April 3, 2022, from a temporary pond located in Făget forest, Cluj-Napoca, Cluj county, Romania (46°41'48.57'' N 23 °32'46.80''E, altitude 682m, Someş river basin). The three clutches were kept separately, in 20L plastic containers filled with dechlorinated water, until the end of the hatching period (April 14, 2022). With the beginning of the larval feeding stage (Smith-Gill and Berven, 1979) of circa Gosner 25 (Gosner, 1960), we selected larvae from each litter (88 in total, 24/treatment) and distributed them randomly to form the two study groups - control and experimental. Larvae excluded from the experiment were released at the site of capture. The study animals were kept in 4L containers (9x17x26cm), with eight larvae/container, in three liters of dechlorinated water (Craioveanu *et al*., 2019). This density corresponds to low densities in natural populations (Glennemeier and Denver, 2002; Rot-Nikcevic *et al*., 2005). A substrate of approximately 1 cm thickness was added to each container, consisting of sterilized forest soil from the collection area of the initial clutches. The water temperature was kept constant at 20 °C (+/- 1) at a natural circadian rhythm. Each container had an oxygen supply provided by electric pumps. Every three days, we changed about 90% of the water with fresh, dechlorinated tap water. We cleaned the organic residues daily by siphoning.

With the emergence of the forelimbs (approx. Gosner 42), we reduced the amount of water to 2 L, and the containers were tilted so that a dry area formed at the raised end (Craioveanu *et al*., 2021). From this point, exposure to the experimental stimuli and feeding ceased. When reaching about Gosner 45 developmental stage, the animals got out of the water towards the dry end of the container and were relocated one by one in containers specific to the terrestrial environment (starting with May 18, 2022), and later released in the collection area.

We considered Gosner stage 45-46 as the moment of metamorphosis, as it corresponds to the total absorption of the larval tail.

The larval diet consisted of Spirulina (Organic Spirulina 500 mg, protein 63.5%, carbohydrates 16.1%, and lipids 8.2%. Origin: China) and pelleted rabbit food (Versele Laga Cuni fit pure, protein 15%, carbohydrates 15% lipids 3%. Origin: Hungary), so that the animals could selectively consume their preferred food type. We used Spirulina to simulate the presence of many protein-rich species of micro-algae (e. g. *Chlorella*, *Anabaena*, *Aphanizomenon*, *Scenedesmus*, *Pediastrum*) (Cărăuş, 2012) in the original ecosystem.

2 Measurements and analysis

Length measurements were performed on digital images using the free image analysis software ImageJ (http://imagej.nih.gov/ij). Images were recorded with a Nikon D3200 camera mounted on a stand 30 cm above the specimens.

For the larval phase of the experiment, we measured the following morphological variables: 1) total body length (the distance from snout to tail tip, in mm), and 2) larval development stages according to the Gosner (1960) model. To establish the correct Gosner-stage we used images taken from several angles. In addition to these variables, we recorded the length of the larval period for each group (days) and mortality at metamorphosis (Craioveanu *et al.,* 2019).

As handling is concerned, the larvae were carefully captured using a shallow net and transferred to a petri dish containing water levels approximately equal to the thickness of the animal (Davis et al., 2008). When photographing, we used a Petri dish with a grid scale. The image was taken only when the subject was not moving, and the entire dorsal part was visible, followed by the prompt return to the original container. Due to the small size and fragility of the larvae at the moment of hatching, batch separation and the first measurements were done only on day 4 (April 14, 2022). We performed five measurements at intervals of approximately one week (April 14, 20, 25, May 4, 11).

In the metamorphosis phase of the experiment, we measured the body length (snout to vent in mm) (Altig, 2007) daily during the entire metamorphosis period (May 20 - June 10). We also recorded the mortality (% of dead animals) during metamorphosis.

The normal distribution of the data was tested with Shapiro-Wilk test. To verify if the larvae grow and develop differently between the two groups (control and experimental), we performed two-sample tests for body length and Gosner-stage, for each larval-stage measurement occasion (between April 14 and May 11). Additionally, we compared the length at metamorphosis and duration of development (defined as days to metamorphosis) between groups. For normally distributed data, we used the two-sample t-test, and for non-normally distributed data, we used the Wilcoxon test. The program RStudio (RStudio Team 2022) was used to perform all analyses.

Results

The first measurement performed at the beginning of the experiment showed no differences between groups regarding their body length ($t = 1.2198$, $df = 43.956$, p-value = 0.229). Gosner stages were identical in both groups as they were selected on this criterion from the beginning.

Three succeeding length measurements (2nd -4th) showed significant differences between groups (Table 1, Figs. 1-3).

In the fifth measurement, we saw no significant differences between the body lengths of the larvae in the control and experiment groups (Table 1).

At metamorphosis, we also found a significant difference between the body lengths of the larvae in the control and experiment groups (Table 1, Fig. 4).

Table 1. Length measurements and statistical tests performed on the two groups of *R. temporaria* larvae.

Measurements	Normality test result (Shapiro-Wilk)	Comparison test result (t-test/ Wilcoxon test)
1st measurement (14th of April)	W = 0.99, p-value = 0.972	t = 1.22, df = 43.96, p-value = 0.229
2nd measurement (20th of April)	W = 0.85, p-value < 0.001	W = 395, p-value = 0.027
3rd measurement (25th of April)	W = 0.83, p-value < 0.001	W = 412, p-value = 0.010
4th measurement (4th of May)	W = 0.80, p-value < 0.001	W = 417, p-value = 0.008
5th measurement (11th of May)	W = 0.89, p-value < 0.001	W = 344, p-value = 0.255
Metamorphosis measurement (20th of May - 10th of June)	W = 0.96, p-value = 0.358	t = 2.10, df = 28.02, p-value = 0.045

In all cases where we recorded significant differences, the control group had higher values than the experiment group.

Gosner's development stages and duration of metamorphosis did not differ significantly between groups on all measurement occasions (in all comparisons p-value > 0.05).

At metamorphosis, the control group had a mortality of 33%, and the experiment group had 37.5%. Before metamorphosis, we recorded no deaths.

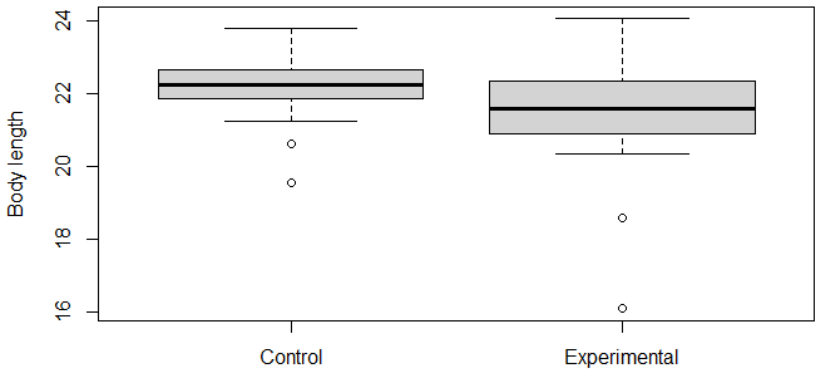

Figure 1. Comparison of body lengths of *R. temporaria* larvae in the second measurement occasion. Boxplots represent median (thick line inside the box), interquartile interval (whiskers) and outlier values (empty dots).

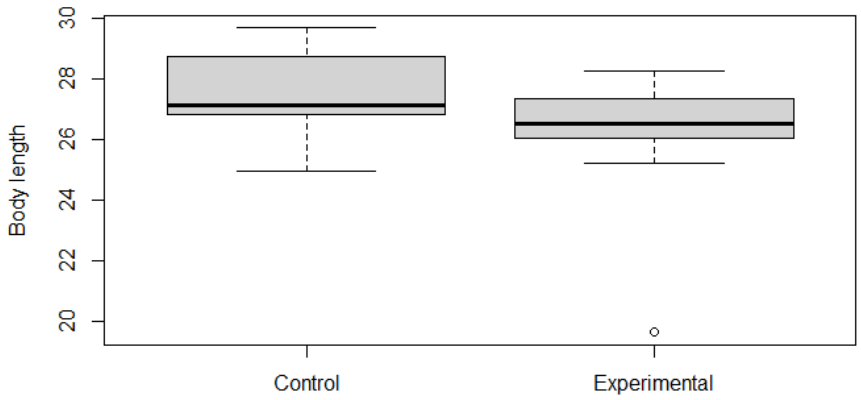

Figure 2. Comparison of body lengths of *R. temporaria* larvae in the third measurement occasion. Boxplots represent median (thick line inside the box), interquartile interval (whiskers) and outlier values (empty dots).

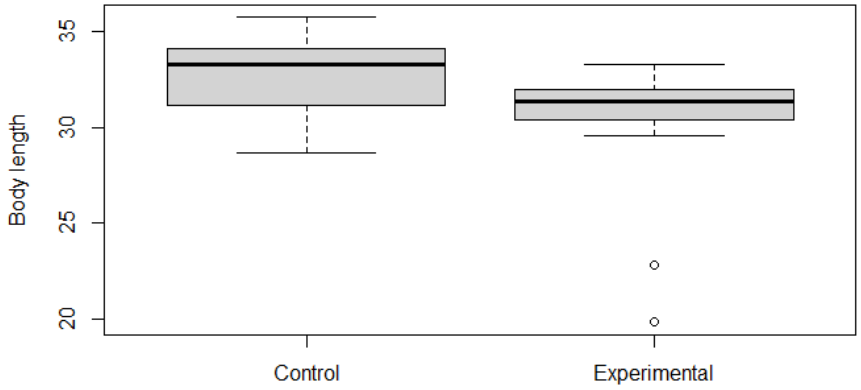

Figure 3. Comparison of body lengths of *R. temporaria* larvae in the fourth measurement occasion. Boxplots represent median (thick line inside the box), interquartile interval (whiskers) and outlier values (empty dots).

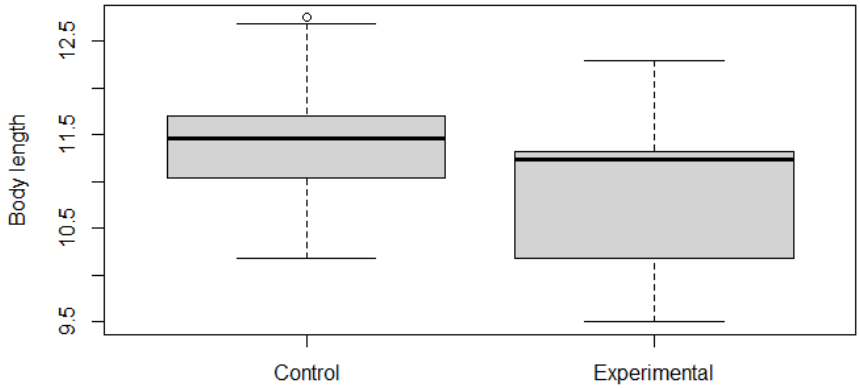

Figure 4. Comparison of body lengths of *R. temporaria* larvae at metamorphosis. Boxplots represent median (thick line inside the box), interquartile interval (whiskers) and outlier values (empty dots).

Discussion

Environmental conditions to which amphibian larvae are exposed can produce delayed effects on the individual performance of postmetamorphic adults. Thus, adult performance is affected by environmental factors long before metamorphosis. The quality of postmetamorphic animals, along with the survival rate at metamorphosis, and postmetamorphic environmental conditions are essential elements that will determine the future dynamics of the entire population (Craioveanu *et al.,* 2019; Beckerman et al., 2002). The metamorphosis is a critical process in the development of amphibians, as they invest the energy acquired and stored as tadpoles in terrestrial maturity, with a complete reconfiguration of their appearance and physiology (Wilbur and Collins, 1973; Semlitsch *et al.,* 1988; Denver *et al.,* 1998).

In this study, we investigated the effect of vibration, mechanical waves, and high turbidity on the larval development of *Rana temporaria*. According to our results, the stimuli we used on the experimental group had no significant effects on the **development rate** (Gosner stages), the **length of the larval period**, and the **mortality at metamorphosis**. The significant difference between the control and experimental groups was the **size of the animals**. Thus, the experimental group had consistently and significantly a smaller size (total length) both during the larval period (measurement sessions 2, 3, and 4) and at metamorphosis (body length).

After reaching the minimum body size required to initiate metamorphosis, anuran larvae are capable of timing the moment of metamorphosis, depending on the growth opportunities, or the risk of mortality in the larval habitat (Wilbur and Collins, 1973). This timing is modulated by the production of thyroid hormone - the main inducer of metamorphosis - along with the production of corticosteroid stress hormones - causing the actual transformation of tissues during metamorphosis. In response to environmental stressors, these two hormones are released earlier and in increased amounts, accelerating metamorphosis (Denver, 2021). Although this mechanism promotes immediate survival in degraded larval habitats, the short and medium-term cost is a reduced tadpole size during the larval period and reduced size at metamorphosis.

The comparisons between our control and experimental groups indicate that this stress-response mechanism was activated as an effect of the experimental treatment.

Although individual size at metamorphosis varies greatly even within the same population (Storer, 1925; Jones *et al.,* 2005; Denver *et al.,* 1998), the smaller size of metamorphs negatively affects performance and survival in the terrestrial stage (Szekely *et al.,* 2020). Thus, smaller specimens are much more affected by predation (Lawlor et al., 1999), cannibalism by conspecific animals of larger size (Alvarez, 2013), and have lower lipid reserves (Scott, 1994) which lead to a decreased ability to cope with variations in the environment.

Our findings largely confirm the initial hypotheses of this study. Seismic-type disturbances and increased water turbidity affected the experimental animals. Also, the experimental group had consistently smaller body sizes than the control group, but the larval period was not significantly different between the two groups.

Conclusion

The stimuli represented by mechanical waves and high water turbidity affected Rana temporaria individuals in the experimental group, resulting in the decreased postmetamorphic performance of the animals.

Even though our laboratory experiment could not precisely reproduce the effects of general traffic, the results indicate that motor-vehicle traffic through the Făgetul Clujului - Valea Morii protected area most likely harms the resident *Rana temporaria* population and can be of major concern regarding its survival in the future.

Our results also intend to raise awareness regarding the effect of traffic-related ground vibrations on amphibian populations.

Limitations of our study

A major limitation of our study is that we only visually quantified the disturbances caused by vehicles in the natural ponds and due to the lack of the proper technology, we were unable to measure them and experimentally reproduce their consequences. Our results, even if experimental, suggest that understanding the 'wavescape' generated and maintained by illegal traffic in the natural systems can be a genuine scientific goal since tadpoles of a common amphibian species responded to such stimuli in laboratory conditions.

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Differential expression of hsa_circ_0064357 and hsa_circ_0064358 between oral squamous cell carcinoma and oral lichen planus

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Abstract. Background/aims: Reliable biomarkers with high specificity and sensitivity and the potential to discriminate precancerous or early lesions from oral cancer improve scientific assessment and early detection. Dysregulated circRNAs play a critical role in the occurrence and progression of malignant biological behaviors of OSCC. The study of potential diagnostic roles of hsa_circ_0064357 and hsa_circ_0064358 in early diagnostic of precancerous lesions such as OLP to OSCC as the most common type of head-and-neck squamous cell carcinoma (HNSCC) was the focus of present research. **Methods:** The differential expression of hsa_circ_0064357, hsa_circ_0064358, and *RAF1* target gene predicted using CircInteractome and Circbase databases between OSCC (n=30), OLP (n=10) tissues and their adjacent normal tissues were evaluated by qRT-PCR. The potential diagnostic value of circRNAs was identified by receiver operating characteristic (ROC) curve analysis. **Results:** hsa_circ_0064357 and hsa_circ_0064358 were identified to be lowly expressed, while RAF1 was upregulated in OSCC and OLP tissues more than adjacent normal tissues. Low expression of circRNAs was markedly correlated with TNM stages of OSCC patients. ROC analysis revealed AUC of 0.962 and 0.965 for hsa_circ_0064357 and hsa_circ_0064358, respectively, suggesting that circRNAs can serve as novel diagnostic biomarkers for early detection of OSCC. Conclusion: hsa_circ_0064357 and hsa_circ_0064358 might be involved in the progression and metastasis of OSCC and could be used as promising novel biomarkers for early diagnosis and the clinical monitoring of the malignant transformation of OLP into OSCC.

Keywords: hsa_circ_0064357, hsa_circ_0064358, OSCC, OLP, *RAF1* gene

Introduction

Oral squamous cell carcinoma (OSCC) is the most common type of malignant tumor of the oral and maxillofacial region (More than 90% of cancer cases) and the sixth most common neoplasm arising in the oral mucosa worldwide (Lin *et al*., 2021). The Global Cancer Observatory (GCO) estimated the annual incidence of OSCC at 377,713 cases and 177,757 deaths globally in 2020 (Ali, 2022). In Iran, lip and oral cavity cancer exhibit similar incidence to India and Pakistan, with an estimated incidence and age-standardized incidence rates (ASIR) of 1.4 and 1.3 per 100,000 individuals among men and women, respectively, in 2020 (Zendehdel, 2021). Among patients diagnosed with OSCC, approximately 60% present advanced locoregionally disease (stage III or IV), and 30-50% have local or distant metastasis probably due to invasion ability and lymphangio genesis of tumor cells (Biswas *et al*., 2019; Xu *et al*., 2021). Detection of late-stage or fully developed tumors and irregular follow-up in the progression of the malignant transformation of precancerous lesions such as oral lichen planus (OLP), which are amenable to resection, are markedly associated with the poor 5-year survival rate of OSCC patients (60%) (Ferlini *et al*., 2013; Tampa *et al*., 2018). Therefore, identifying biomarkers and molecular alterations in OLP as predictors of OLP malignant transformation is imperative.

Raf1 (C-Raf or c-Raf-1) is the integral serine-threonine kinase and downstream effector of the central signal transduction mediator (Ras) belonging to the ERK/MAPK (Extracellular signal-regulated kinase /mitogen-activated protein kinase) pathway, a critical signaling network responsible for regulating diverse physiological processes, including cell proliferation, differentiation, and apoptosis (Guo *et al*., 2020). Several lines of evidence demonstrated that dysregulated upregulation of wild-type Raf-1 and oncogenic raf-1 mutations, particularly those leading to cellular proliferation and increased cell survival, are associated with carcinogenesis and tumor invasion in various cancer types such as colorectal prostate and thyroid cancer (Gollob *et al*., 2009; Tian *et al*., 2018). Circular RNAs (circRNAs) are a class of endogenous non-coding RNAs that, unlike linear RNAs, have unique circular covalently bonded structures without polarity or a polyadenylated tail, which give them stability and higher tolerance to exonuclease (Meng *et al*., 2017). In recent decades, circRNAs have been widely discovered in human, animal, and plant species through high-throughput sequencing (Huang *et al*., 2022). Abnormal expression of circRNAs is reportedly correlated with the onset and development of various cancers, suggesting their potential role as biomarkers in molecular diagnosis and predicting the development of tumors (Wang *et al*., 2021).

However, to the best of our knowledge, the differential expression of hsa_circ_0064357 and hsa_circ_0064358 in OSCC and OLP have not been clarified. Therefore, in this experimental study, we aimed to identify the expression of circRNAs and their predicted target gene (*RAF1*) in OSCC and OLP samples compared to their normal adjacent tissues. Further, we determined whether the circRNAs and *RAF1* level are associated with clinicopathological characteristics in OSCC patients.

Materials and methods

Clinical samples

In this study, a total of 40 samples (30 patients with OSCC and 10 patients with OLP) and their related-adjacent normal oral epithelial tissue specimens were collected from surgical specimens by the Iran National Tumor Bank, Cancer Institute, Imam Khomeini Hospital, Tehran, between October 2020 and January 2021. All patients have a precise histologic diagnosis of OSCC based on diagnostic criteria of the World Health Organization (Pindborg *et al*., 1997). All protocols of the present study were approved by the Research Medical Ethics Committee of Imam Khomeini Hospital (IR.IAU.SRB.REC.1399.093). Every sample is obtained with the patient's informed consent, and none of the patients had previously undergone chemotherapy and any local or systemic treatment before surgery. The tissue samples were confirmed by experienced pathologists, frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Clinicopathological information such as age, gender, tumor stage, and other variables was collected from patients' medical records. The degree of tumor differentiation was classified into well, moderately, and poorly differentiated squamous cell carcinoma, according to World Health Organization (Pindborg *et al*., 1997).

RNA extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA extraction was performed from tissues with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and the concentration, quality, and integrity of extracted RNAs were evaluated using 1% agarose gel electrophoresis and NanoDrop spectrophotometer (Thermo scientific-Nanodrop 2000), respectively. The reverse transcription (RT) reaction in 20 µL of reaction mixture containing 1 µg of total RNA was performed with oligo-dT primers using a BioFACT cDNA (complementary DNA) Synthesis kit (Daejeon, South Korea). For circRNAs, Total RNA was incubated with RNase R for 15 min at 37 °C to deplete the linear RNAs and cDNA was synthesized from

2 µg of total RNA with random hexamer primers by a High-Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, USA) to determine the hsa_circ_0064357 and hsa_circ_0064358 levels. qRT-PCR analysis of circRNAs and *RAF1* gene was performed on a LightCycler TM 96 (Roche) using a SYBR Green Master Mix (TAKARA, Japan) as per the manufacturer's instructions. PCR was performed using the following program: holding stage at 95°C for 5 min, cycling stage comprising 40 cycles (95°C for 15 s, 60°C for 30 s, 72°C for 20 s), melt curve stage at 60°C for 1 min, and 95°C for 15 s. All the primers were designed using Primer3plus software, and sequences have been shown in Tab. 1. Relative quantification of circRNAs and *RAF1* expression was compared with *ACTB* internal standards and were measured using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Table 1. Primer sequences used for qRT-PCR

Target transcript	Primer type	Sequence (5'→3')
has_circ_0064357	Forward	CCCTTTCTCCAGAGGCAGAA
	Reverse	TCCACTTGCGCATCTACAGA
has_circ_0064358	Forward	ATCATCTTCATGGTGGGCC
	Reverse	CCTCTTCATTGCTTTGGGGC
RAF1	Forward	AGATGGCGGGAGTAAGAGGA
	Reverse	CATCGTAGCAAACGCGCTC
ACTB	Forward	GATCAAGATCATTGCTCCTCCTG
	Reverse	CTAGAAGCATTTGCGGTGGAC

Prediction of circRNAs and target gene

In the preliminary experimental screening, OSSC-associated circRNAs, hsa_circ_0064357 and hsa_circ_0064358, and the potential circRNA target gene, *RAF1*, were selected using the online circRNA bioinformatics databases such as Circinteractome (https://circinteractome.nia.nih.gov/) and Circbase (http://www.circbase.org/). Then, the selected gene for both circRNAs was subjected to analysis.

Statistical analysis

All experiments were repeated three times, and numerical data were expressed as means ± standard deviation (SD). The data obtained were all statistically analyzed using GraphPad Prism software 5.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS (GraphPad Prism 5 software (version 18.0; SPSS, Inc., Chicago, IL, USA). Expression data were controlled for normal

distribution by one-sample Kolmogorov-Smirnov (K–S test). A one-way ANOVA was used to determine statistical differences in *RAF1* gene expression levels. The associations between hsa_circ_0064357 and hsa_circ_0064358 and *RAF1* levels and clinic-pathological parameters of OSCC patients were assessed using independent sample test and independent-sample Kruskal-Wallis test. The correlation between variables was performed by Pearson correlation analysis. The diagnostic value, sensitivity and specificity of circRNAs were determined by ROC curve analysis. A p-value less than 0.05 (≤0.05) was considered statistically significant.

Results

Hsa_circ_0064357 and hsa_circ_0064358 was downregulated in OSCC and OLP

Hsa_circ_0064357 and hsa_circ_0064358 expression in OSCC and OLP tissues was measured using RT-qPCR. As shown in figures, hsa_circ_0064357 (11.7-fold), and hsa_circ_0064358 (8.5-fold) were expressed at significantly lower levels in OSCC tissues than in the corresponding non-tumorous tissues (n=30, p <0.0001)(Fig. 1a, b). Moreover, we found that the expression levels of hsa_circ_0064357 (12.8-fold) and hsa_circ_0064358 (4.4-fold) were lowly expressed in OLP tissues in comparison to that in normal tissues (n=10, p <0.05, Fig. 1 c, d). Specifically, compared to OLP, OSCC tissues showed 52.1 and 704.6-fold decreases in mRNA expression of hsa_circ_0064357 and hsa_circ_0064358, respectively (p < 0.001).

Correlation between clinicopathological features and RAF1, hsa_circ_0064357 and hsa_circ_0064358 expression levels

RAF1 was predicted as a potential target gene of studied circRNAs via Circinteractome and Circbase online bioinformatics databases. By qRT-PCR analyses, we determined the expression level of *RAF1* gene in each pair of OSCC, OLP tissues, and related adjacent normal tissue; *RAF1* was expressed at significantly higher and lower levels in OSCC (3.09-fold, n=30, p<0.001) and OLP (0.48-fold, n=10, p<0.001) tissues than in the corresponding non-tumorous tissues (Fig. 2 a, b). Pearson's correlation analysis revealed weak, mainly non-significant negative correlations between hsa_circ_0064357 (r = – 0.0897; p = 0.6371) and hsa_circ_0064358 (r = – 0.0683; p = 0.7195) expression levels and *RAF1* overexpression in OSCC tissues (Fig. 3 a, b).

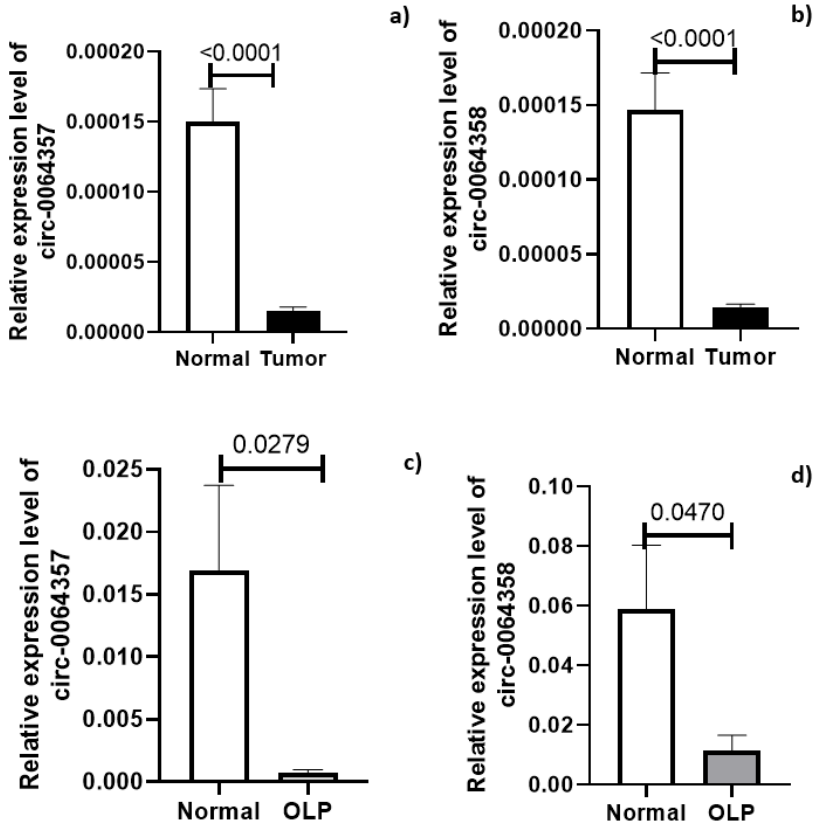

Figure 1. Quantitative RT-PCR analysis of hsa_circ_0064357 and hsa_circ_0064358 expressions in OSCC tissues and adjacent normal tissues (n=30) (a and c), OLP (Oral lichen planus), and adjacent normal tissues (n=10) (b and d). Transcript levels were normalized to *ACTB* expression. Data are presented as means ± SD. *** indicates a statistically significant difference ($p < 0.0001$).

The association of hsa_circ_0064357 and hsa_circ_0064358 and *RAF1* expression with several clinicopathological characteristics of OSCC patients were analyzed (Tab. 2). We found that *RAF1* level does not correlate with any of the clinicopathological factors, possibly due to insufficient sample sizes ($p>0.05$). hsa_circ_0064357 expression levels were positively correlated with age (P=0.002), gender (P=0.042), vascular invasion (P=0.043), clinical stage (P=0.005), histologic grade (P=0.000) and metastasis (P=0.000). However, we did not find significant differences in the tumor size, necrosis, and perineural and lymphatic invasion of hsa_circ_0064357 expression. Moreover, hsa_circ_0064358 levels were related to

age (P=0.020), tumor size (P=0.018), lymphatic and vascular invasion (p=0.000 and p=0.031, respectively). We also found a significant association with the presence of clinical stage (P=0.016), histologic grade (P=0.000), and metastasis (P=0.005). In contrast, no significant differences were observed concerning other clinicopathological factors (gender, lymphatic invasion, and necrosis (P>0.05) (Tab. 2).

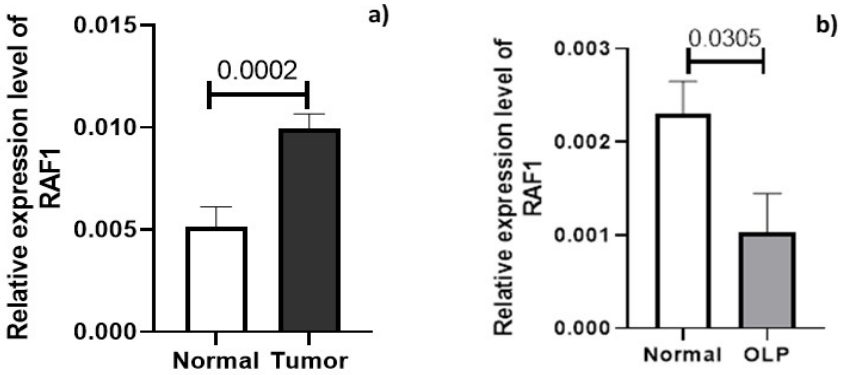

Figure 2. Quantitative RT-PCR analysis of *RAF1* expression in OSCC tissues and adjacent normal tissues (n=30) (a), OLP (Oral lichen planus), and adjacent normal tissues (n=10) (b). Transcript levels were normalized to *ACTB* expression. Data are presented as means ± SD. ***indicates a statistically significant difference ($p < 0.0001$).

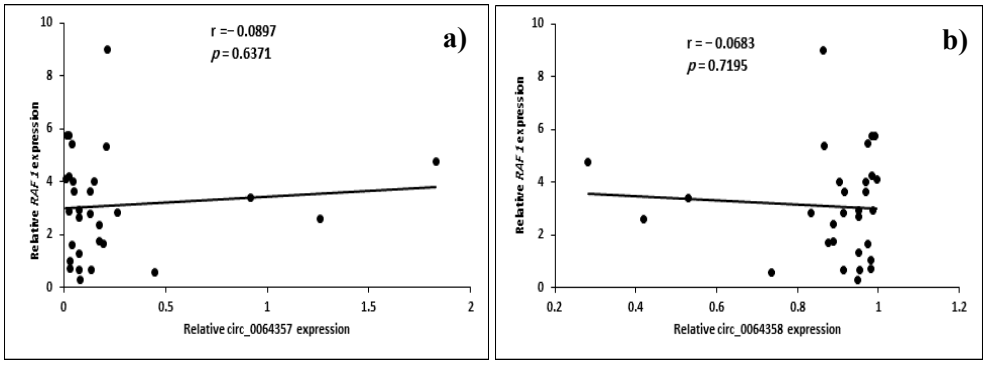

Figure 3. Correlations between hsa_circ_0064357, hsa_circ_0064358 and *RAF1* expression were measured using Pearson's correlation coefficient (r), and the significance levels are reported as follows: *p < 0.05; ***p < 0.001. Non-significant and very weak negative correlation was found between the expression of *RAF1* and circRNAs in OSCC patients' tissues.

Table 2. Association of hsa_circ_0064357, hsa_circ_0064358 and *RAF1* target gene expression with clinicopathological characteristics in OSCC

Clinical features	Case No. (%)	hsa_circ_0064357		hsa_circ_0064358		*RAF1*	
		Mean±SD	*P* value	Mean±SD	*P* value	Mean±SD	*P* value
Gender			**0.042**		**0.020**		0.443
Female	8	0.06±0.05		0.05±0.04		1.75±1.43	
Male	22	0.28±0.45		0.28±0.45		3.58±1.93	
Age (Years)			**0.002**		0.134		0.883
≥ 40	26	0.18±0.28		0.25±0.45		3.08±2.01	
< 40	4	0.52±0.86		0.44±0.77		3.18±1.96	
Size (cm)			0.095		**0.018**		0.105
≥ 5	9	0.12±0.13		0.08±0.06		3.30±2.80	
< 5	21	0.27±0.47		0.36±0.57		3.00±1.56	
Perineural invasion			0.631		0.076		0.878
Present	12	0.27±0.39		0.43±0.63		2.84±2.29	
Absent	18	0.19±0.42		0.17±0.37		3.26±1.77	
Vascular invasion			**0.043**		**0.000**		0.335
Present	6	0.39±0.54		0.61±0.87		3.18±1.62	
Absent	24	0.18±0.36		0.19±0.33		3.07 ±2.07	
Lymphatic invasion			0.308		**0.031**		0.159
Present	5	0.32±0.52		0.49±0.88		4.78±2.65	
Absent	25	0.20±0.38		0.23±0.39		2.75±1.68	
Necrosis Presence			0.636		0.157		0.244
Present	7	0.24±0.45		0.42±0.74		3.09±4.76	
Absent	23	0.22±0.40		0.23±0.40		3.09±2.13	
Stage			**0.005**		**0.016**		0.250
Low (I and II)	13	0.09±0.08		0.16±0.18		3.35±1.94	
High (III and IV)	17	0.32±0.51		0.37±0.63		2.89±2.03	
Grade			**0.000**		**0.000**		0.737
I	16	0.08±0.07		0.16±0.17		3.08±2.13	
II	13	0.41±0.56		0.44±0.71		3.31±1.72	
Status unknown	1						
Clinical Metastasis			**0.000**		**0.005**		0.396
M0	29	0.17±0.27		0.23±0.43		3.03±1.98	
M1	1	1.82		1.60		4.77	

Potential Diagnostic Values of hsa_circ_0064357 and hsa_circ_0064358 in OSCC

ROC curve analysis was performed to estimate the potential diagnostic value of hsa_circ_0064357 and hsa_circ_0064358 in OSCC for distinguishing OSCC tissues from paired adjacent normal tissues. The area under the ROC curve (AUC) for hsa_circ_0064357 expression in OSCC tissues was 0.962 (95% confidence interval (CI), 0.923- 1.000; $P<0.0001$), the cut-off value was 15.15, and the sensitivity and specificity were 83.33% and 96.67%, respectively (Fig. 4a, b). As shown in figure 4c, d-D, AUC for hsa_circ_0064358 was found to be 0.965 (95% CI, 0.926- 1.000; $P<0.0001$), and at the cut-off value of 15.18, the optimal sensitivity and specificity were 93.33% and 94.57%, respectively. A higher AUC indicates a higher accuracy of the diagnostic value of the tested variable. These results indicated that hsa_circ_0064357 and hsa_circ_0064358 have high diagnostic values for discriminating OSCC patients from healthy controls.

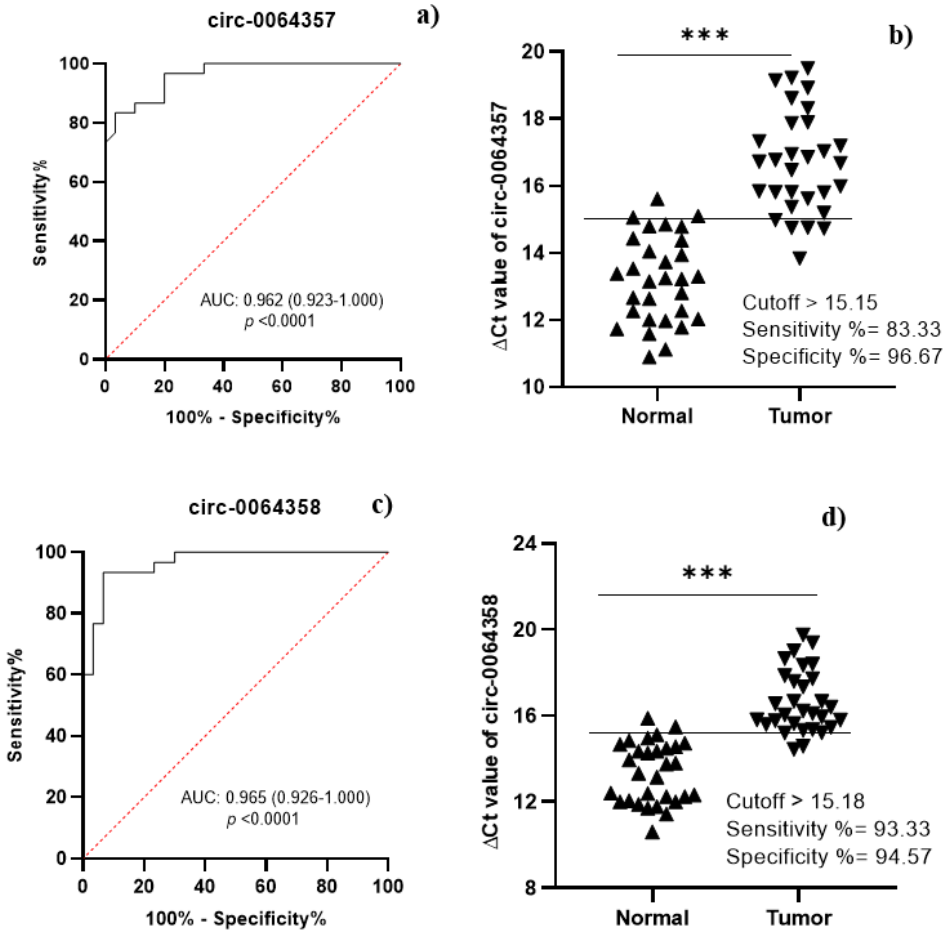

Figure 4. ROC curve analysis for the diagnostic value of hsa_circ_0064357 and hsa_circ_0064358 in OSCC. (A) The AUC for hsa_circ_0064357 and hsa_circ_0064358 were 0.962 (95% CI =0.923–1.000; P<0.0001) (A), and 0.965 (95% CI, 0.926- 1.000; P<0.0001) (C), respectively. The cut-off of circ-0082737(B) and circ-0082738 (D) was 15.15 and 15.18. Data are presented as means ± SD; ***P<0.0001.

Discussion

OSCC is often characterized at the late stages (III/IV) due to the inherent ability of lymph node metastasis of the oral cavity (Biswas *et al*., 2019). Accurate and reliable detection of oral precancerous or early lesions such as OLP may

help prevent their potential for malignant transformation and onset of symptoms of severe dysplasia and even squamous cell carcinoma, where treatment is less screening methods based on measuring the expression of cancer-associated molecular biomarkers improve the accuracy, performance sensitivity, and specificity of oral cancer screening over the conventional ocular inspection of the oral cavity and histopathological assessment of biopsy tissue (Yardimci *et al*., 2014; Tampa *et al*., 2018). Cumulative research has demonstrated that the expression level of circRNAs differs substantially between tumor and normal tissues and can be involved in the regulation of tumorigenesis. Therefore, the research of the circRNAs renders favorable as molecular biomarkers to enhance the efficacy of cancer diagnosis (Su *et al*., 2019).

In OSCC, abnormal expression of circRNAs has been implicated in cancer development and progression (Zhou *et al*., 2020). For instance, using high-throughput microarray analysis, Deng *et al*. (2019) found that 213 circRNAs were differentially expressed in 3 pairs of OSCC and matched normal tissues, including 124 up-regulated and 89 down-regulated. In another study, over expression of circRNA_100290 regulate the function of cyclin-dependent kinase 6 (CDK6) by sponging miR-26 in OSCC tissues (Chen *et al*., 2017). circDOCK1 (hsa_circ_100721) is up-regulated in OSCC tissues and can inhibit miR-196a-5p by competing with BIRC3, thereby suppressing apoptosis (Wang *et al*., 2018). Li *et al*. (2018) identified that hsa_circ_0008309 is significantly downregulated in OSCC and could increase ATXN1 (Ataxin 1; components of the Notch signaling pathway) expression through inhibition of miR-136-5P and miR-382-5P expression in the OSCC cell lines. Wang and his colleagues demonstrated that circ_000334, circ_006371, and circ_006740 were significantly downregulated in OSCC and could act as ceRNA (competing endogenous RNA), affecting the development of OSCC (Wang *et al*., 2018). Similarly, other circRNA, such as hsa_circRNA_100533 and hsa_circ_0003829, have also been reported to exhibit low expression levels in OSCC, and their overexpression can effectively inhibit OSCC proliferation, migration and extend cell apoptosis (Zhu *et al*., 2018; Zhang *et al*., 2020). Su *et al*. proved hsa_circ_0055538 was significantly downregulated in OSCC tissue. Also, knockdown of hsa_circ_0055538 correlated with the malignant biological behavior of OSCC by regulation of the p53/Bcl-2/caspase signaling pathway (Su *et al*., 2019).

Most recent studies have focused on the potential prognostic significance of cancer-related circRNAs (Wang *et al*., 2021; Kristensen *et al*., 2022). In contrast, accurate recognition and monitoring of malignant transformation in oral potentially malignant disorders (OPMD) such as OLP with malignant transformation rates ranging from 0.44 to 1.4% through differential expression patterns of circRNAs is a challenging issue that has not been addressed (Tsushima *et al*., 2021). Therefore, in the present study, we focused on the differential expression

of hsa_circ_0064357 and hsa_circ_0064358 in OLP and OSCC patients. The circRNAs hsa_circ_0064357 and hsa_circ_0064358 are encoded by *RAF1* gene and located on chromosome 3 at chr3:12625099-12626752 (spliced sequence length of 1324 bp) and chr3:12626345-12626480 (spliced sequence length of 1354 bp) respectively from UCSC and circBank database; however, the function of these circRNAs in OSCC progression has remained unknown. Here, we carried out qRT-PCR analyses to compare circRNAs expression levels in OSCC (n=30) and OLP (n=10) tissues with those in normal adjacent tissues. We found that hsa_circ_0064357 and hsa_circ_0064358 were significantly downregulated in OSCC and OLP relative to their matched normal tissues (p <0.001). A notable observation was the significantly lower expression of circRNAs in OSCC compared to OLP tissue (p <0.001). Consistent with our previous report, the expression of circ_0045638 and circ_0045639 was significantly downregulated in OSCC and OLP tissues relative to their matched-adjacent normal tissues, and Low expression of circRNAs was also found in OSCC compared with OLP tissues (Jahangiri *et al*., 2022). Thus, this discrepancy may help the precise detection of precancerous lesions at screening programs and follow-up on their malignancy potential and down-staging the disease.

Increasing reports have shown that circRNAs play multiple regulatory roles in various cellular events critical in cancer development and progression, such as the abnormal expression of important downstream components of cancer-related signaling pathways, including MAPK/ERK and Wnt/β-catenin signaling and PTEN/PIK3/AKT pathways (Garlapati *et al*., 2021; Xue *et al*., 2021). Dysregulation of Raf1 as a core regulatory signaling molecule in the ERK/MAPK pathway due to aberrant circRNA expression has been reported to activate this pathway in various cancer types (Cheng *et al*., 2022). For example, circ_CDR1 depletion suppresses hepatocellular carcinoma cell (HCC) proliferation and metastasis *in vivo* via regulating miR-1287/Raf1 pathway (Zhang *et al*., 2020). ciRS-7 can interact directly with miR-7, resulting in the upregulation of RAF-1/PIK3CD expression and enhancing the metastatic progression of OSCC (Dou *et al*., 2020).

In the present study, using the online circular RNA databases (CircInteractome and Circbase) for target gene prediction, *RAF1* was identified as the potential targeted gene of hsa_circ_0064357 and hsa_circ_0064358 and the level of *RAF1* in 30 pairs of OSCC and 10 pairs of OLP tissues are detected. Our present findings are consistent with those of Kordi-Tamandani *et al*. (2014) in that compared with healthy samples, the expression level of *RAF1* was significantly up and down-regulated in OSCC and OLP tissues, respectively. In addition, we found a very weak negative and non-significant correlation between *RAF1* overexpression and circRNAs levels in OSCC (Figures 3a, b), and whether

those circRNAs are associated with elevated levels of *RAF1* should be the focus of the further investigation. Recent studies have revealed that abnormal expression of Raf1 are closely correlated with tumor invasion, lymph node metastasis, and T stage in thyroid cancer (Wang *et al*., 2015), non-small cell lung cancer (NSCLC)(Tian *et al*., 2018), prostate (Ren *et al*., 2012), colorectal (Slattery *et al*., 2012; Borovski *et al*., 2017). A study by Li *et al*. (Li *et al*., 2022) showed that up-regulation of *RAF1* can promote tumor growth and lymphatic metastasis by targeting LAGE1 in hypopharyngeal carcinoma.

Our present findings were not in line with previous reports in that we did not find any significant correlations between the up-regulation of *RAF1* expression and the clinicopathological characteristics of OSCC. More importantly, a comparison of hsa_circ_0064357 and hsa_circ_0064358 expression levels with clinicopathological features of OSCC patients revealed that decreased expression of the circRNAs was significantly associated with the TNM stage, an important factor in evaluating the prognosis of OSCC. These results indicate that hsa_circ_0064357 and hsa_circ_0064358 might be involved in the progression and metastasis of OSCC and could be used as promising novel biomarkers for early diagnosis and new therapeutic targets for OSCC. In addition, the ROC analysis indicated that the hsa_circ_0064357 and hsa_circ_0064358 expression levels with high sensitivity and specificity exhibited a potential diagnostic value in distinguishing OSCC tissues from healthy samples.

Conclusion

In summary, the findings of the present study provided the first evidence that hsa_circ_0064357 and hsa_circ_0064358 were downregulated in both OSCC and OLP tissues and were significantly associated with TNM stage in OSCC patients, suggesting that the studied circRNAs may serve as prognostic markers for early detection and new target treatment in OSCC. hsa_circ_0064357 and hsa_circ_0064358 expression levels of OSCC and OLP tissues exhibited significant differences that may facilitate accurate and early discrimination of OSCC from suspicious OLP. However, further studies with larger sample sizes are needed to elucidate the functions and mechanisms of hsa_circ_0064357 and hsa_circ_0064358 in OSCC tumorigenesis and metastasis.

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Phylogenetic analyses of the proteins involved in encapsulation signaling pathways in ants

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Abstract. One of the major evolutionary transitions is the shift from solitary to social lifestyle, which involved a plethora of behavioral and physiological changes in social entities. Group living has several advantages as the evolution of collective defense mechanisms. It may also affect the individual immune system either due to the efficiency of social immune defenses or because of the high transmission frequency of pathogens. Individual defense consists of the innate and acquired immune components. In insects, there are two signaling pathways (Toll and Jak/Stat) that result in the expression of specific immune genes, which, in their turn, encode peptides, proteins and activate innate immune responses like encapsulation. The main aim of our study was to verify whether transition to eusocial lifestyle is reflected in proteins involved in immune responses. We carried out phylogenetic analyses of 15 proteins involved in encapsulation signaling pathways in ants. We also included four other social insect groups, bees, sweat bees, social wasps, and termites, and three solitary insect groups, as fruit flies, braconid wasps, and megachilid bees. Ants grouped separately from other insect groups in most cases, however, there were some notable exceptions mostly in the case of pattern recognition proteins, probably correlating

with differences in potential pathogens. No major differences were revealed though between solitary and social insects with respect to proteins involved in encapsulation.

Keywords: eusociality, evolution, immune response, innate immunity, insects, social immunity

Introduction

Multicellular organisms are continuously exposed to different pathogens, like viruses, bacteria, fungi, which can harm the host, may cause behavioural, morphological, and functional changes, or even lead to its death (Schmid-Hempel, 1998; Verble *et al.,* 2012; Csata *et al.,* 2014; Hughes *et al.,* 2016; Csata *et al.,* 2017a, Csata *et al.,* 2017b; Csata *et al.,* 2018; Csősz *et al.,* 2021; Csata et al. 2023). In animals, the innate (non-specific) and acquired immune systems are employed internally against pathogens (Schmidt, 2001). The innate immune response is short-term, general, and inhibits the spread of pathogens within the host's body through physical and chemical defense mechanisms (Cotter and Kilner, 2010). On the other hand, the acquired immune response is long-term, highly specialized, and is based on immune memory since after being challenged by specific pathogens, the organism can recognize these for a longer period (Janeway *et al.,* 2001). While in vertebrates both innate and acquired immune systems are present, invertebrates are considered to lack entirely the acquired component. There are though some notable exceptions as is the case of the *Anopheles gambiae* mosquito (Yan *et al.,* 1997; Kurtz and Armitage, 2006), the fruit fly *Drosophila melanogaster* (Flemming, 2017), and the ant *Lasius neglectus* (Konrad *et al.,* 2012), where a certain degree of immune priming had been shown.

In insects, the innate immune system is made up of physical barriers, cellular and humoral responses (Rosales, 2017). If pathogens or parasites pass the physical barrier (cuticle, peritrophic membrane) then the cellular and humoral immune responses are activated. The cellular immune responses include: phagocytosis, nodulation, coagulation, melanization, and encapsulation. The humoral immune responses are related to some pattern recognition proteins (PRPs), which after the invasion recognize the pathogen-associated molecular patterns (PAMPs) and thus activate immune signaling pathways

(Toll, Imd, Jak/Stat). The activated pathways elicit the synthesis of antifungal and/or antibacterial peptides in the fat bodies, and these peptides will be emitted into the hemolymph (Dubovskiy *et al.,* 2016). Gram-positive bacteria and fungi activate the Toll pathway, Gram-negative bacteria the Imd pathway, while stress/injury activates the Jak/Stat pathway (Broderick *et al.,* 2009; Fig. 1).

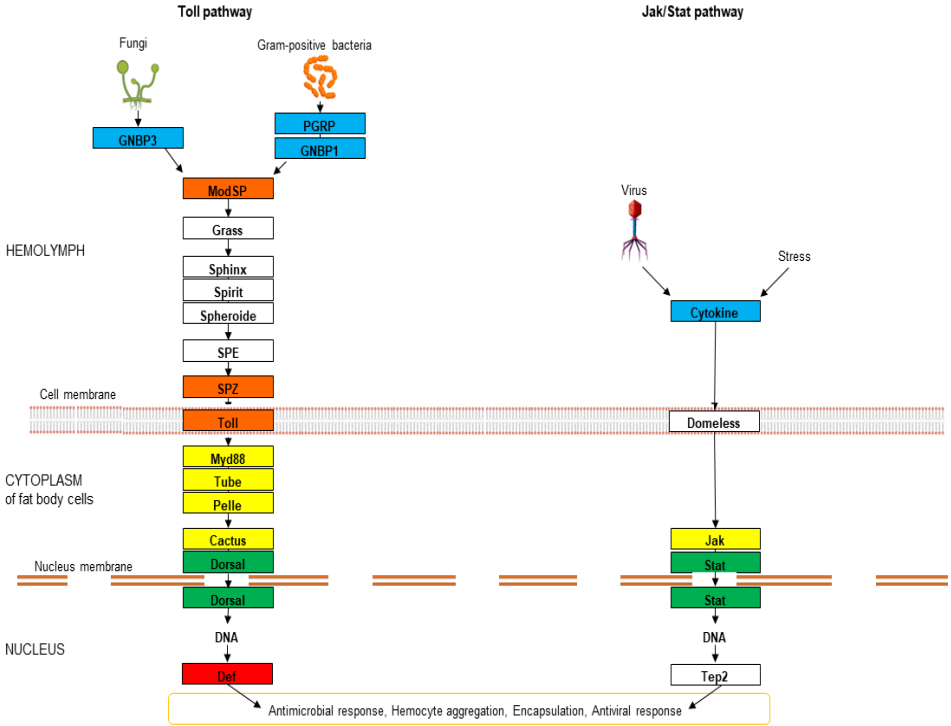

Figure 1. Key proteins of the Toll and Jak/Stat signaling pathways: coloured boxes stand for analyzed proteins; white boxes are for the non-analyzed proteins; dark blue - recognition molecules; orange - the signaling molecules in the haemolymph; yellow - signaling molecules in the cytoplasm; dark green - transcription factor; red - the antimicrobial peptid.

The haemocytes are binding to the target forming a multilayer capsule around the invader, which is melanized by the proPO (prophenoloxidase) cascade (Marmaras and Lampropoulou, 2009; Dubovskiy *et al.,* 2016; Rosales, 2017). The invader within the capsule is then killed and destroyed by asphyxia or by reactive cytotoxic radicals (ROS, RNS) (Nappi *et al.,* 1995; Nappi and Ottaviani, 2000; Carton *et al.,* 2009).

The Toll signaling pathway is regulating the melanization process, and its activation results in the expression of immune genes like drosomycin, and defensin, which encode antimicrobial peptides and prophenoloxidase-activating enzymes. The Jak/Stat pathway's activation results in the expression of immune genes such as Turandot, Tep2 (Rolff and Reynolds, 2009).

While individual defense strategies, both immunological (e.g. encapsulation) and behavioural (e.g. grooming), are quite straightforward to study, in the case of social organisms the social context should also be considered due to the existence of emergent social defensive strategies, which might interfere with the individual immune system. As formulated by Cremer and Sixt (2009) the collective defense of a group comprises all individual defenses of the group members and their interaction. In comparison to solitary lifestyle, there are some mechanisms developed in the social context, particularly in eusocial animals, mostly insects, to fight off parasites, pathogens and/or reduce their spread inside the host body/system: the production of diverse antibiotic secretions, mutual grooming, collective broodcare, removal/exclusion of infected individuals, nest hygienic behaviour (Cremer *et al.,* 2007; Cremer and Sixt, 2009; Meunier, 2015). Two major hypotheses could be formulated as to how sociality could change individual immune responses: (a) individual immunity could be less efficient in social insects due to the compensatory effect of emerging social immunity, (b) or individual immune system should function at a higher level than in solitary animals due to higher risk of infection conferred by high frequency of interactions within the social system (Castella *et al.,* 2008; Cremer and Sixt, 2009; Stroeymeyt *et al.,* 2014). Under both hypotheses switching from solitary lifestyle to sociality could have resulted in evolutionary changes in the level of immune system.

In ants all species are eusocial, and sociality emerged only once during their evolution (Ward, 2006). Therefore, they constitute an intriguing study subject with regard to how sociality might have influenced the immune system. As presented above, we hypothesize there could be differences between solitary and social insects, such as ants, with regard to proteins involved in the immune signaling pathways. Thus, we proposed to analyze the phylogeny of proteins of the Toll and Jak/Stat signaling pathways in ants by considering a wide array of different ant species alongside other social and solitary insects.

Methods

Sequence selection, data sets

Amino acid sequences of the proteins involved in the encapsulation signaling pathways were downloaded from GenomeNet (http://www.genome.jp) and from NCBI (May 2019–October 2020). We used amino acid sequences for the phylogenetic analyses because amino acid sequences are more conserved than the corresponding nucleotide sequences, and they exhibit far less random homoplasy, than DNA sequences. We obtained the amino acid sequences for 15 proteins, key components of the Toll and Jak/Stat signaling pathways, out of a total of 22 proteins (Broderick *et al.,* 2009, Bechsgaard *et al.,* 2016) (Fig. 1). For the other 7 proteins (Grass, Sphinx, Spirit, Speroide, SPE, Domeless, Tep2) the GenomeNet and NCBI research did not get any results, thus we excluded these proteins from our analyses. We used homologue sequences instead of orthologs. After taking a look at OMA (Orthologous matrix) Orthology database (Altenhoff *et al.,* 2021) to determine the orthologs for our species set, we realized the lack of many orthologs. We found that most of the species genomes have not been sequenced, and this is why many of the sequences we use are from transcriptomes rather than genomes. We used the query sequences of the ant species *Atta cephalotes* Linnaeus 1758, for all of 15 proteins to obtain the homologue sequences. Homologues of protein sequences were identified with blastp (protein Blast) search (Altschul *et al.,* 1997), from the non-redundant database. We used it as a common practice and selected the longest isoform annotated from each protein to do the phylogenetic reconstruction. Besides ants (47 species), we also included species from four different social insect families as Apidae (35 species), Halictidae (1 species), Vespidae (2 species) (order Hymenoptera), and Termitidae (15 species) (order Blattodea), and from three solitary insect families as Braconidae (4 species), Megachilidae (2 species) (order Hymenoptera), and Drosophilidae (24 species) (order Diptera) (Table S1). Blast search hits with E-values higher than $1e^{-05}$ were discarded. In the case of the PGRP protein we obtained partial homologous sequences for 8 *Myrmica* ants species, but the query cores (>70%) and the E-values (> $1e^{-05}$) of the sequences were adequate so we used these protein sequences to generate the phylogenetic tree. In the case of the Drosophilidae family, more than 5 homologue sequences were found for each protein, and we chose 5 sequences with the best E-values and query cores (Table 1). For the transmembrane receptor Domeless (Fig. 1), we did not get any results by NCBI protein sequence search.

Phylogenetic trees

Phylogenetic trees were reconstructed by using Phylogeny.fr (http://www.phylogeny.fr), an online web service for analyzing phylogenetic relationships between sequences (Dereeper *et al.,* 2008). We used the Clustal Omega, Muscle, Mafft, and T-coffee softwares for the multiple sequence alignment (Madeira *et al.,* 2019). Then we used the TCS (T-coffee package), a new multiple sequence alignment reliability measure to estimate alignment accuracy and improve phylogenetic tree reconstruction (Chang *et al.,* 2014). From the four multiple sequence alignment programs the T-coffee gave us for each protein the highest score, thus in the following analyses we used the alignments generated by this program. To eliminate the poorly aligned and/or gap positions, such as the nonconserved segments from the alignments we used G-blocks 0.91b (Castresana, 2000; Talavera and Castresana, 2007) with these settings: (i) minimum number of sequences for a conserved position (50% of the number of sequences + 1), (ii) minimum number for a flank position: 85% of the number of sequences, (iii) maximum number of contiguous nonconserved positions: 8, (iv) minimum block length: 10, (v) no gaps in final blocks. Phylogenetic trees were reconstructed using the PhyML 3.0. software (Guindon *et al.,* 2010) based on the Maximum Likelihood method. The default substitution model was LG (Le and Gascuel, 2008). The standard bootstrapping was replaced by a faster approximate Likelihood-ratio test (aLRT), which provides the exact statistical evaluation of branch support and values that bootstrap ones. We used TreeDyn for graphical editing (Chevenet *et al.,* 2006). There are generally accepted values of confidence: 70% or above is considered as statistically significant support (Soltis and Soltis, 2003).

Phylogenetic trees were successfully built for each of the 14 proteins studied with the exception of Defensin, where after multiple alignments and elimination of gap positions, the available amino acid sequences were too short (10 aa positions). The phylogenetic analysis depends on the sequence length, and on the alignment of the sequences. If the amino acid sequence is small, the probability of the wrong alignment is higher and this changes the phylogenetic tree, thus in the case of the Defensin the obtained result was not relevant.

Results

We identified several homologue sequences from a high number of species in the studied insect groups (Table 1, Table S1). The alignments generated by the T-coffee program yielded the best scores, therefore we used these to generate phylogenetic trees.

Table 1. Number of protein homologue sequences identified in five social (Termitidae, Apidae, Halictidae, Formicidae, Vespidae) and three solitary insect families (Megachilidae, Braconidae, Drosophilidae).

Protein	Function	Insect families							
		Termitidae	Apidae	Megachilidae	Halictidae	Formicidae	Vespidae	Braconidae	Drosophilidae
Toll signaling pathways									
PGRP	Recognition	2	28	2	1	30	2	3	5
GNBP1		13	15	2	1	19	2	3	5
GNBP3		15	13	2	1	21	2	3	5
ModSP	Signalling	2	5	2	1	13	2	3	5
Spz		2	11	2	1	20	2	3	5
Toll		2	11	2	1	22	2	3	5
Myd88		2	11	2	1	22	2	3	5
Tube		2	10	2	1	21	2	3	5
Pelle		2	11	2	1	22	2	3	5
Cactus		2	12	2	1	20	2	3	5
Dorsal	Transcription factor	2	10	2	1	22	2	3	5
Def	Antimicrobial response	1	23	2	1	38	2	4	5
Jak/Stat signaling pathway									
Cytokine	Recognition	2	11	2	1	22	2	3	5
Jak	Signalling	2	10	2	1	22	2	3	5
Stat	Transcription factor	2	10	2	1	20	2	3	5

Toll signaling pathway

We reconstructed the phylogenetic trees of the 11 proteins involved in the Toll signaling pathway (Figs 2-3, see supplementary files: Figs S1–S9). The different insect families grouped separately in well-defined clades in most phylogenetic trees (bootstrap proportion 70-99%) (Figs 2-3, see supplementary files: Figs S1–S9).

In the case of Pelle and Dorsal proteins, the phylogenetic tree structure followed the general insect phylogeny with Hymenoptera grouping separately. The different members of the order belonged to a well-supported clade, while species of the other two orders (Blattodea, Diptera) formed outgroups on the phylogenetic trees (Fig. 3, see supplementary files: Fig. S8).

The other proteins of the Toll signaling pathway (PGRP, GNBP1, GNBP3, ModSP, Spz, Toll, Myd88, Tube, Cactus) did not follow the classical insect phylogeny, and they neither did reflect differences in social lifestyle (Fig. 2, see supplementary files: Figs S1–S7, S9).

Ants formed a single well-supported clade in the case of ModSP, Toll, Myd88, Tube, and Dorsal (Fig. 3, see supplementary files: Fig. S3, Figs. S5–S7), whereas in the case of PGRP, GNBP1, GNBP3, Spz, Pelle, and Cactus they did not group together. Phylogenetic trees of recognition proteins as PGRP, GNBP1, and GNBP3 were especially diverse.

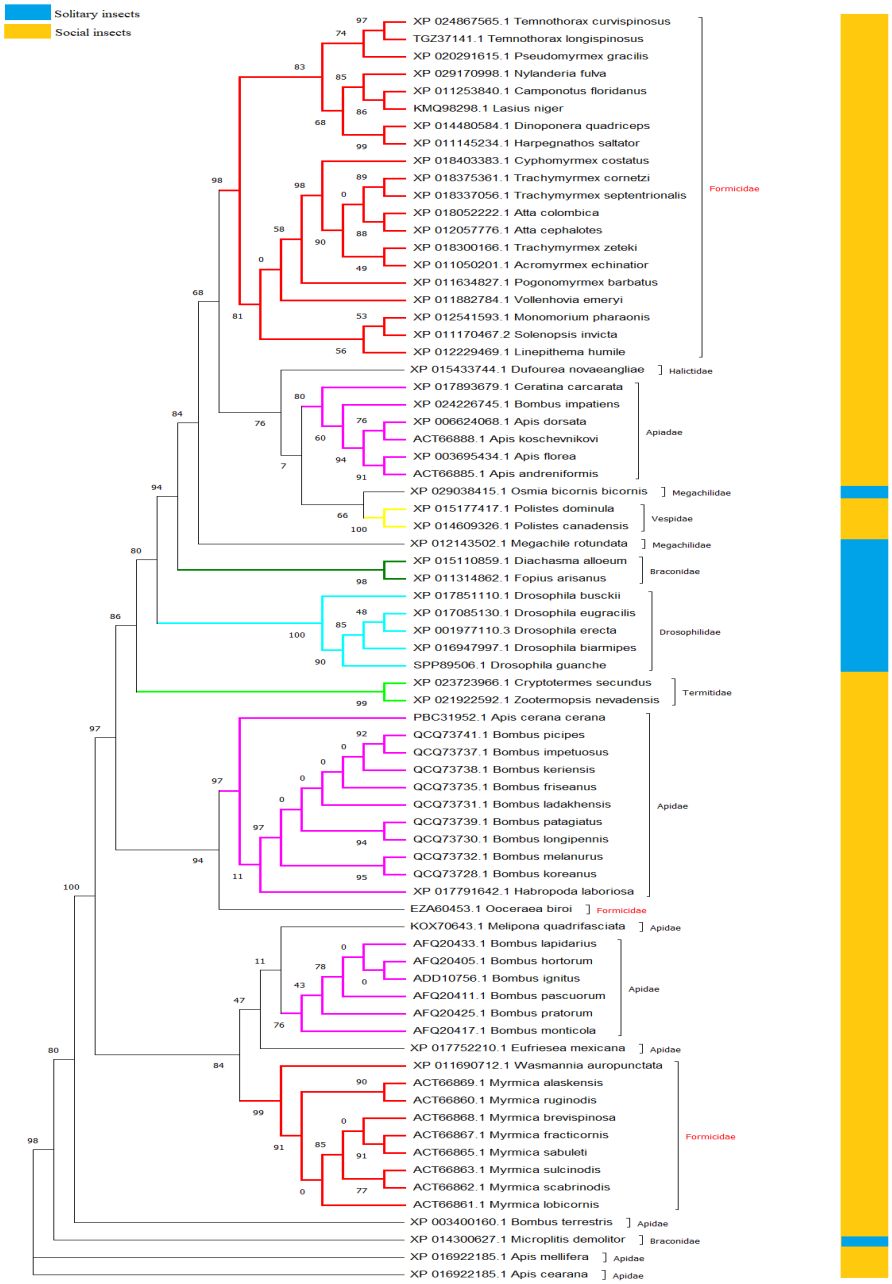

Figure 2. Phylogenetic maximum-likelihood PhyML tree of the peptidoglycan recognition protein (PGRP) based on a LG model. The tree was calculated with 73 sequences and 139 aa positions.

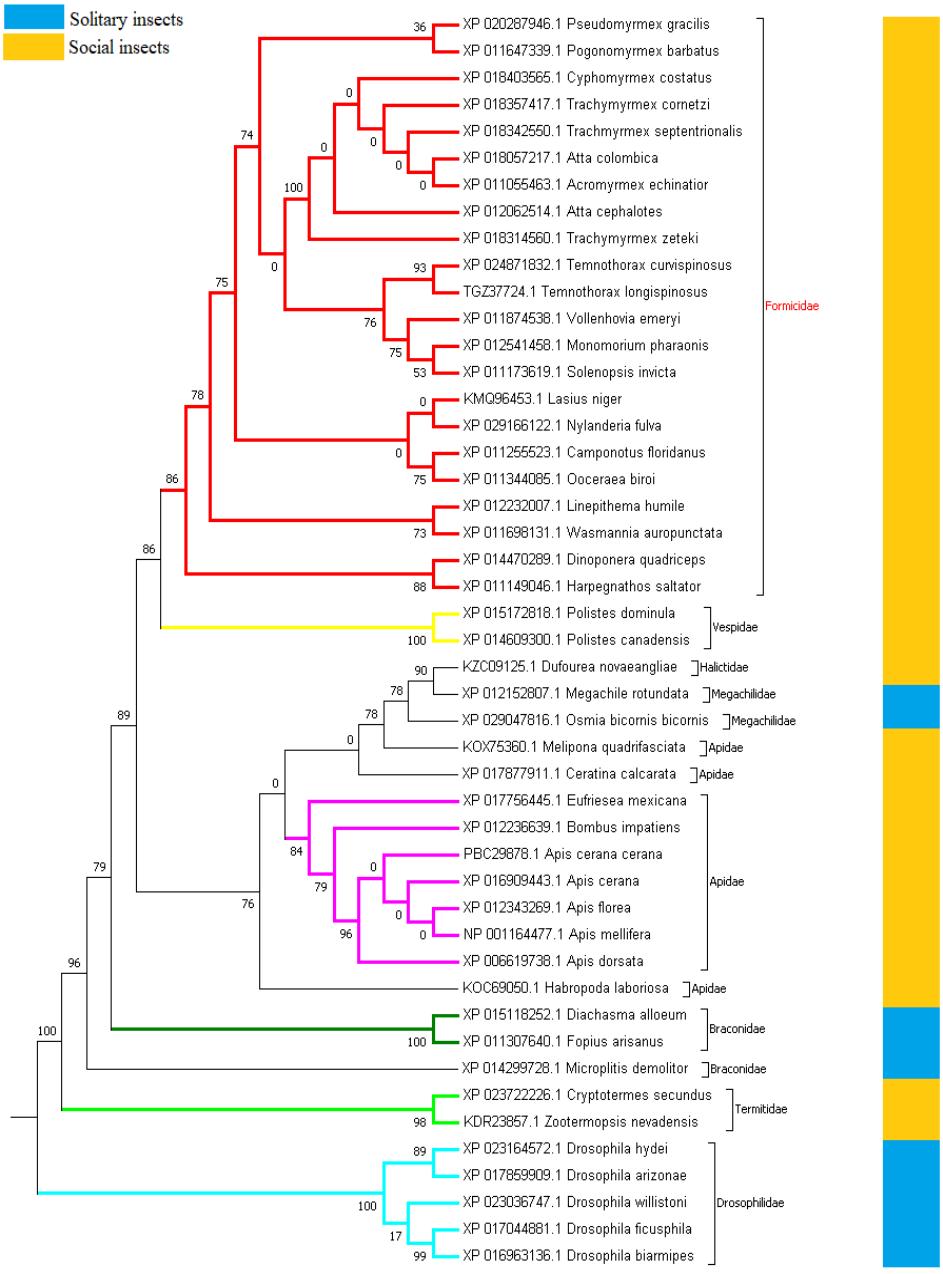

Figure 3. Phylogenetic maximum-likelihood PhyML tree of the Dorsal protein based on a LG model. The Dorsal tree was calculated with 47 sequences and 229 aa position.

Jak/Stat signaling pathway

The different insect families grouped in well-defined separate clades in the majority of phylogenetic trees (Figs. 4-5, see supplementary files: Fig. S10).

In the case of the recognition protein Cytokine receptor, we found that the phylogenetic tree structure followed the general insect phylogeny, thus there was a well-supported clade, of the different social and solitary Hymenoptera species, with two outgroups as Blattodea and Diptera (Fig. 4). The phylogenetic trees of the signaling molecule Jak and the transcription factor Stat did not show the same structure and did not reflect differences with regards to lifestyle either (Fig. 5, see supplementary files: Fig. S10).

All things considered, ants did not group together in a single well-supported clade in any of the studied proteins.

Discussion

The evolutionary success of any species depends, amongst others, on the efficiency of its immune system to properly recognize pathogens and respond adequately (Akira, 2009). Besides species-specific differences, geographical location (Wikelski *et al.,* 2004; Ayres *et al.,* 2010; Matson and Beadell, 2010; Lobato *et al.,* 2017), climatic factors (Jin *et al.,* 2011; Inbaraj *et al.,* 2016; Filipe *et al.,* 2020), and sexual features (Kurtz *et al.,* 2000; Ruggieri *et al.,* 2016) play a major role in shaping the immune responses. Transition from solitary to social life could also account for changes in the immune system of individuals due to e.g., the emergence of efficient social strategies to combat infections.

We studied the phylogeny of proteins involved in immune responses in ants to reveal whether there are ant-specific and sociality specific patterns, respectively. While in many cases proteins grouped according to larger systematic groups, e.g. Formicidae (ants), transition to social life did not seem to shape the phylogeny of these proteins in insects. We did not find evidence that the eusociality or solitary lifestyle affected/explain the separation of the different insect clades, in the studied proteins' phylogenetic trees.

The Toll pathway is activated by infection with Gram-positive bacteria and fungi, whereas the Jak/Stat pathway is activated by stress/injury, viral infection (Broderick *et al.,* 2009). The proteins of both signaling pathways seem to be conserved in the studied solitary and social insects. In the case of PGRP tree, *Myrmica* ants formed a clearly separated, well-defined clade, this separation could be explained by the fact that we had partial protein sequences for these *Myrmica* species, and despite corresponding E-values and query cores, this sequence partiality could result in their separate placement.

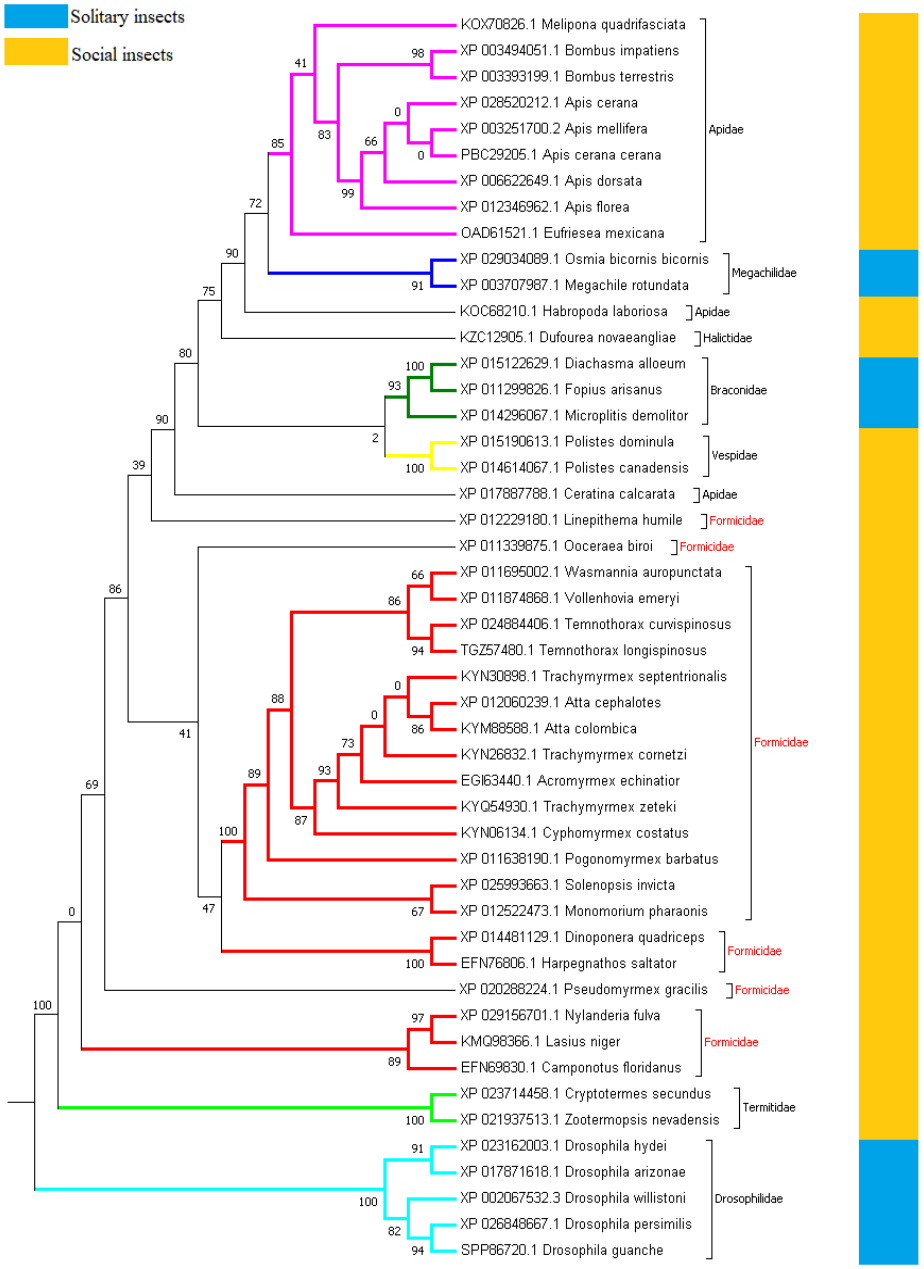

Figure 4. Phylogenetic maximum-likelihood PhyML tree of the Cytokine receptor (Cyt) based on a LG model. The Cyt tree was calculated with 48 sequences and 194 aa positions

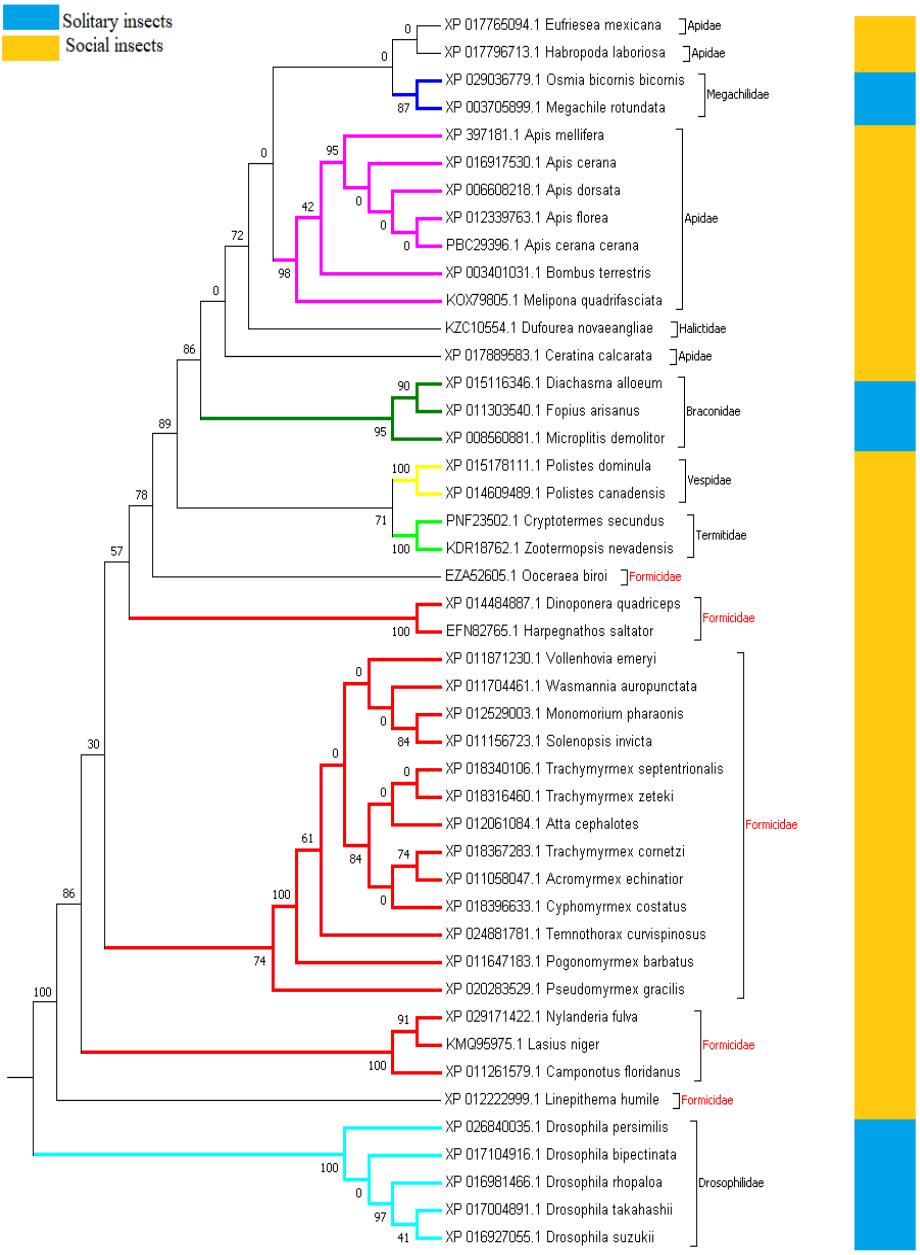

Figure 5. Phylogenetic maximum-likelihood PhyML tree of the signal transducer and activator of transcription protein (Stat) based on a LG model. The Stat tree was calculated with 45 sequences and 426 aa positions.

Recognition proteins (PGRP, GNBP1, GNBP3, Cytokine receptor) show diverse phylogenetic trees in both pathways. Insects do have a very large repertoire of pattern recognition receptors (PRR) (Wang *et al.,* 2019), and the secreted and/or transmembrane PRRs are the most diverse components of the different immune signaling pathways. For example, the cytokine receptor was highly diverse, similar to mammals, where 40 different cytokine receptors are known, suggesting an evolutionary tendency for the diversification of recognition proteins (Murray, 2007). The recognition proteins detect the molecular patterns of the pathogens, and after recognition, they activate the host defense mechanisms (Janeway *et al.,* 2001). Thus, we would expect that species with different lifestyles, with different habitats, should have different pathogens that they need to recognize adequately. There are e.g. several ant-parasitic fungal species with different lifestyles, and each species has a very well-defined set of hosts, which are not necessarily overlapping (see Espadaler and Santamaria, 2012; Csata *et al.,* 2013), therefore differences are expected to occur in the immune system accordingly, and these could present themselves mostly on the level of recognition proteins, but also on the level of immune genes and signaling proteins, transcription factors.

The phylogenetic trees of signaling proteins, and transcription factors showed though less diversity. The same was found by Khush *et al.* (2001) who compared the Toll pathways in *Drosophila* and mammals, and they found that the signal transduction and transcription systems' proteins are conserved. Bechsgaard *et al.* (2016) analyzed the genome of the arthropods and they found that the genes which encode the immune proteins of the Toll and Jak/Stat signaling pathways are conserved. In the case of the cell-type specific signaling pathways, cellular processes like gene expression have the lowest variability. Furthermore, the protein variability decreases from the recognition proteins to the transcription factors. The signal transduction proteins usually have a fundamental role, thus they are conserved, and show less diversity (Schaefer *et al.,* 2014).

Conclusion

There are several factors influencing the immune system of organisms. The building blocks of immune pathways could react differentially, some might show fine-tuned adaptations, while others could be conserved. Exposure to change could be heavily determined by the function of the proteins. Future works should analyze the potential effect of ecological background factors such as geographical distribution, lifestyle, colony structure (e.g. polygyny),

ecological characteristics (e.g. invasiveness), etc. which could, eventually, explain some of the uncovered diversity in ants with regard to their immune system, but also generally in social insects.

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SUPPLEMENTARY MATERIAL
Phylogenetic analyses of the proteins involved in encapsulation signaling pathways in ants

Kincső Orbán-Bakk[1,2]✉, Enikő Csata[3], Bálint Markó[1,4] and Ferencz Kósa[1]

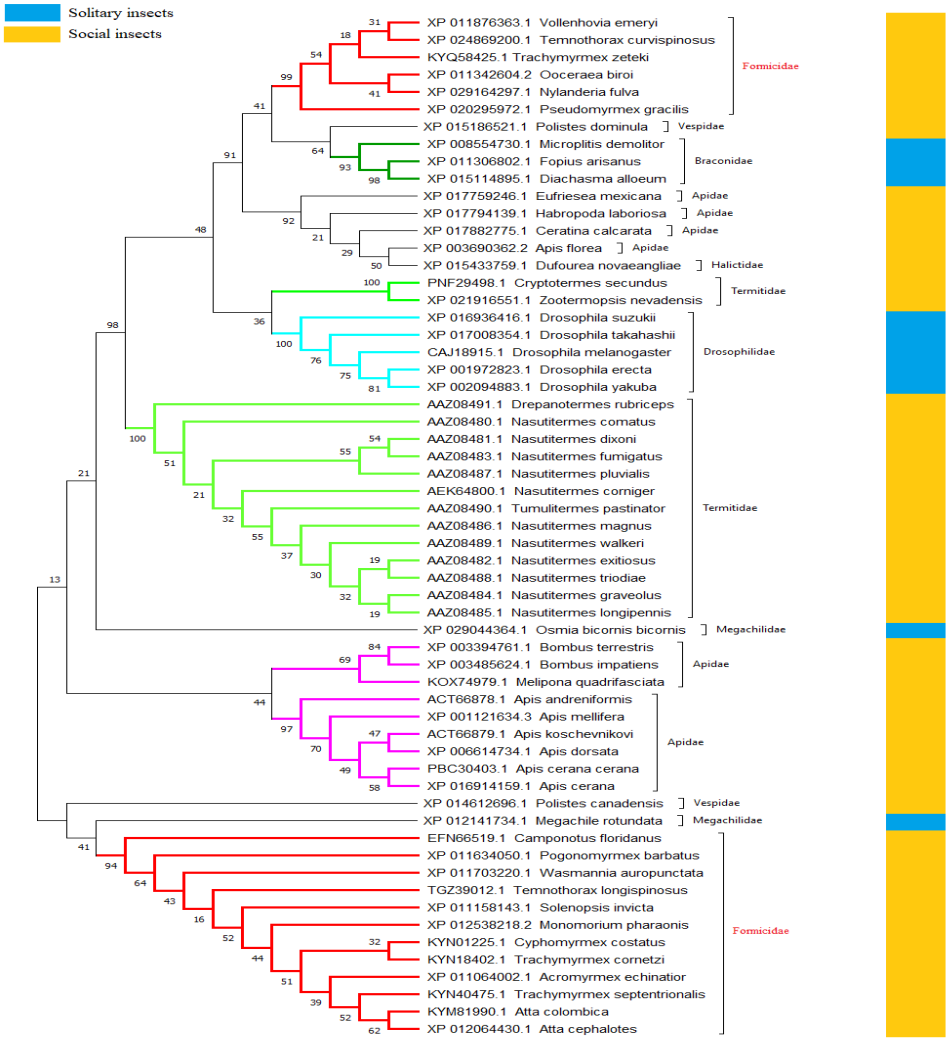

Figure S1. Phylogenetic maximum-likelihood PhyML tree of the Gram-negative binding protein 1 (GNBP1) based on a LG model. The tree was calculated with 59 sequences and 87 aa positions.

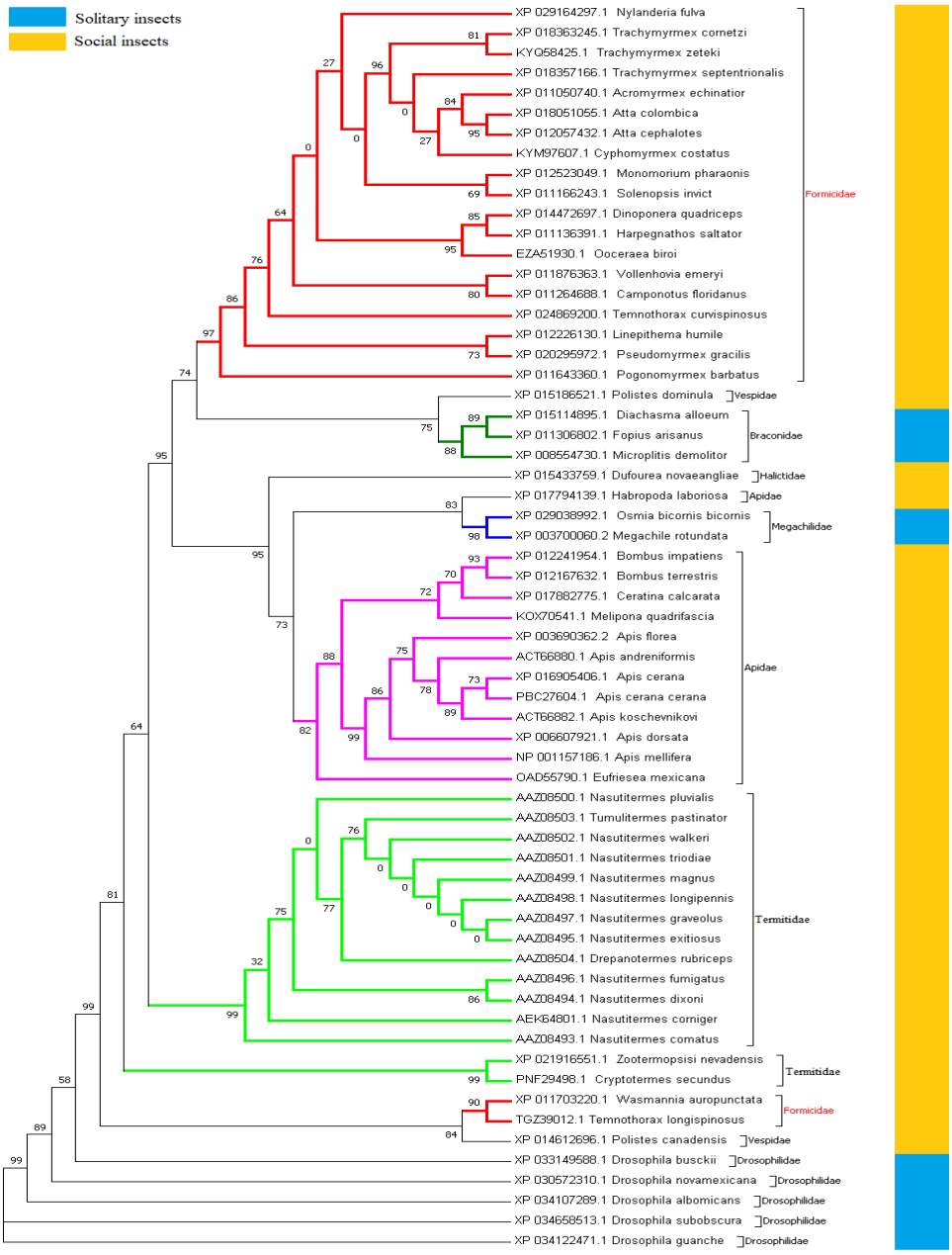

Figure S2. Phylogenetic maximum-likelihood PhyML tree of the Gram-negative binding protein 3 (GNBP3) based on a LG model. The tree was calculated with 62 sequences and 84 aa positions.

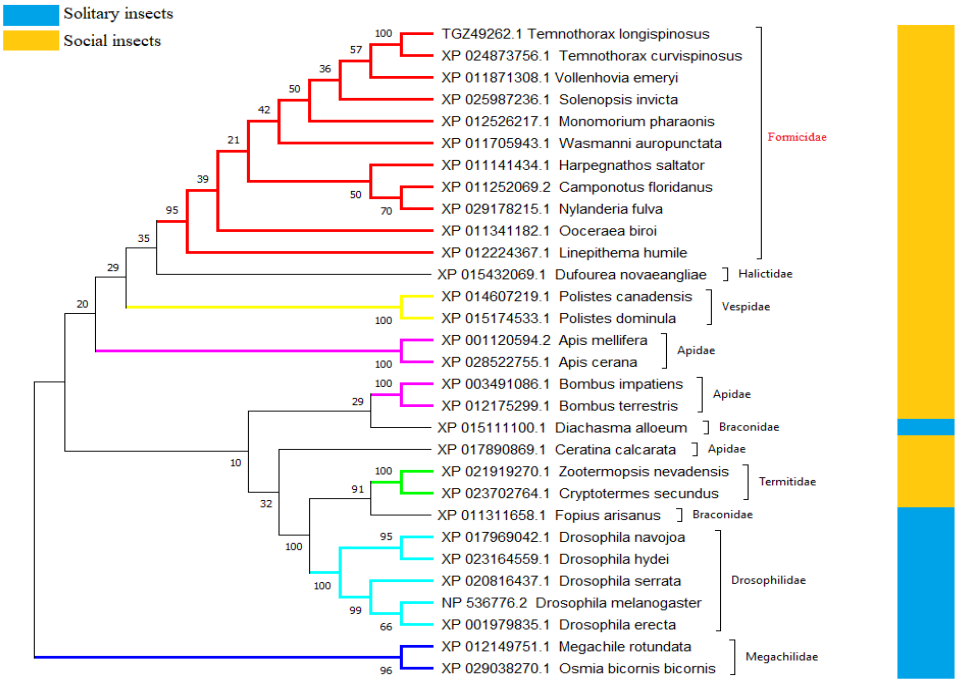

Figure S3. Phylogenetic maximum-likelihood PhyML tree of the modular serine protease (ModSP) based on a LG model. The tree was calculated with 30 sequences and 154 aa positions.

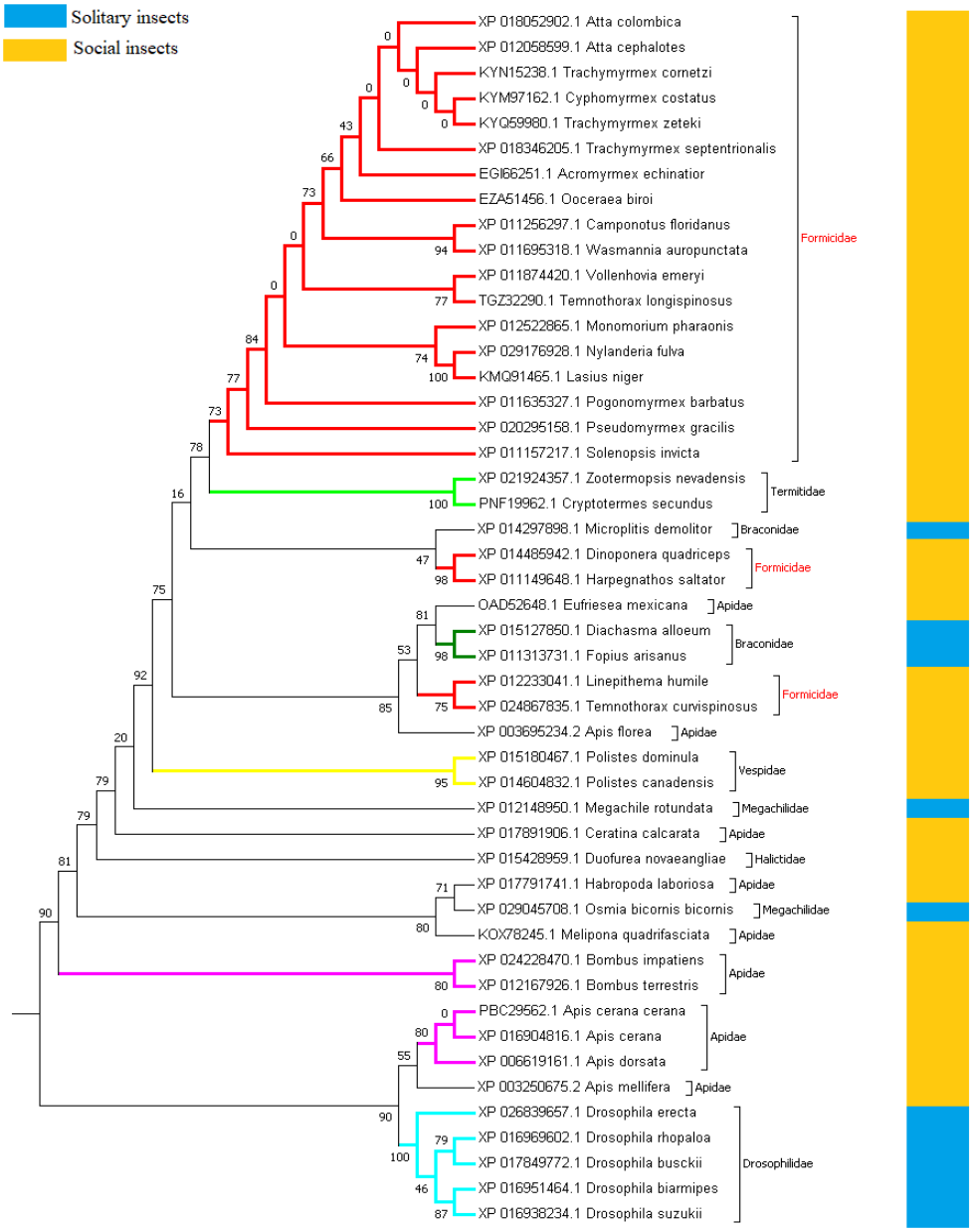

Figure S4. Phylogenetic maximum-likelihood PhyML tree of the Spatzle protein (Spz) based on a LG model. The tree was calculated with 48 sequences and 58 aa positions.

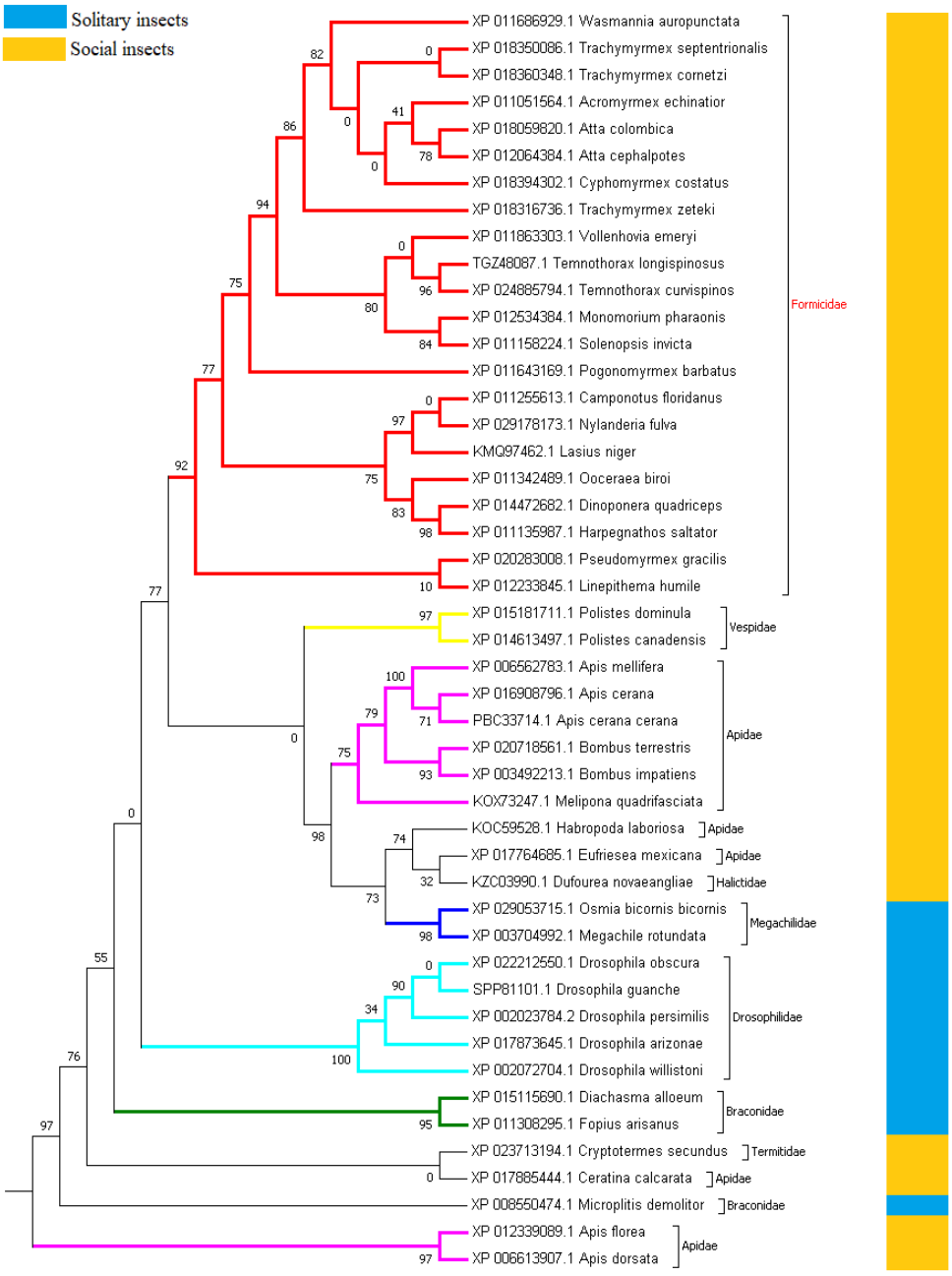

Figure S5. Phylogenetic maximum-likelihood PhyML tree of the Toll protein based on a LG model. The tree was calculated with 47 sequences and 60 aa positions.

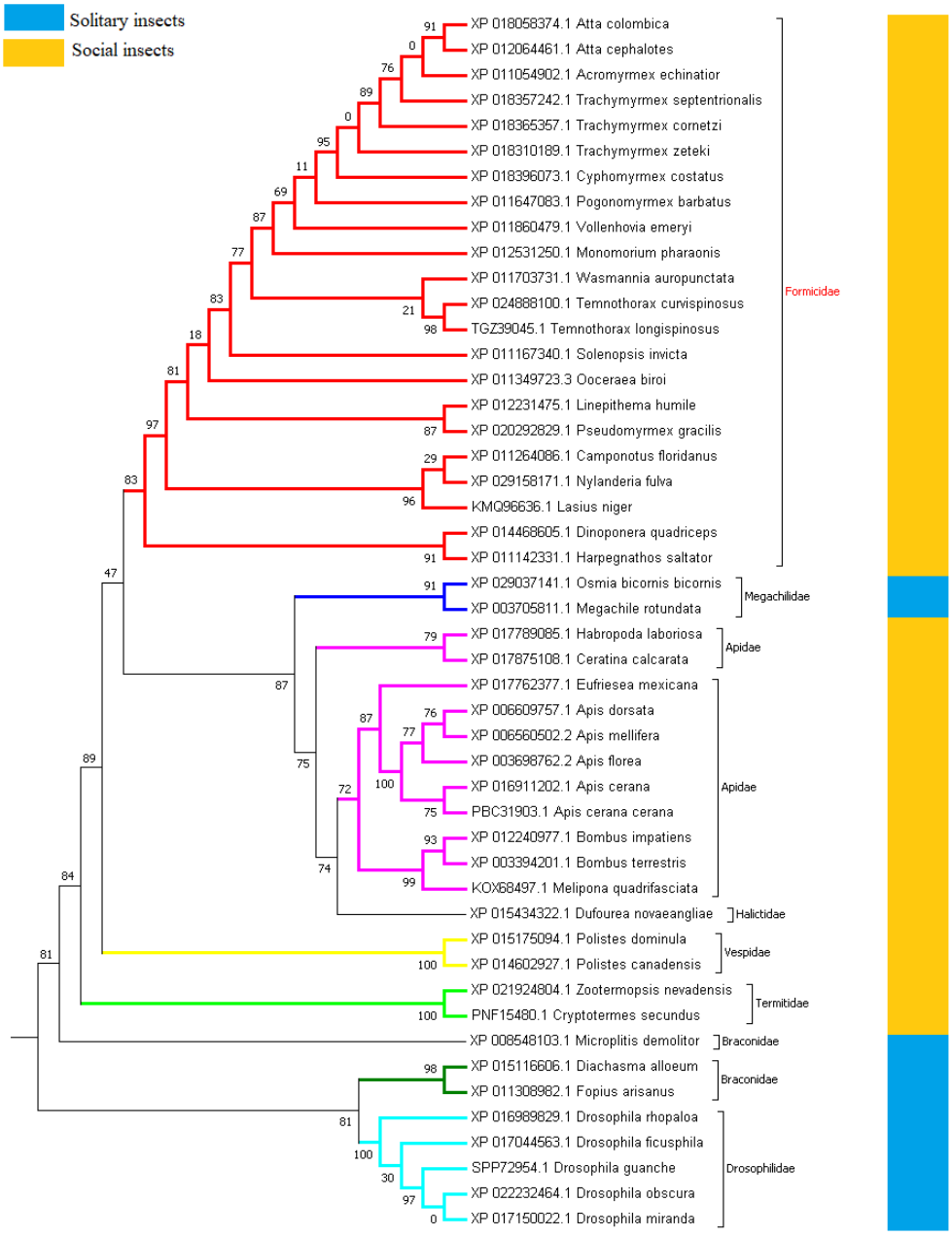

Figure S6. Phylogenetic maximum-likelihood PhyML tree of the Myeloid differentiation primary response 88 protein (MyD88) based on a LG model. The tree was calculated with 48 sequences and 129 aa positions.

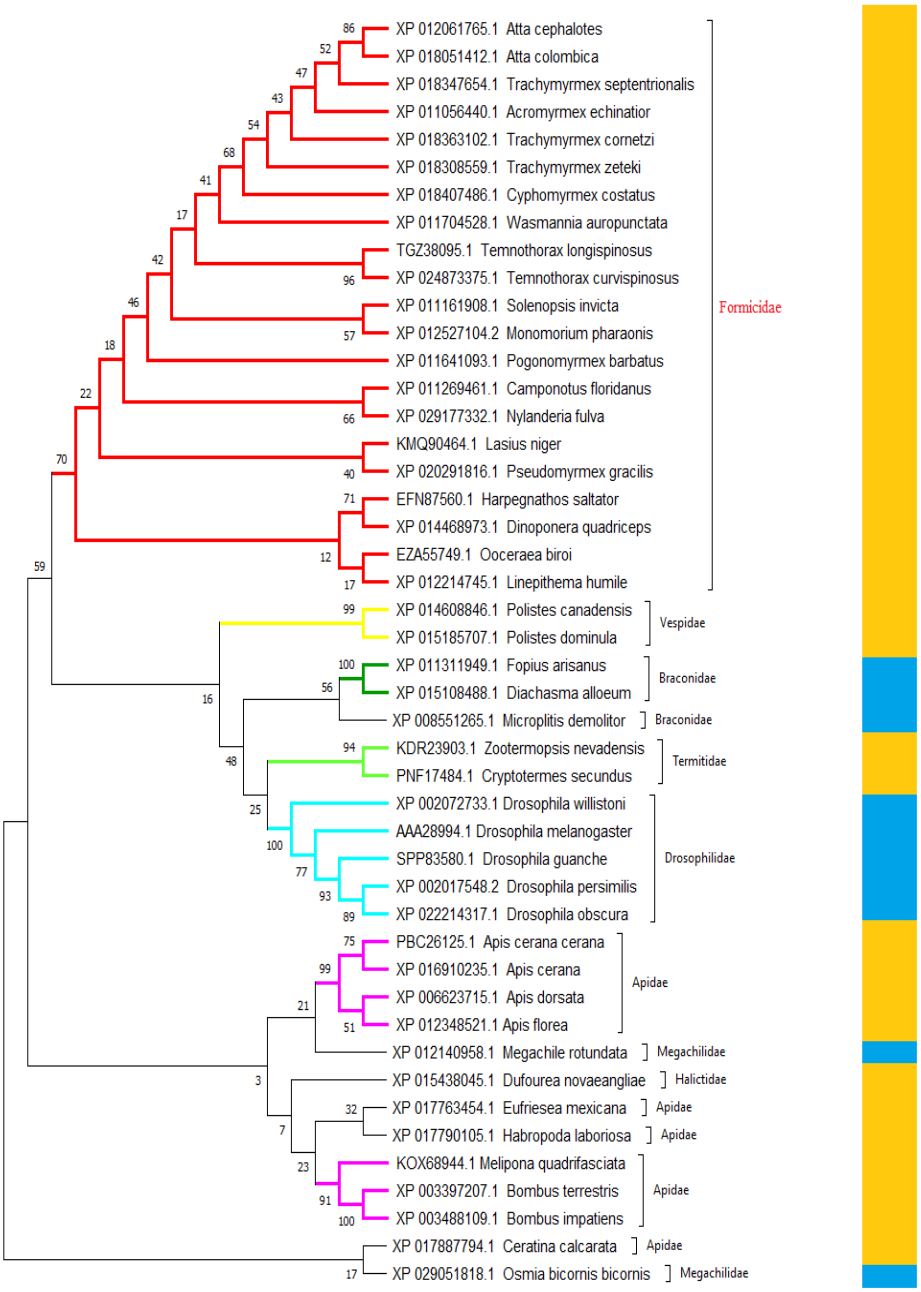

Figure S7. Phylogenetic maximum-likelihood PhyML tree of the Tube protein based on a LG model. The Tube was calculated with 46 sequences and 96 aa positions.

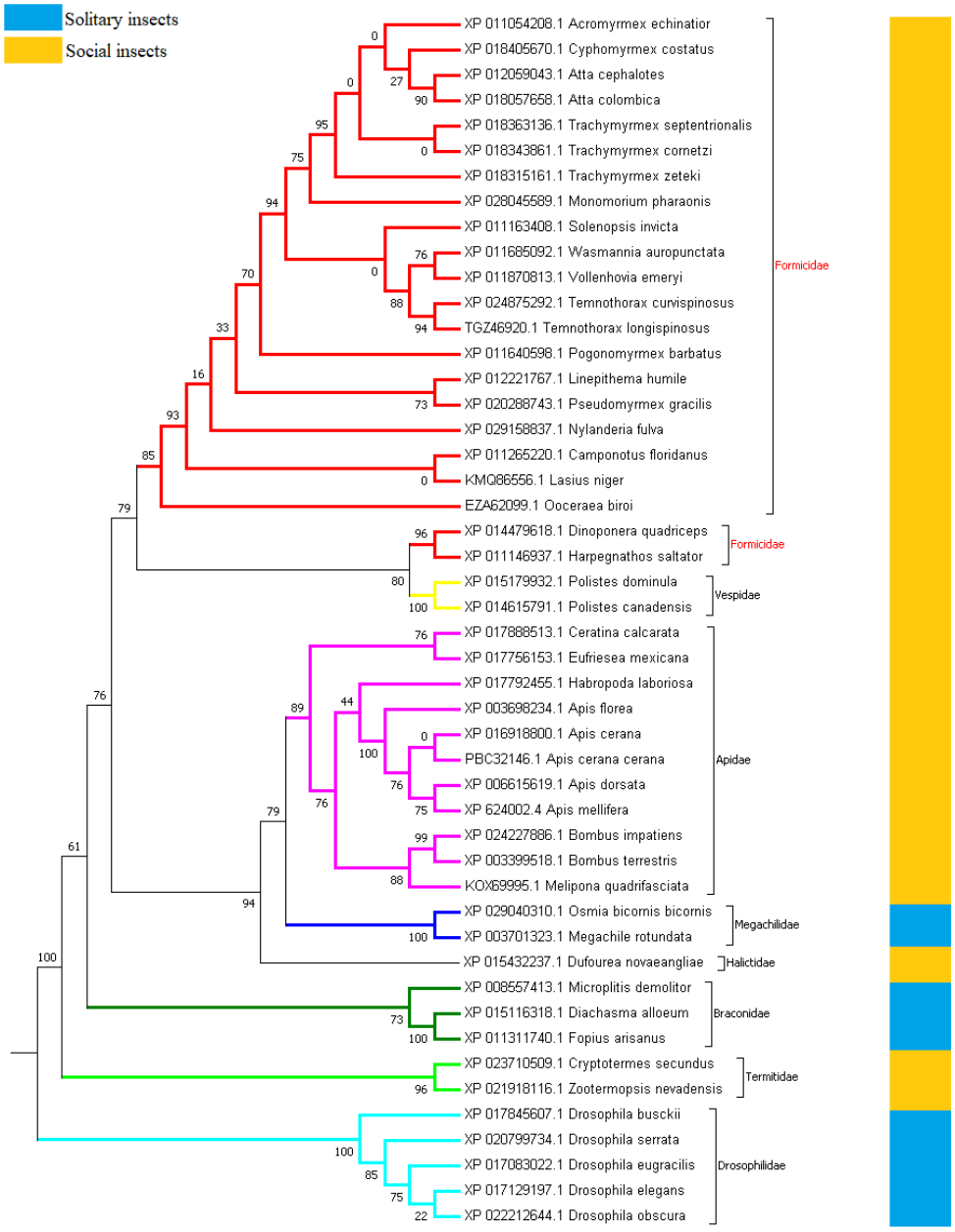

Figure S8. Phylogenetic maximum-likelihood PhyML tree of the serine/threonine-protein kinase Pelle based on a LG model. The Pelle tree was calculated with 48 sequences and 210 aa positions.

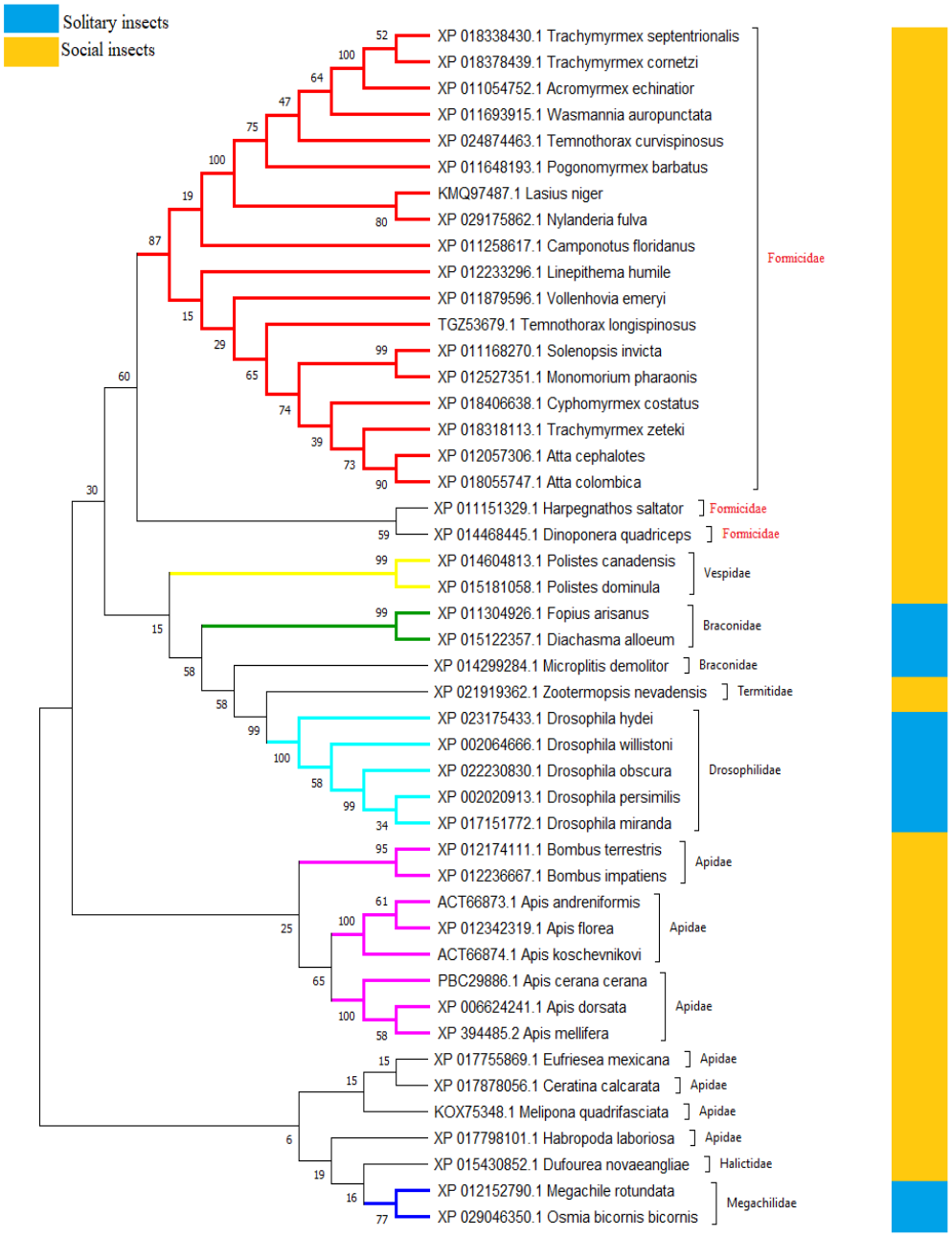

Figure S9. Phylogenetic maximum-likelihood PhyML tree of the Cactus protein based on a LG model. The Pelle tree was calculated with 48 sequences and 172 aa positions.

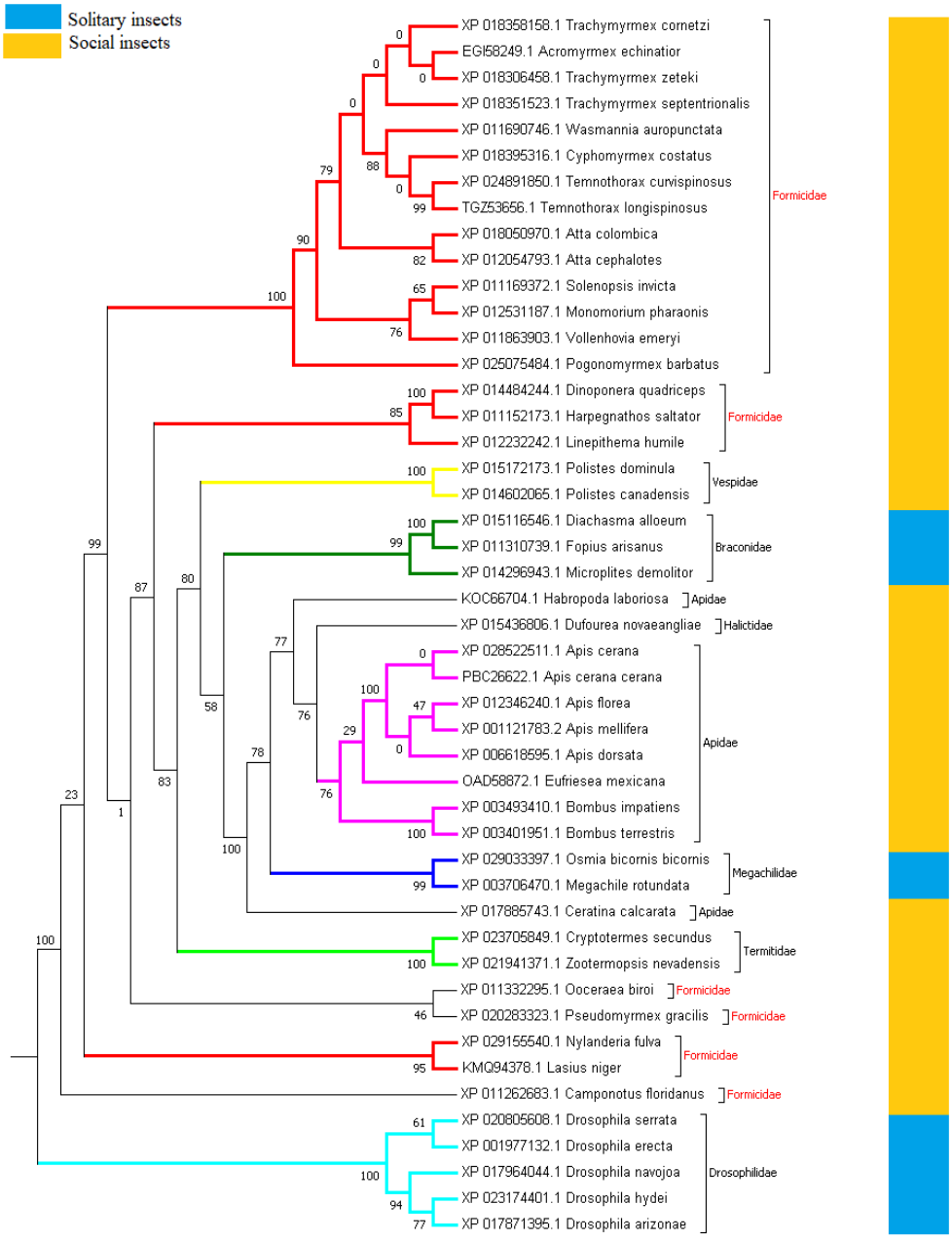

Figure S10. Phylogenetic maximum-likelihood PhyML tree of the Janus kinase (Jak) based on a LG model. The Jak tree was calculated with 47 sequences and 386 aa positions

Table S1. Insect species used in the study and the identified proteins.

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Formicidae															
Acromyrmex echinatior (Forel, 1899)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Atta cephalotes (Linnaeus, 1758)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Atta colombica Guérin-Méneville, 1844	+	+	+		+	+	+	+	+	+	+	+	+	+	
Camponotus floridanus (Buckley, 1866)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cataglyphis velox Santschi, 1929												+			
Cyphomyrmex costatus Mann, 1922	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Dinoponera quadriceps Kempf, 1971	+		+		+	+	+	+	+	+	+	+	+	+	+
Harpegnathos saltator (Jerdon, 1851)	+		+	+	+	+	+	+	+	+	+	+	+	+	+
Formica polyctena Foerster, 1850												+			
Formica rufibarbis Fabricius, 1793												+			
Formica sanguinea Latreille, 1798												+			
Formica uralensis Ruzsky, 1895												+			
Lasius austriacus Schödl & Seifert, 2003												+			
Lasius emarginatus (Olivier, 1792)												+			
Lasius flavus (Fabricius, 1782)												+			
Lasius japonicus Santschi, 1941												+			
Lasius lasioides (Emery, 1869)												+			
Lasius niger (Linnaeus, 1758)	+	+			+	+	+	+	+	+	+	+	+	+	+
Lasius sakagamii Yamauchi & Hayashida, 1970												+			
Linepithema humile (Mayr, 1868)	+		+	+	+	+	+	+	+	+	+	+	+	+	+

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Manica rubida (Latreille, 1802)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Monomorium pharaonis (Linnaeus, 1758)	+														
Myrmica alaskensis Wheeler, 1917	+														
Myrmica brevispinosa Wheeler, 1917	+														
Myrmica fracticornis Forel, 1901	+														
Myrmica lobicornis Nylander, 1846	+														
Myrmica rubra (Linnaeus, 1758)	+											+			
Myrmica ruginodis Nylander, 1846	+														
Myrmica rugulosa Nylander, 1849	+											+			
Myrmica sabuleti Meinert, 1860	+														
Myrmica scabrinodis Nylander, 1846	+											+			
Myrmica sulcinodis Nylander, 1846	+														
Notostigma carazzii (Emery, 1895)	+											+			
Nylanderia fulva (Mayr, 1862)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Ooceraea biroi (Forel, 1907)	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Pogonomyrmex barbatus (Smith, 1858)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Polyergus rufescens (Latreille, 1798)	+											+			
Pseudomyrmex gracilis (Fabricius, 1804)	+	+	+		+	+	+	+	+		+	+	+	+	+
Rossomyrmex minuchae Tinaut, 1981	+											+			
Solenopsis invicta Buren, 1972	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Temnothorax curvispinosus (Mayr, 1866)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Temnothorax longispinosus (Roger, 1863)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Trachymyrmex cornetzi (Forel, 1912)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Trachymyrmex septentrionalis (McCook, 1881)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Trachymyrmex zeteki Weber, 1940	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Vollenhovia emeryi Wheeler, 1906	+	+	+	+	+	+	+		+	+	+	+	+	+	+
Wasmannia auropunctata (Roger, 1863)	+	+	+	+	+	+	+	+	+	+	+		+	+	+
Apidae															
Apis andreniformis Smith, 1858	+	+	+							+		+			
Apis cerana Fabricius, 1793	+	+	+	+	+	+	+	+	+		+	+	+	+	+
Apis cerana cerana Fabricius, 1793	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Apis cerana japonica (Radoszkowski, 1877)												+			
Apis dorsata Fabricius, 1793	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Apis florea Fabricius, 1787	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Apis koschevnikovi Enderlein, 1906	+	+	+							+					
Apis mellifera Linnaeus, 1758	+	+	+	+	+	+	+		+	+	+	+	+	+	+
Bombus ardens ardens Smith, 1879												+			
Bombus bohemicus Seidl, 1838												+			
Bombus friseanus Skorikov, 1933	+														
Bombus hortorum (Linnaeus, 1761)	+														
Bombus hypocrita sapporensis Cockerell, 1911												+			
Bombus ignitus Smith, 1869	+											+			
Bombus impatiens Cresson, 1863	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Bombus impetuosus Smith, 1871	+														
Bombus keriensis	+														

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Morawitz, 1887															
Bombus koreanus (Skorikov, 1933)	+														
Bombus ladakhensis Richards, 1928	+														
Bombus lapidarius (Linnaeus, 1758)	+											+			
Bombus longipennis Friese, 1918	+														
Bombus lucorum (Linnaeus, 1761)												+			
Bombus melanurus Lepeletier, 1835	+														
Bombus monticola Smith, 1849	+														
Bombus patagiatus Nylander, 1848	+														
Bombus pascuorum (Scopoli, 1763)	+											+			
Bombus picipes Richards, 1934	+														
Bombus pratorum (Linnaeus, 1761)	+														
Bombus ruderatus corsicola Strand, 1917												+			
Bombus rupestris (Fabricius, 1793)												+			
Bombus terrestris (Linnaeus, 1758)	+	+	+	+	+	+	+	+	+	+		+	+	+	+
Ceratina calcarata Robertson, 1900	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Eufriesea mexicana (Mocsáry, 1897)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Habropoda laboriosa (Fabricius, 1804)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Melipona quadrifasciata Lepeletier, 1836	+	+	+		+	+	+	+	+	+	+	+	+		+
Halictidae															
Dufourea novaeangliae (Robertson, 1897)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Vespidae															
Polistes canadensis (Linnaeus, 1758)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Polistes dominula (Christ, 1791)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Termitidae															
Cryptotermes secundus (Hill, 1925)	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Drepanotermes rubriceps (Froggatt, 1898)		+	+												
Nasutitermes comatus (Hill, 1942)		+	+												
Nasutitermes corniger (Motschulsky, 1855)		+	+												
Nasutitermes dixoni (Hill, 1932)		+	+												
Nasutitermes exitiosus (Hill, 1925)		+	+												
Nasutitermes fumigatus (Brauer, 1865)		+	+												
Nasutitermes graveolus (Hill, 1925)		+	+												
Nasutitermes longipennis (Hill, 1915)		+	+												
Nasutitermes magnus (Froggatt, 1898)		+	+												
Nasutitermes pluvialis (Mjöberg, 1920)		+	+												
Nasutitermes triodiae (Froggatt, 1898)		+	+												
Nasutitermes walkeri (Hill, 1942)		+	+												
Tumulitermes pastinator (Hill, 1915)		+	+												
Zootermopsis nevadensis (Hagen, 1874)	+	+	+	+	+		+	+	+	+	+		+	+	+
Braconidae															
Cotesia vestalis (Haliday, 1834)												+			

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Diachasma alloeum (Muesebeck, 1956)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fopius arisanus (Sonan, 1932)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Microplitis demolitor Wilkinson, 1934	+	+	+		+	+	+	+	+	+	+	+	+	+	+
Drosophilidae															
Drosophila albomicans (Duda, 1923)			+												
Drosophila arizonae Ruiz, Heed and Wasserman, 1990						+					+		+	+	
Drosophila biarmipes Malloch, 1924	+				+						+				
Drosophila bipectinata Duda, 1923															+
Drosophila busckii Coquillett, 1901	+		+		+				+						
Drosophila elegans Bock and Wheeler, 1972									+						
Drosophila erecta Tsacas and Lachaise, 1974	+	+		+	+									+	
Drosophila eugracilis Bock and Wheeler, 1972	+								+			+			
Drosophila ficusphila Kikkawa and Peng 1938							+				+	+			
Drosophila guanche Monclús, 1977	+		+			+	+	+					+		
Drosophila hydei Sturtevant, 1921				+						+	+		+	+	
Drosophila melanogaster Meigen, 1830		+		+				+				+			
Drosophila miranda Dobzhansky 1935							+			+					
Drosophila navojoa Ruiz, Heed and Wasserman 1990				+										+	
Drosophila novamexicana Patterson, 1941			+												
Drosophila obscura Fallén, 1823						+	+	+	+	+					
Drosophila persimilis						+		+		+			+		+

Families and species	**Proteins**														
	PGRP	GNBP1	GNBP3	ModSP	Spz	Toll	Myd88	Tube	Pelle	Cactus	Dorsal	Def	Cyt	Jak	Stat
Dobzhansky and Epling, 1944 *Drosophila rhopaloa* Bock and Wheeler, 1972					+		+								+
Drosophila serrata Malloch, 1927				+					+					+	
Drosophila subovscura Collin, 1936			+												
Drosophila suzukii (Matsumura, 1931)		+			+							+			+
Drosophila takahashii Hsu, 1949		+										+			+
Drosophila willistoni Sturtevant, 1916						+		+		+	+		+		
Drosophila yakuba Burla, 1954		+													
Megachilidae															
Megachile rotundata (Fabricius 1787)	+	+		+	+	+	+	+	+	+	+		+	+	+
Osmia bicornis bicornis (Linnaeus, 1758)	+	+		+	+	+	+	+	+	+	+		+	+	+

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Exploring protein - protein interaction in cell physiology by reviewing the role of dynein-dynactin interaction as a representative example

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Abstract. Protein-protein interactions are essential for the normal function of cells and are involved in various cellular processes. These interactions can occur through a variety of mechanisms, including hydrogen bonding, ionic interactions, and hydrophobic interactions. Changes in protein-protein interactions can alter the normal function of the cell and lead to various diseases. Understanding protein-protein interactions is important for the development of therapeutic approaches targeting these interactions for the treatment of diseases. In this article, I will discuss the role of protein-protein interactions in normal cellular function, the consequences of changes in these interactions, and the importance and significance of understanding these interactions by using the example of dynein-dynactin.

Keywords: protein-protein interactions, dynein, dynactin, dysregulation, cargo transport

Introduction

Protein-protein interactions refer to the binding of one protein to another, which can occur through a variety of mechanisms such as hydrogen bonding, ionic interactions, and hydrophobic interactions (Alberts, 2014, Athanasios *et al*., 2017). These interactions are essential for the normal function of cells and are involved in various cellular processes, including signal transduction,

gene regulation, and protein synthesis. Protein-protein interactions are crucial for the proper function of cells and are involved in a wide range of biological processes. For example, enzymes require specific substrate proteins to function properly and perform their catalytic function. Protein-protein interactions also play a role in the regulation of gene expression, as transcription factors bind to specific DNA sequences to regulate the expression of genes (Alberts, 2014). Changes in protein-protein interactions can alter the normal function of the cell and lead to various diseases. For example, mutations in proteins that disrupt their ability to interact with other proteins can lead to abnormal cellular function and contribute to the development of diseases such as cancer and neurodegenerative disorders. The importance and significance of understanding protein-protein interactions lie in the potential for therapeutic approaches targeting these interactions (Rao *et al*., 2014). By targeting specific protein-protein interactions, it may be possible to modulate their function and correct abnormal cellular function in diseases.

Protein-protein interactions

Cell-to-cell contacts, metabolic regulation, and control of developmental processes are only a few of the biological activities that are handled by protein-protein interactions (Rao *et al*., 2014). Protein-protein interaction is increasingly one of system biology's key goals. Protein folding, protein assembly, and protein-protein interactions are all based on noncovalent interactions between the side chains of the residues (Ofran and Rost, 2003). Many interactions and connections between the proteins are brought about by these contacts. Protein-protein interactions may be categorised in a number of ways based on their differing structural and functional traits (Nooren and Thornton, 2003). They can be homo- or heterooligomeric depending on their interaction surface, obligatory or nonobligate depending on their stability, and transitory or permanent depending on their persistence (Zhang, 2009). Any combination of these three distinct pairs may make up a given protein-protein interactions (Rao *et al.,* 2014).

Protein-protein interactions can be classified into several types based on the nature of the interaction (Alberts, 2014). Non-covalent interactions, such as hydrogen bonding and ionic interactions are reversible and can be disrupted by changes in the environment (Alberts, 2014). Covalent interactions, such as disulfide bonds and isopeptide bonds, are more stable and are less likely to be disrupted by environmental changes (Alberts, 2014). It does not exist in the References list). Protein-protein interactions are crucial for the proper function of cells and are involved in a wide range of biological processes (Alberts, 2014).

For example, enzymes require specific substrate proteins to function properly and perform their catalytic function (Alberts, 2014). Protein-protein interactions also play a role in the regulation of gene expression, as transcription factors bind to specific DNA sequences to regulate the expression of genes (Alberts, 2014). Changes in protein-protein interactions can alter the normal function of the cell and lead to various diseases (Alberts, 2014).

While temporary contacts would create signalling pathways, long-term interactions would result in a protein complex that is stable. While executing their tasks *in vivo*, proteins often hardly ever behave as separate species (Yanagida, 2002). Around 80% of proteins have been found to function in complexes rather than alone (Berggård *et al*., 2007). Proteins participating in the same biological processes are consistently discovered to interact with one another, according to a thorough review of verified proteins (von Mering et al., 2002). Protein-protein interactions research is crucial for determining how proteins behave inside of cells. On the basis of the evidence of their interaction with a protein whose function has previously been established, the functioning of unidentified proteins can be anticipated. For example, mutations in proteins that disrupt their ability to interact with other proteins can lead to abnormal cellular function and contribute to the development of diseases such as cancer and neurodegenerative disorders (Alberts, 2014). The importance and significance of understanding protein-protein interactions lie in the potential for therapeutic approaches targeting these interactions (Alberts, 2014). By targeting specific protein-protein interactions, it may be possible to modulate their function and correct abnormal cellular function in diseases (Alberts, 2014). Changes in these interactions can alter the normal function of the cell and lead to various diseases (Alberts, 2014). The importance and significance of understanding protein-protein interactions lie in the potential for therapeutic approaches targeting these interactions for the treatment of diseases (Alberts, 2014). Finding knowledge about protein-protein interactions aids in the selection of therapeutic targets (Dunker et al., 2005). Families of enzymes, transcription factors, and intrinsically disordered proteins, among others, have been demonstrated in studies to be proteins with more connections (Sarmady *et al.,* 2011). Protein-protein interactions, however, have a wider regulatory reach and more complex procedures involved (Rao *et al.,* 2014). One must recognise different interactions and ascertain the effects of the interactions in order to more accurately comprehend their significance in the cell (Zhang, 2009). Protein-protein interactions data have recently been improved by high-throughput experimental techniques that are assured, including two-hybrid systems, mass spectrometry, phage display, and protein chip technology (Zhang, 2009). These experimental resources have been used to construct extensive protein-

protein interactions networks (Rao *et al*., 2014). The quantity of protein-protein interactions data is creating a difficulty for laboratory validation, though. Understanding the roles of undiscovered proteins is becoming more and more dependent on computational study of protein-protein interactions networks. Protein-protein interaction is currently one of the important areas of study for the advancement of contemporary biological systems.

Dynein-dynactin interaction

One specific example of a protein-protein interaction that is necessary for the normal function of a particular cellular process is the interaction between the proteins dynein and dynactin. The dynein-dynactin interaction plays a crucial role in various cellular processes (Devine *et al*., 2016). Dynein is a motor protein complex that moves along microtubules (MTs) in cells and plays a crucial role in the transport of various cellular cargos, including organelles and vesicles (Hirokawa *et al*., 1998). Dynein is composed of several subunits and has two main types: cytoplasmic dynein, which transports cargos towards the cell centre, and axonal dynein, which transports cargos towards the cell periphery. Dynein is involved in a wide range of cellular processes, including organelle transport, chromosome segregation during cell division, and cargo transport to the synapse in neurons (Hirokawa *et al*., 1998). The principal cargo transporter for the minus ends of MTs, cytoplasmic dynein-1 is involved in a wide range of cellular functions (Reck-Peterson *et al.,* 2018). A complex autoinhibition mechanism is present in the multi-subunit dimer dynein, which has a mass of 1.4 MDa (Zhang *et al.,* 2017). It requires the cooperation of a 1.1 MDa complex, dynactin, and a coiled-coil cargo adapter in order to function completely (McKenney *et al.,* 2014). Activating adaptors are cargo adaptors that can both bind cargo and stimulate dynein-dynactin motility (Reck-Peterson *et al.,* 2018). The coiled coils of these adaptors are positioned along the filament of dynactin, connecting and placing dynein in a conformation that frees it from autoinhibition, as demonstrated by cryo-electron imaging of the active complex (Urnavicius *et al.,* 2015).

According to earlier researches (Urnavicius *et al.,* 2018, Elshenawy *et al.,* 2019), certain adaptors are able to recruit two dyneins per dynactin, boosting the force and speed of the complex and enabling it to defeat kinesin in a tug-of-war. Adaptors engage with dynein and dynactin numerous times in order for the complex to form (Urnavicius *et al.,* 2015, Gama *et al.,* 2017, Urnavicius *et al.,* 2018). These include interactions between the adaptor coiled-coil and the pointed end complex of dynactin (Urnavicius *et al.,* 2015, Gama *et al*., 2017),

sites at the adaptor N-termini that bind to the flexible end of the dynein light intermediate chain (LIC), and multiple interactions with the dynein heavy chains (Urnavicius *et al.,* 2015). The motor domains of dynein, which attach the dynein-dynactin-adaptor complex to MTs, are responsible for enzymatic cycle-driven dynein motility. Six AAA+ domains form a ring around each motor, and unlike other AAA+ family proteins, they are not interchangeable (Roberts *et al.,* 2009). A coiled-coil stalk transmits the nucleotide status of the first AAA+ domain (AAA1) across a considerable distance to theMT binding domain (Chaaban and Carter, 2022, Gibbons *et al.,* 2005). The powerstroke of dynein is defined by changes in the linker domain's bent and straight conformations, which are regulated by the ATPase cycle (Schmidt and Carter, 2016). The nucleotide states of AAA3 and AAA4 operate as a gate for this communication, which may be influenced by interactions between nearby dyneins in the assembled complex (Bhabha *et al.,* 2014, DeWitt *et al.,* 2015). Dynactin is a protein complex that assists dynein in its cargo transport function (Devine *et al*. 2016). The dynein-dynactin complex is a large molecular machine composed of multiple subunits, with a total molecular weight of over 2 million Da. The complex is composed of two main subunits: the MT-binding dynein motor domain and the dynactin subunit. The dynein motor domain contains the ATPase and MT-binding domains, while the dynactin subunit contains the actin-binding domains. Additionally, the complex also contains several other subunits that are involved in regulation, localization, and stabilization of the complex.

The dynein-dynactin complex plays a crucial role in the movement of cilia and flagella. Cilia and flagella are microtubule-based structures that are involved in various physiological processes, such as cell motility, fluid flow, and sensory signaling (Wheway *et al*., 2014). The dynein-dynactin complex is responsible for the movement of these structures by providing the necessary force for the sliding of microtubules. Recent studies have also revealed that the dynein-dynactin complex is involved in the regulation of cilia and flagella function. For example, it has been shown that the complex is involved in the regulation of the number and length of cilia and flagella, as well as the regulation of their beating patterns (Wheway *et al*., 2014). Recent progress in the field has provided insights into the molecular mechanism of the dynein-dynactin interaction. It has been shown that the dynein motor domain binds to the dynactin subunit through a specific interaction between the dynein intermediate chain and the p150Glued subunit of dynactin (Ross *et al*., 2006). The intermediate chain is a component of the dynein motor domain that binds to theMT and regulates the activity of the ATPase domain. The p150Glued subunit is a component of the dynactin subunit that binds to actin filaments. Additionally, it has been shown that the dynein-dynactin interaction is regulated by several

other proteins, such as the dynactin-associated proteins dynactin-binding protein 1 (Dab1) and dynactin-binding protein 2 (Dab2) (Ross *et al*., 2006). These proteins are thought to play a role in the localization and stabilization of the dynein-dynactin complex.

Dynein and dynactin are also involved in the transport of various cargos in cells, including organelles, vesicles, and proteins (Hirokawa *et al*., 1998). In neurons, dynein and dynactin are involved in the transport of cargos towards the cell periphery, which is essential for the function of synapses (Devine *et al*., 2016). In order to start cargo transport, dynein often has to be recruited to MT plus ends because it is the main minus end-directed motor (Liu, 2017). Single dynein molecules in the cytoplasm are shown to bind to and diffuse along MTs by fluorescently tagged dynein in fission yeast (Ananthanarayanan *et al*., 2013). In fly and human cells, contact with p150 and the +TIPs (MT plus-end-interacting/tracking proteins) EB1 and CLIP-170 (Dixit et al., 2009). is necessary for direct recruitment of dynein from the cytoplasm to the plus end (Liu, 2017). Recent in vitro reconstitution studies using pure recombinant proteins have shown how EB1 and CLIP-170 sequentially recruit dynactin and dynein to MT plus ends (Duellberg *et al.,* 2014). Transport via kinesin is a second method of dynein localisation to MT plus ends. Kinesin is necessary for the targeting of dynein to MT plus ends, according to research on mouse and human dorsal root ganglia (DRG) neurons (Carvalho et al., 2004). In vitro reconstitution studies using proteins isolated from yeast have shown that EB1 and CLIP-170, which act as processivity factors for kinesin to overcome the intrinsic minus-end-directed motility of dynein, couple dynein to kinesin for its transport towards the plus end. Lis1 (also known as NudF, a member of the nuclear distribution (Nud) family), a regulator of dynein motility, couples (Liu, 2017). The majority of current research also shows that kinesin-1 directs dynein's sluggish axonal transport to MT plus ends in mammalian neurons (Twelvetrees et al., 2016). Before detaching, dynein must travel down the MT track several times in order to carry payloads across significant intracellular distances. MTs are moved processively by dynein with the assistance of dynactin and other regulatory elements. According to in vitro motility experiments, dynactin lengthens the average run length of dynein but not its velocity (King and Schroer, 2000). The basic MT-binding domain at the N-terminus of p150, not the CAP-Gly domain, has been shown in studies with recombinant segments of dynactin p150 and chick brain-purified dynein-dynactin to increase dynein processivity by anchoring the motor to MTs. Nevertheless, dynein-driven transport of membranous payloads in Drosophila S2 cells is unaffected by the deletion of the p150 MT-binding domains, suggesting that control of dynein processivity in vivo is more complex than that in vitro. Subsequent investigations

utilising recombinant dynein and dynactin isolated from yeast show that the MT-binding domains are not necessary, but the coiled-coil -helical dynein-binding domain is.

The inherent characteristics of vesicular payloads control dynein activity. According to the cargo load, the MT track's dynein's step size and duration of force production change (Mallik *et al.,* 2004). Dynein creates a stronger force and a smaller step size while under stress, according to *in vitro* optical intra protein-protein interaction of dynein-coated beads (Mallik *et al*., 2004). Multiple motors generate large collective forces in response to higher load, not only by adjusting step size to bunch together and share the load better but also by reducing the detachment rate from MTs through a "catch-bond" conformational state, as shown by optical intra protein-protein interaction experiments on phagosomes in live macrophage cells and on dynein-absorbed beads *in vitro* (Rai *et al.,* 2013). The association of particular cargo molecules with particular dynein subunits controls dynein activity as well. Two ICs and two LICs separately bind to DHC in vertebrates. Separate locations are used by the 3 distinct LCs to bind to the IC. 7 The IC, LIC, and LC subunits are each encoded by at least two genes. Additionally, alternative splicing produces a variety of IC and LC isoforms. It is thought that the assembly of various isoforms of the subunits with the dynein HC results in unique dynein complexes, which serve as a mechanism for cargo selectivity and the control of motor activity. It is true that dynein drives the movement of endosomes that communicate neurotrophins. The Trk (Tropomyosin Receptor Kinase) family of receptor tyrosine kinases is the target of neurotrophins, which bind and activate their cognate transmembrane receptors (e.g., NGF (Nerve Growth Factor) binds to TrkA and BDNF (Brain-Derived Neurotrophic Factor) binds to TrkB) to activate downstream signalling cascades and When neurotrophins bind to receptors, the ligand-receptor complex is endocytosed and transported retrogradely from the nerve terminal to the cell body in the form of signalling endosomes to mediate further signalling processes. In vitro direct binding experiments demonstrate that TrkA directly binds to Tctex-1, and all three Trk receptors are associated with the Tctex-1 subunit of dynein LC in the brain (Yano *et al.,* 2001). Moreover, research shows that whereas TrkA signalling endosomes connect with dynein complexes carrying the widely expressed IC-2C, TrkB signalling endosomes are carried by dynein complexes having the neuron-specific IC-1B isoform. 94 It's interesting to note that NGF stimulation of cells activates TrkA, its cognate receptor, as well as increasing the connection of activated TrkA with dynein and increasing the frequency and speed of retrograde motion of vesicles associated with dynein (Ha et al., 2008). Alternative splicing of ICs is thought to produce distinctive phosphorylation sites that might help

control dynein activity. So, another layer of control for the motor activity by a particular cargo may be provided by the phosphorylation of IC and LIC isoforms by some downstream effector(s) of active Trk (Ha *et al.,* 2008).

The dynein motor domain binds to microtubules, while the dynactin subunit binds to actin filaments. This interaction allows the complex to move along microtubules and transport organelles, such as vesicles, endosomes, and lysosomes, towards the minus end of the microtubules. The dynein-dynactin complex also plays a crucial role in the transport of mRNA and proteins during the cell cycle. It has been shown that the complex is involved in the transport of mRNA and proteins from the nucleus to the cytoplasm during interphase and the transport of chromosomes during mitosis (Reck-Peterson *et al.,* 2018) Dysregulation of the dynein-dynactin interaction can lead to defects in the transport of cargos to synapses, which can affect neurotransmitter release and disrupt normal brain function (Devine *et al*., 2016). The interaction between dynein and dynactin is necessary for the normal function of dynein and is involved in the transport of cargos towards the cell periphery in neurons. The interaction also helps to anchor dynein to cargos and stabilize the interaction between dynein and microtubules, allowing for efficient cargo transport (Devine *et al*., 2016).

Effect of dynein-dynactin interaction in mitochondrial dynamics

Mitochondria are essential for energy production in cells and are transported along microtubules by dynein (Hirokawa *et al*., 1998). Fission-fusion dynamics in mitochondria help to maintain the quantity and quality of mitochondrial DNA as well as the replenishment of proteins in this organelle (Scheibye-Knudsen et al., 2015). Mitophagy, mitochondrial DNA loss, and loss of oxidative potential have all been associated with disrupted mitochondrial fission or fusion. Although mitochondrial fusion and fission both involve the active separation of dividing organelles, axonal transport of mitochondria is closely related to mitochondrial dynamics (Drerup *et al*., 2017). At the molecular level, the connection between mitochondrial dynamics and transport is clearly clear: Mitofusin, a protein required for mitochondrial fusion, takes part in the anterograde transport of this organelle (Misko *et al*., 2010). Moreover, altering the dynamin-like protein Drp1, which is required for mitochondrial fission, affects where mitochondria are located (Smirnova *et al*., 2001). The proteins Milton TRAK1/2 (Stowers *et al.,* 2002, Drerup *et al*., 2017) and Miro RhoT1/2 (Guo *et al.,* 2005) enable the principal anterograde mitochondrial motor, Kinesin-1, to bind to mitochondria. When exposed to large concentrations of

this ion, the calcium-sensitive EF hands in Miro shift their orientation, which causes the Kinesin-1 motor to decouple from the microtubules (Saotome *et al.,* 2008). This process permits mitochondria to congregate at regions of strong synaptic activity in conjunction with substances that anchor mitochondria (Kang *et al*., 2008). Yet, the absence of either protein affects both anterograde and retrograde mitochondrial mobility, indicating that the Miro/Milton transport mechanism is not specialised for anterograde transport (Guo *et al.,* 2005; Saotome *et al.,* 2008). The cytoplasmic dynein complex is recognised to be essential for retrograde mitochondrial transport (Pilling *et al.,* 2006). During retrograde axonal transport, the core dynein motor (Holzbaur and Vallee, 1994) is frequently paired with dynactin, a multiprotein complex (Drerup *et al.,* 2017). It has been demonstrated that dynactin, especially its p150 subunit, promotes dynein processivity and acts as an anchor for dynein at microtubule plus ends, promoting cargo loading (Lloyd *et al.,* 2012). P150 interacts with the tails of the dynein intermediate chains to connect the dynactin accessory complex to dynein (Vaughan and Vallee, 1995). Arp1 and Actr10, two actin-related proteins, are found in dynactin in addition to p150 (Eckley *et al.,* 1999). Actr10 is a component of the dynactin pointed end complex, which is projected to be in an optimum position for cargo binding together with p25, p62, and p27 (Yeh *et al.,* 2012). Dysregulation of the dynein-dynactin interaction can lead to defects in the transport of mitochondria, which can affect energy production in cells and lead to various diseases such as neurodegenerative disorders and cancer (Vona *et al*., 2021).

Impact of dysregulation of dynein-dynactin interaction

Dysregulation of the dynein-dynactin interaction has also been linked to various neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and Huntington's disease (Gunawardena and Goldstein, 2004, Lipka *et al*., 2013). In neurons, dynein and dynactin are involved in the transport of cargos towards the cell periphery, which is essential for the function of synapses. Dysfunction of dynein and dynactin can lead to defects in the transport of cargos to synapses, which can affect neurotransmitter release and disrupt normal brain function (Devine *et al*., 2016). In cancer, dysregulation of the dynein-dynactin interaction can lead to defects in the transport of tumor suppressor proteins, which can contribute to the development and progression of cancer (Harris and Levine, 2005, Vona *et al*., 2021). Defects in the dynein-dynactin interaction have been linked to several human diseases, such as primary ciliary dyskinesia (PCD) and spinal muscular atrophy (SMA) (Ross *et al*., 2006). PCD

is a genetic disorder characterized by defects in cilia and flagella function, leading to respiratory and reproductive problems. SMA is a neurodegenerative disorder characterized by the loss of motor neurons. Recent studies have shown that mutations in genes encoding the dynein-dynactin complex subunits are associated with PCD (Ross *et al*., 2006). Additionally, defects in the dynein-dynactin complex have also been linked to SMA (Ross *et al*., 2006). These findings suggest that the dynein-dynactin complex plays a crucial role in the development and progression of these diseases. The dynein-dynactin complex plays a crucial role in various cellular processes, including intracellular transport, cell division, and cilia and flagella function. Therefore, a better understanding of the dynein-dynactin complex and its interactions could provide insights into the development of new therapies for these diseases.

One example of this is the development of small molecules that modulate the activity of the dynein-dynactin complex. A study by Stamenovic *et al*. (2002) found that small molecules can bind to specific sites on the dynein-dynactin complex and modulate its activity. This suggests that small molecules could be developed as therapeutics to specifically target the dynein-dynactin complex in diseases such as PCD and SMA. Another approach to developing new therapies for diseases associated with defects in the dynein-dynactin interaction is gene therapy. For example, studies have shown that the delivery of functional dynein-dynactin complex subunits to cells can rescue the defects in intracellular transport and cilia and flagella function in PCD (Stamenovic *et al*., 2002). In addition, recent studies have also focused on developing targeted therapies for PCD by identifying specific subunits or domains of the dynein-dynactin complex as potential therapeutic targets. For example, Fliegauf *et al*. (2007) have identified the p150Glued subunit of the dynactin complex as a potential therapeutic target for PCD. By using a small molecule inhibitor that specifically binds to p150Glued, the authors were able to rescue the defects in cilia and flagella function in PCD patient-derived cells. Moreover, Rompolas *et al.* (2012) have shown that the tail domain of the dynein intermediate chain is a critical site of interaction between dynein and dynactin, and also a potential therapeutic target. They have generated a small molecule that binds specifically to the tail domain of the intermediate chain and disrupts the dynein-dynactin interaction, leading to the rescue of defects in intracellular transport in PCD patient-derived cells. These studies demonstrate the potential of targeting specific subunits or domains of the dynein-dynactin complex as a strategy for developing new therapies for PCD and other diseases associated with defects in the dynein-dynactin interaction.

Conclusion

Like intricate interactions of molecules, protein-protein interactions orchestrate the symphony of cellular life, weaving together the intricate tapestry of essential functions and unlocking the secrets of biological complexity. Changes in these interactions can alter the normal function of the cell and lead to various diseases. The importance and significance of understanding protein-protein interactions lie in the potential for therapeutic approaches targeting these interactions for the treatment of diseases. Further research on protein-protein interactions can provide valuable insights into the role of these interactions in normal cellular function and the consequences of changes in these interactions. This information can be used to develop therapeutic approaches targeting protein-protein interactions for the treatment of diseases. Recent studies have provided insights into the molecular mechanism of the dynein-dynactin interaction, and it's clear that defects in this interaction are linked to several human diseases such as PCD and SMA. In the intricate web of molecular connections, scientific studies have illuminated a path towards hope. By harnessing the power of protein-protein interactions, small molecules have emerged as beacons of promise, rescuing the delicate machinery of cilia and flagella, restoring order within PCD patient-derived cells. This ground-breaking research beckons us to delve deeper into the complex dance of molecules, unveiling potential therapies and paving the way towards a brighter future. As we unravel the intricacies of these interactions, we unravel the mysteries of diseases, unravel the possibilities of healing, and ultimately, unravel the very fabric of life itself.

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