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Standard approach to isolate and purify microsporidian spores from orange belted bumble bee (*Bombus trifasciatus*) from Kashmir (India)

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Abstract

Known for its beauty across the globe, Kashmir is considered as one of the best places for bumble bees' occurrence because of its cold temperature and favourable climatic conditions. Bumble Bees act as significant pollinators in Himalayan region and are playing essential role in maintaining life on earth, however bee populations are declining day-by-day due to several factors like genetic drift, shelter demolishment, lack of flowers etc. One main reason behind this threat is the parasite- Microsporidia which is adversely affecting wild bumble bee physiology, foraging capabilities and frequency resulting in weaker colonies. The present study was aimed to determine occurrence of the parasite by isolating and purifying spores from *Bombus trifasciatus.* Study helped in attaining knowledge related to morphological characters of microsporidian sp. However, species identification is only possible by DNA analysis using PCR.

Keywords: standard approach, purification, nosema spores, bumble bees, homogenisation, percoll, density gradient centrifugation

Introduction

Situated in the northern most region of India, Kashmir is considered to be the best place for bumble bees' occurrence because of its temperature, climate and favourable environmental conditions (Dar, 2019) [5]. Due to their attractive colours, robust shape and necessary abilities to accomplish vital pollination services in several crops, bumble bees have always been a matter of fascination for humanity (Shankar, 2013) [14]. They have been recognized for pollination of numerous crops which are generally incapable of being pollinated by honey bees with additional aids in pollination where they visit more flower per minute as their long tongue favours flower with long corolla tube that gives more nectar to them (Wahengbam, 2019) [21]. The species diversity of bumble bees has been studied in several countries of the world including Japan, France, Germany, Italy, Korea, U.K, Sweden, Canada, New Zealand, U.S.A, Finland and China (Shankar, 2013) [14]. They are typically prevalent on temperate areas, though the population has been deteriorating in current years and some species are in danger to get extinct (Wahengbam, 2019) [21]. One of the limitations in Kashmir is the pervasiveness of parasite and disease infection in bee colonies (Dar, 2019) [5]. Nosemosis is one of the serious bee diseases (Fries, 2010) which is caused by an obligate intracellular microsporidian parasite of genus *Nosema* that produce spores of 4.1 – 6.0 x 2.0 – 4.0 µm size. These are a diverse group of organisms comprising more than 1200 species belonging to 144 genera (Weiss & Keohane 1999) [22] which parasitize a huge number of vertebrates as well as invertebrates' hosts.

Microsporidia have long been known to be a causative agent of disease in insects. Amongst 1200 species more than 200 were assigned to the *Nosema* (Sprague 1982) [15]. The presence of polar filaments in spores, places the parasite under the phylum- Microsporidia (Issi 1986). Earlier microsporidia were placed in the kingdom– Protista but now, after analysing the clear evidence by studying phylogenetic analysis using protein coding genes suggest, that microsporidia share the same origin that of fungi (Hirt *et. al* 1999) [7]. Bumblebees are infected by the microsporidia *Nosema bombi*, which shows major effects on the bumble bees' fitness (Otti and Schmid-Hempel 2007; Schmid-Hempel 2001) [10, 12]. These microsporidian spores can cause emerging infectious disease (EIDS) in the bumble bees (Fatham and Porter 1913). *N. apis* and *N. bombi* are presently considered to be identical, but they diverge not only in their tissue specificities and spore sizes but also in their merogony and in the number of polar filament coils (Kashyap, 2018) [9]. According to Veronique (2021) the spores existing in the lumen of the digestive tract of an insect develop and discharge their polar filaments that instinctively puncture epithelial cells of gut epithelium, through which the sporoplasm finds its way to the host cell and starts to multiply. Declining of bumble bees' population may have led to lower genetic diversity and subsequent higher susceptibility to get infection which enables microsporidia to increase in prevalence (Stentiford *et al*, 2016) [16]. The present study was designed to isolate and purify microsporidian spores from wild bumble bees to study the morphological characters using dark field microscopy however, species identification is not possible without molecular characterization of isolated spores.

Materials and Methodology

To perform an efficient isolation of spores and purification from bumble bee samples, following strategy was planned and executed-

1. **Sample collection:** Total of seven hundred and forty-

four (744) bumble bees were captured from 5 different places of Kashmir (India) *viz*. Sonamarg, Gulmarg, Phalgham, Kokanmarg and Kashmir University (Srinagar) in the month of June- July (late spring and early summer) using insect collecting net, bee covers and hand-picking method (wherever required). For an ample amount of collection few other regions were also explored (Betab Vally, Awantipora, (Pulwama) and Simthan Top) but got no bumble bees in those regions. Captured bees were transferred to collection tube and brought to the Advanced Parasitology Laboratory of Kashmir University, Srinagar.

2. **Identification of bee species:** Bumble bee species were identified using the available literature and books in the university library. After identification, total of 121 *Bombus trifasciatus* bee samples were transferred to another collection tube. Tubes were allowed to cool in -20°C for 25 minutes to immobilize all the bee samples. After 25 minutes, bees were allowed to thaw and prepared for homogenisation.

3. **Homogenisation of bee samples:** Abdomen of bees were carefully removed using forceps and brush. Abdomens were washed thrice using ddH_2O to remove extra dirt, pollen grains and plant parts from bee body and were homogenised using pestle-mortar and later with electronic homogeniser (Ikon Instruments) in 0.6% K_2CO_3 solution (freshly prepared) with a vigorous shake to the tube after every 1 minute (Kashyap, 2018) [9].

4. **Sample preparation:** The mascaraed solution was firstly sieved through a musclin cloth to remove insect body parts and then sieved using cell strainer having 70 µm mesh size (Falcon ®) to remove cellular components greater than 70 µm. The clear translucent solution was transferred to Eppendorf tubes. Tubes were labelled and centrifuged at 300g for 5 minutes to form two phases- Upper strata having supernatant fluid and lower strata having most of the spores. Transferred the supernatant into another tube using micropipette and resuspended the lower strata in ddH_2O. Centrifuged the supernatant containing tube again at 300g for five minutes and repeated the process for 5 times. At last, combined all the 5 resuspended lower strata obtained from each centrifugation to yield a spore suspension with maximum purity (Cole, 1970) [4].

5. **Purification using Percoll ®:** For purification, pallet was resuspended in TE buffer and spores were purified by 90% percoll solution (P1644 Sigma-Aldrich). Stock Isotonic Percoll (SIP) solution was prepared by adding 9 parts of Percoll (v/v) (undiluted) to 1 part (v/v) of 1.5 NaCl. Density gradient centrifugation was performed (with slight modifications) at 20,000 g for 30 minutes. Two bands (A and B) were formed in the tube out of which the band at the bottom (band B) of the tube was carefully transferred to fresh eppendorf tube and washed several times in Tris-EDTA buffer (Tsai, 2003; Tsui, 1988). The spore pallet was further examined for the presence of spore by using dark field mode on EVOS XL Imaging system with a magnification at 100 X.

Percentage calculation of infected samples: Prevalence of infection is calculated as follows-

$$\text{Percentage of microsporidian infection} = \frac{\text{Number of samples infected}}{\text{Total number of bees captured}} \text{ X } 100$$

Results

The present study gave a clear expression of microsporidian infected bumble bees. However, there were no visible symptoms found on the external morphology of bees but their crawling behaviour in meadows propelled us to further examine them for parasitic infection. Out of 121 collected bees, infected bees were 98 in number meanwhile prevalence % of microsporidian infection is about 80.99% in region. Here, figure 1 is showing the selected species of bumble bee- *Bombus trifasciatus* (Orange belted bumble bee). Figure 2- shows the occurrence of two bands after density gradient centrifugation using Percoll solution. The lighter and developing meronts formed the upper band (Band A) and heavier matured spores settled at the bottom of the centrifuge tube to form Band B (heavier band). Band formation occurred due to difference in the densities in the microsporidian sub particles that got separated at high speed. Figure 3- demonstrates about the prevalence of spores in bumble bee homogenate. Band B was taken under consideration as it bears the matured and developing sporonts separated by density gradient centrifugation. Observed spores shown a Brownian motion (a continuous vibration at axis) and greenish luminescence under dark field microscope (figure- 3). Also, their external morphology explains about ovo- cylindrical shape. During the work various developmental stages of microspordian spores were observed. The sporonts divide to become sporoblast or young sporonts which further matures into spores. Dividing sporonts were frequently detected than dividing meronts. Fresh smear also showed very tiny spores that were nearly spherical in appearance with low or almost no luminescence. The dividing sporont gives rise to two sporoblasts or young sporonts. The very first stage observed was merogonial stage that is spherical and very slightly oval in shape. When life cycle proceeds these meronts undergo binary fission and form primary meronts that are also known as binucleate meronts BM (figure -3). When this further divide they form four nuclei per cell condition known as tetranucleated meronts; TM). Diplokaryotic stage was seen in meronts only and not in matured sporonts. Graph 1 shows a pie chart explaining about the overall percentage infection of microsporidian spores in *B. trifasciatus* bees of Kashmir. Data suggests that out of 121 bees, 98 were infected thus 80.99 % infected bees are struggling for life in the wild conditions.

Fig 1: Selected Bumble bee species *Bombus trifasciatus* (Orange belted bumble bee) for microsporidian spore isolation.

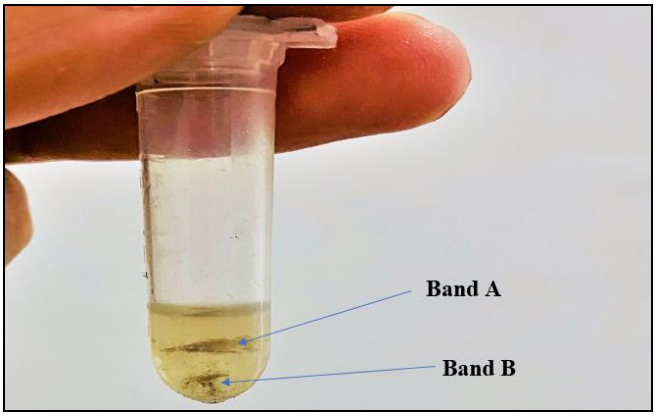

Fig 2: Density gradient centrifugation using Percoll (90%). Two band formations clearly seen. Band A containing developing meronts and band B containing matured developed sporonts.

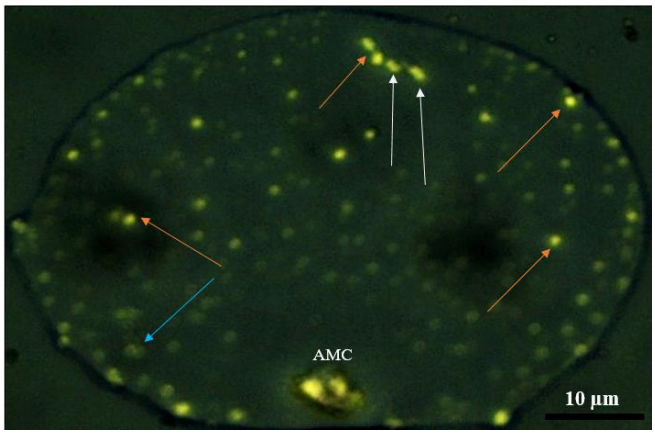

Fig 3: A drop of band- B on microscopic slide (unstained). Matured spores (sporonts) are marked by orange arrows and showing bioluminescence (green shine) when observed under dark field microscope at 100 X magnification (EVOS XL live imaging system). A slight constriction in between the spore (marked by white arrow) showing spores undergoing binary fission (BF). Blue arrow showing the binucleated meronts (BM) with low bioluminescence. Amorphic spore clumping (AMC) is imparting huge bioluminescence due to clustering of spores.

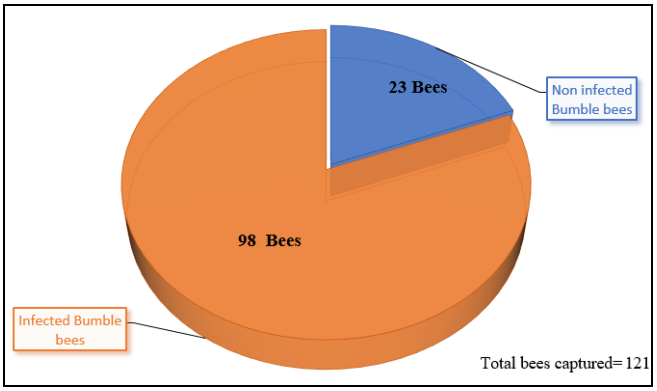

Fig 4: Pai chart showing overall percentage of infection. Out of 121 bees, 98 bees were infected with the parasite and 23 bees were found to be non-infected. Prevalence percentage is 80.99%.

Discussion

The microsporidia detected in the gut epithelium of orange belted bumble bees is an intracellular, eukaryotic obligate parasite that multiplies in the host cell. In our study the occurrence of microsporidia in *Bombus sp*. showed that it is a developing pathogen but due to lack of knowledge and expertise, these intracellular obligate parasites remained to be unknown in many regions of India. The Indian species of *Bombus* has usually been limited to higher elevations particularly Himalayan ranges (Shankar, 2013) [14]. Saini (2007) [11] provided insights on seven different bumble bee species residing in Himalayan ranges with respect to taxonomy and their preferred plant species where they like to visit more often. Bushmann *et.al.*, (2012) [1] determined the pervasiveness of *Nosema bombi* contamination as higher in wild bumblebees (*Bombus* spp.) caught in lowbush blueberry areas with a past history of commercial bumblebee usage compared with the bumblebees caught in areas with no prior history of commercial bumblebee usage explaining about the economic importance of bumble bees.

The genus *Nosema* is considered as a constant pathogen of both useful and harmful insects being prevalent particularly in Lepidoptera (Cheung and Wang, 1995) [3].

Morphological characterisation of microsporidia is not confined to any area, region or country. Due to its detrimental effects on bees' populations, studies have been performed across the globe since decade. The most efficient way to purify spores from any insect is density gradient centrifugation. First finding related to purification was performed by Seleznev in 1995. They performed fractionation of different life stages of microsporidia *Nosema grylli* isolated from crickets *Gryllus bimaaculatus* by centrifugation in percoll density gradient for Biochemical Research and did a noble work in the field. Similar study was performed by Feng *et al*, (2011) [2] in which they determined the morphological characters of spores by isolating them from *Phyllobrotica armata Baly* collected in China. Also, Tsai (2003) [17] purified and characterised the microsporidian isolates (particularly belonging to Nosimatidae: *Nosema*) from five important lepidopteran pests *viz Spodoptera exigua*, *Spodoptera litura*, *Plutella xylostella*, *Helicoverpa armigera*, and *Pieris spp*. which were compared on the basis of spore morphology in Taiwan. Cai (2011) [2] separated and purified various life stages of *Nosema bombycis*, the pathogen causing pebrine disease in silkworms by an efficient method of Percoll-density centrifugation to identify the spore wall protein of *N. bombycis* corresponding to spore phagocytosis. Reported works support the fact that percoll is the best suited chemical to separate developmental stages of microsporidia.

Our data revealed 80.99% infection percentage in Orange belted bumble bees. Data can be supported with the finding of Dar (2019) [5] that says it might be due to the mountainous climate of Kashmir and humidity in late spring and early summer that may favoured *Nosema* spreads.

The morphological characters illustrated in the study show a ray of an interrelation of microsporidian spores with genus *Nosema* (Feng 2011) [2]. The prevalence of these parasites permits further investigation and characterisation in different insect order from varied areas of Kashmir (India) also. Finding of spores is based on light microscopic observation. The restrictive populations of pollinators and abrupt colony deterioration of bees connects it with the growing dispersion of microsporidian parasites. The disease has become gradually more complex as a greater number of microsporidian strains has been recognized resulting from chronic infection. Therefore, an attempt has been made to successfully isolate and purify the microsporidian spores to perform molecular characterisation. Present study can be considered fruitful as it helped in successful isolation of

microsporidian spores and provided an efficient method to purify them.

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