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Pathogens of bark beetles (Coleoptera: Curculionidae) in Bulgarian forests

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Abstract The occurrence and prevalence of bark beetle pathogens in forest stands in Bulgaria were investigated in 944 specimens belonging to 21 bark beetle species. Protozoa, microsporidia, fungi and nematodes occurred in 19 of all investigated species. The infections were found in the gut (nematodes, gregarines, microsporidia), gonads (microsporidia) and hemolymph (nematodes) of the infected insects. Protozoan species (*Gregarina typographi*, *Gregarina* spp.) were detected in eight bark beetle species. Morphometric data about *G. typographi* and *Gregarina* spp. are presented. The prevalence of the gregarines varied between 1.4% and 64.2%. Microsporidia of the genera *Nosema* and *Chytridiopsis* were revealed in three bark beetle species. The prevalence of microsporidia ranged between 1.5% and 11.8%. This is the first report of a microsporidium in *Taphrorychus villifrons* and of gregarines in *T. villifrons, Pityogenes bistridentatus, P. conjunctus,* and *Orthotomicus erosus.* The fungus *Beauveria bassiana* was found in 3.4% of *Hylurgops palliatus* specimens. Nematodes (in gut and haemolymph) were revealed in 19 bark beetle species and their prevalence varied between 10% and 98.5%.

Keywords Entomopathogens · Fungi · Gregarines · Microsporidia · Nematodes

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Introduction

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are among the most dangerous insect pests in forests. Usually they colonize recently dry-topped and physiologically exhausted trees.

There is still lack of knowledge about the potential of pathogens in population regulation or as biological control agents of bark beetles (Händel *et al.* 2003). So far the most commonly used controls against these pests are sanitation measures, which are limited to the removal of infested host trees (Wermelinger 2004).

The investigations of bark beetle pathogens started at the beginning of last century when Fuchs (1915) described the first protozoan, *Gregarina typographi,* parasitizing *Ips typographus*. Later several authors studied bark beetle pathogens, e.g. Händel *et al.* (2003), Holuša *et al.* (2009), Wegensteiner (2004), and Yaman (2007).

In Bulgaria, economically important bark beetle species of coniferous trees (*Pinus sylvestris*, *Pinus nigra*, *Picea abies*) are the European spruce bark

beetle (*Ips typographus* L.), the engraver beetle (*Ips acuminatus* Gyllenhal), the six-toothed bark beetle (*Ips sexdentatus* Borner), the pine shoot beetle (*Tomicus piniperda* L.), the lesser pine shoot beetle (*Tomicus minor* Hartig) and the black pine bark beetle (*Hylastes ater* Paykull) (Georgiev 2006; Rosnev *et al.* 2006; Tsankov *et al.* 1997).

To explore the possibilities of using biological agents against forest pests it is necessary to investigate the diversity, distribution, biology and host interactions of their pathogens. Therefore an investigation of pathogens of *I. typographus* and other bark beetle pests (in total 22 species) was started in Bulgaria in 2003. As a result, Takov *et al.* (2006, 2007) and Nedelchev *et al.* (2008) reported 14 pathogen species (four protozoa, two microsporidia, one virus and seven nematodes) of bark beetles from Bulgaria.

The aim of the present study was to obtain new data about the occurrence of pathogens and their prevalence in a variety of bark beetle species from different forest stands in Bulgaria.

Materials and methods

Adult offspring beetles of 21 species were collected from April to September 2009 at 11 sites from various regions in Bulgaria (Fig. 1, Table 1). Beetles were collected from wind-thrown trees by peeling off the bark manually and removing the insects from the maternal galleries and nuptial chambers. In the laboratory, collected beetles were refrigerated at 4°C to reduce movement and prevent horizontal transmission of any pathogens. Bark beetles were dissected and fresh preparations of the gonads, Malpighian tubules, fat body and the entire gut from the host were examined for the presence of pathogens under a light microscope (160–400 x) according to Wegensteiner *et al.* (1996). *Dryocoetes autographus* was found mainly in the larval stage and only several adult beetles were observed; therefore mostly larvae of this host were investigated. When pathogens were observed, Giemsa-stained smears were made of the infected tissues (Weiser 1977). Sizes of spores of microsporidia and gregarine trophozoites (protomerite and deutomerite) were measured with an ocular micrometer at 200 and 400 x magnifications.

Dead beetles with symptoms of mycosis were placed in a moist chamber at 25°C to allow the development of hyphal and reproductive structures of the fungus. Some parts of cadavers were prepared as permanent preparations using lactophenol with aniline blue; others were prepared as smears stained with methylene-blue. Small amounts of insect cadavers after surface sterilization were used to isolate the fungal pathogens in pure cultures on SDAY (Sabouraud dextrose agar with yeast extract). Morphological characters of the fungal pathogens on the host and on media were studied under a light microscope in order to determine their taxonomic status and were identified according to Samson *et al.* (1988) and Humber (1997).

Statistical analysis was performed using the computer program STATISTICA, version 7.0 (StatSoft Inc. 1999).

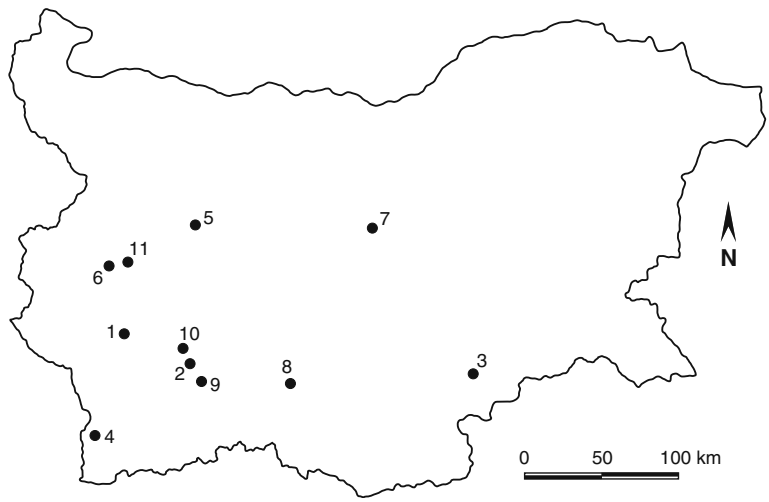

Fig. 1 Collection localities in Bulgaria (data about localities are presented in Table 1). 1—Rila Mt., Skakavitsa, 2—Rhodope Mt., Yundola Vill., 3—Sakar Mt., Zvezdata Place, 4—Maleshevska Mt., 5—Pravets, 6—Lyulin Mt., above Gorna Banya, Monastery St. Cyril and Methodius, 7—Balkan Range, above Gabrovo, 8—Rhodope Mt., Byala cherkva, 9—Rhodope Mt., above Velingrad, 10—Rila Mt., Belmeken Dam, 11—Sofia, Borisova gradina Park

Table 1 Number of investigated bark beetles, localities, host plants, found pathogens and their prevalence (%)

Bark beetle host	Locality/Altitude (m)	Host plant	N	*Gregarina typographi* n (%)	*Gregarina* sp. n (%)	*Nosema* sp. n (%)	*Chytridiop-sis* sp. n (%)	*Beauveria bassiana* n (%)	Nematoda n (%)
Dryocoetes autographus (Ratzeburg)	Rila Mt., Skakavitsa/1700	*Picea abies* (L.) Karsten	40	–	–	–	–	–	24 (60.0)
Hylurgops palliatus (Gyllenhal)	Rhodope Mt., Yundola Vill./1350; Lyulin Mt., above Gorna Banya/900; Balkan Range, above Gabrovo/600	*P. abies, Pinus sylvestris* (L.)	58	–	–	–	–	2 (3.4)	24 (41.4)
Hylurgus ligniperda (F.)	Sakar Mt., Zvezdata Place/500	*P. sylvestris*	67	–	–	1 (1.5)	–	–	58 (86.6)
Ips acuminatus (Gyllenhal)	Maleshevska Mt./950	*P. sylvestris*	36	–	–	–	3 (8.3)	–	10 (27.7)
Ips sexdentatus (Borner)	Sakar Mt., Zvezdata Place; Pravets/450; Rhodope Mt., Byala cherkva/1500	*P. sylvestris*	67	43 (64.2)	–	–	–	–	66 (98.5)
Ips typographus (L.)	Rhodope Mt., Yundola Vill.; Lyulin Mt., above Gorna Banya	*P. abies, P. sylvestris*	69	1 (1.4)	–	–	–	–	61 (88.4)
Orthotomicus erosus (Wollaston)	Sakar Mt., Zvezdata Place; Balkan Range, above Gabrovo	*P. sylvestris*	25	–	3 (12.0)	–	–	–	21 (84.0)
Orthotomicus laricis (F.)	Balkan Range, above Gabrovo	*P. sylvestris*	9	–	–	–	–	–	5 (55.5)
Orthotomicus longicollis (Gyllenhal)	Maleshevska Mt.	*P. sylvestris*	20	–	–	–	–	–	2 (10.0)
Orthotomicus proximus (Eichhoff)	Rhodope Mt., Byala cherkva	*P. sylvestris*	26	–	1 (3.8)	–	–	–	18 (69.2)
Pityogenes bidentatus (Herbst)	Balkan Range, above Gabrovo	*P. sylvestris*	10	–	–	–	–	–	2 (20.0)
Pityogenes bistridentatus (Eichhoff)	Balkan Range, above Gabrovo; Rhodope Mt., above Velingrad/1000	*P. sylvestris*	28	–	3 (10.7)	–	–	–	8 (28.6)
Pityogenes chalcographus (L.)	Rhodope Mt., Yundola Vill.	*P. sylvestris*	59	–	5 (8.4)	–	–	–	24 (40.7)
Pityogenes conjunctus (Reitter)	Rila Mt., Belmeken Dam/1940	*P. abies*	22	–	4 (18.2)	–	–	–	8 (36.4)
Pityogenes quadridens (Hartig)	Rhodope Mt., Yundola Vill.	*P. sylvestris*	23	–	–	–	–	–	4 (17.4)
Pityophthorus pityographus	Lyulin Mt., above Gorna Banya; Rhodope Mt.,	*P. sylvestris*	67	–	–	–	–	–	–

Table 1 (continued)

Bark beetle host	Locality/Altitude (m)	Host plant	N	*Gregarina typographi* n (%)	*Gregarina* sp. n (%)	*Nosema* sp. n (%)	*Chytridiop-sis* sp. n (%)	*Beauveria bassiana* n (%)	Nematoda n (%)
(Ratzeburg)	above Velingrad								
Scolytus rugulosus (Muller)	Maleshevska Mt.	*Pyrus communis* L.	30	–	–	–	–	–	–
Taphrorychus villifrons (Dufour)	Lyulin Mt., above Gorna Banya	*Fagus silvatica* (L.)	119	–	30 (25.2)	14 (11.8)	–	–	29 (24.4)
Tomicus minor (Hartig)	Balkan Range, above Gabrovo; Rhodope Mt., above Velingrad	*P. sylvestris*	13	–	–	–	–	–	2 (15.4)
Tomicus piniperda (L.)	Maleshevska Mt.; Balkan Range, above Gabrovo; Rhodope Mt., above Velingrad	*P. sylvestris*	118	–	–	–	–	–	33 (27.9)
Xyleborinus saxesenii (Ratzeburg)	Sofia, Borisova gradina Park/550	*Prunus cerasus* (L.)	38	–	–	–	–	–	7 (18.4)

N- number of investigated beetle individuals, n – number of infected individuals,% - percentage of infected individuals

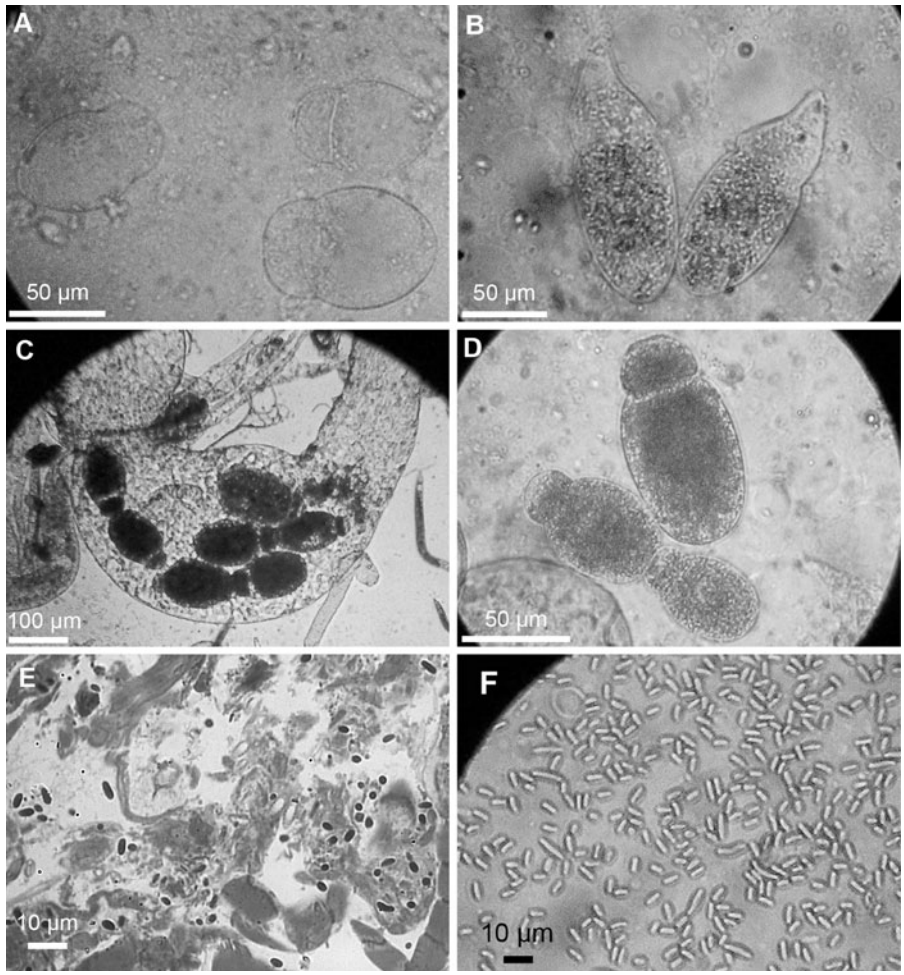

Fig. 2 **a**, Trophoizoites of *Gregarina* sp. in midgut lumen of *Taphrorychus villifrons*; **b**, Trophoizoites of *Gregarina* sp. in midgut lumen of *Orthotomicus erosus*; **c**, Trophoizoites of *Gregarina* sp. in midgut lumen and nematodes in hemolymph of *Pityogenes conjunctus*; **d**, Trophoizoites and syzigia of *Gregarina* sp. in midgut lumen of *Pityogenes bistridentatus*; **e**, Spores of *Nosema* sp. in gonads and fat body of *Taphrorychus villifrons* (semithin section stained with Richardson); **f**, Fresh spores of *Nosema* sp. from midgut of *Hylurgus ligniperda*

Results

Protozoa, microsporidia, fungi and nematodes were detected in 19 (90.4%) of all investigated bark beetle species (Table 1). Protozoan species (Apicomplexa: Gregarina) were detected in the gut system of eight bark beetle species (Table 1). Trophozoites of *Gregarina typographi* were observed in the gut lumen of *Ips typographus, I. sexdentatus* and *Pityogenes chalcographus*. Other *Gregarina* species were found in the gut lumen of *P. conjunctus, P. bistridentatus*, *Orthotomicus proximus*, *O. erosus* and *Taphrorychus villifrons* (Fig. 2). The prevalence of the gregarines varied between 1.5% and 64% but the average prevalence for all investigated host species was 9.5%.

The systematics and taxonomy of the group are based on morphometric parameters such as the sizes of protomerites and deutomerites which form the gregarine trophozoite and the ratios between them. We measured morphometrical data of gregarine trophozoites found in eight hosts (Table 2). The statistical analysis (Figs. 3, 4 and 5) showed that the sizes of *Gregarina* spp. from *P. chalcographus*, *P. bistridentatus*, *O. erosus* and *T. villifrons* are within the limits of the species variability of *G. typographi,* found in *I. typographus* and *I. sexdentatus.* However, the most precise identification of these gregarines would be possible only after a molecular analysis and detailed investigations of life cycle stages.

Microsporidia, which can morphologically be assigned to the genera *Nosema* and *Chytridiopsis,* were revealed in three bark beetle species (*Hylurgus ligniperda, Ips acuminatus, Taphrorychus villifrons*) (Table 1). The prevalence of the microsporidia ranged between 1.5% and 11.8% and the average prevalence for all investigated host species was 1.9%.

Spores and pansporoblasts of *Chytridiopsis* sp. were found in the gut of *I. acuminatus* individuals. The size

Table 2 Morphometric data of *Gregarina* spp. and *G. typographi* from different bark beetle hosts (in μm)

Host species	TL mean (min-max)	LP mean (min-max)	LD mean (min-max)	WP mean (min-max)	WD mean (min-max)	LP/TL mean (min-max)	WP/WD mean (min-max)	LP/WP mean (min-max)
Gregarina spp.								
P. conjunctus (1 specimen)	122	22	100	32	82	0.180	0.390	0.687
P. bistridentatus	75.4 (60–96)	19.7 (18–22)	55.7 (40–74)	27.4 (22–34)	44.6 (38–52)	0.265 (0.229–0.333)	0.612 (0.550–0.684)	0.733 (0.588–0.9)
Orthotomicus proximus	262 (75–400)	27.8 (12.5–40)	234 (62–360)	58.8 (25–92)	80.5 (25–130)	0.125 (0.093–0.166)	0.800 (0.666–1)	0.481 (0.434–0.5)
O. erosus	137 (80–242)	36 (22–50)	98 (50–180)	45 (34–60)	54 (40–90)	0.279 (0.143–0.375)	0.844 (0.555–0.96)	0.804 (0.600–1.08)
Taphrorychus villifrons	82.4 (46–140)	22.4 (12–50)	60 (34–90)	31.2 (16–70)	45.8 (24–70)	0.262 (0.200–0.357)	0.673 (0.480–1)	0.727 (0.514–0.96)
Gregarina typographi								
Ips typographus	111 (80–135)	36 (21–52.5)	75.4 (51.2–94)	44 (25–75)	49.5 (25–87.5)	0.321 (0.226–0.488)	0.895 (0.628–1.1)	0.830 (0.566–1.2)
I. sexdentatus	144 (55–260)	25.8 (10–40)	117.8 (40–237)	45 (20–87.5)	78.8 (35–106)	0.190 (0.080–0.380)	0.590 (0.250–1)	0.605 (0.279–1.25)
Pityogenes chalcographus	67 (50–80)	18 (10–30)	49.3 (40–62.5)	28.4 (20–38)	38.1 (25–47.5)	0.259 (0.160–0.375)	0.800 (0.5–1)	0.607 (0.5–0.789)

mean values (mean); extreme values (min-max); total length (TL), length protomerite (LP), width protomerite (WP), width deutomerite (WD), ratio of LP and TL (LP/TL), WD, ratio of WP and WD (WP/WD), ratio of LP and WP (LP/WP)

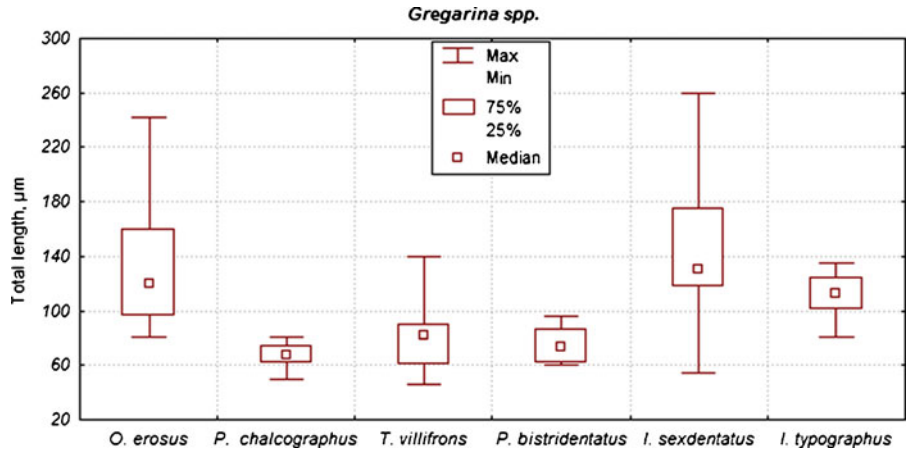

Fig. 3 Total lengths of *Gregarina* spp. from different hosts

of live pansporoblasts varied from 7.2 to 14.4 µm (n=30) and up to 30 spores were observed in the gut.

Microsporidia with morphological features typical of the genus *Nosema* were detected in gonads and fat body tissue of *Taphrorychus villifrons* (Fig. 2). The *Nosema* spores were oval and the length of spores stained with Giemsa varied from 2.4 to 3.9 µm and the width from 1.2 to 3 µm (n=50). Spores of another microsporidium of the genus *Nosema* were observed in the gut epithelium of *Hylurgus ligniperda* (Fig. 2). The length of the oval, live spores varied from 4.8 to 7.2 µm and the width from 1.8 to 3.30 µm (n=30). The length of Giemsa-stained spores varied from 3.6 to 5.1 µm and the width from 1.95 to 3.44 µm (n=35). Furthermore, the tissue localization of the two *Nosema* microsporidia, isolated from *Hylurgus ligniperda* and *Taphrorychus villifrons,* is different, and therefore we suppose that they belong to two different species. However, for accurate species determination, additional investigations and especially detailed molecular characterization are needed.

The fungus *B. bassiana* was found in 3.4% of the investigated *Hylurgops palliatus* specimens (Table 1).

Nematodes were revealed in 19 bark beetle species. The determination of the nematodes in this study was very difficult. In most cases only nematode larvae without a completed sexual system, which is important for species determination, were found in the gut and the hemolymph of the bark beetles. Their prevalence was high and varied between 10% and 98.5% and the average prevalence for all investigated host species was 43% (Table 1).

Individuals of *S. rugulosus* and *P. pityographus* were also investigated but no pathogens were revealed.

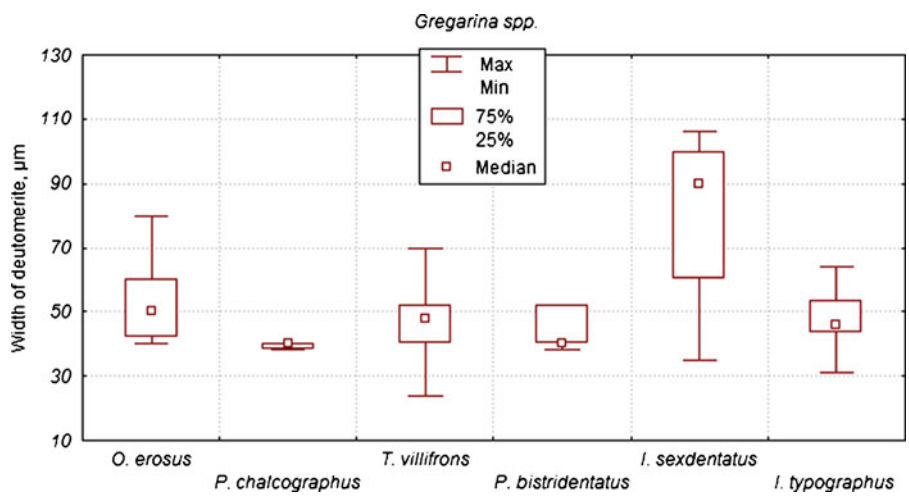

Fig. 4 Widths of deutomerites of *Gregarina* spp. from different hosts

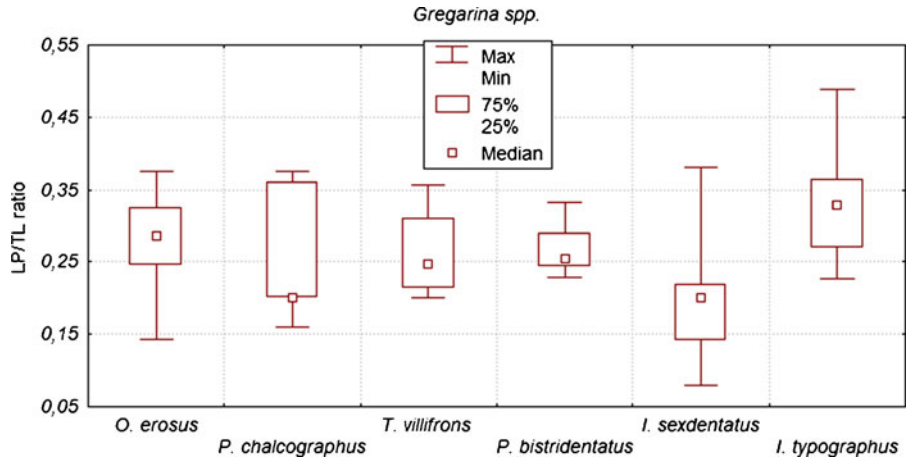

Fig. 5 Ratios between lengths of protomerite and total lengths of *Gregarina* spp. from different hosts

Discussion

Pathogens (*G. typographi*, *Gregarina* spp., *Chytridiopsis* sp., *Nosema* spp. and *B. bassiana*) were found in 19 of the 21 investigated bark beetle species. Four species are new hosts for *Gregarina* spp., and a *Nosema* sp. was recorded for the first time in *T. villifrons*.

Gregarina typographi and *Gregarina* spp. were the most frequently recovered pathogens. *G. typographi* is a polymorphic species and was described by Fuchs (1915) in *I. typographus* and reported in *I. sexdentatus* by Theodorides (1960). The pathogen was found in Austria, Czech Republic and Bulgaria (Takov *et al.* 2006, 2007; Wegensteiner 1994). The investigations of bark beetle pathogens conducted in Austria by B. Haidler (Diploma thesis,1998, Vienna Univ., Austria) and U. Händel (Ph.D. thesis, 2001, Boku Univ., Vienna, Austria) revealed the presence of *G. typographi* in *Hylastes cunicularius* and *Dryocoetes autographus* and of *G. cf. typographi* in *Hylurgops glabratus*, *Pityogenes chalcographus* and *I. amitinus*. Takov *et al.* (2007) conducted a morphometrical characterization of this gregarine and showed its variability.

The *Chytridiopsis* sp. we recovered in *I. acuminatus* is possibly *Chytridiopsis typographi*. It was reported also from Bulgaria by Takov *et al.* (2007) and from Austria, Czech Republic and Norway by P. M. Zitterer (Diploma thesis, 2002, Boku Univ., Vienna, Austria). The size of the pansporoblasts measured by us corresponds to the size recorded by Zitterer in his thesis, but he did not identify the microsporidium to species level. According to Wegensteiner (2004), *C. typographi* has a broader specificity and it might be possible that it infects also *I. acuminatus*. More detailed study of additional material is needed for the precise identification of *Chytridiopsis* sp. found in this study.

Several microsporidia of the genus *Nosema* were described in different bark beetles: *N. typographi*, which infects fat body and Malpighian tubules of *I. typographus* (Weiser 1955) and also *Hylurgops palliatus* (Purrini 1978). *N. curvidens* was found in the fat body, hypoderm and connective tissue of *Pityokteines curvidens* (Weiser 1961), *N. scolyti* in the gut epithelium, Malpighian tubules and hemocytes of *Scolytus scolytus, S. multistriatus, S. pygmaeus* and *S. ensifer;* and *N. dendroctoni* in the fat body of *Dendroctonus pseudotsugae* (Weiser 1970). Händel *et al.* (2003) discovered a *Nosema* sp. in the fat body and gut epithelium of *H. palliates* and Takov *et al.* (2007) found a *Nosema* sp. in the gut epithelium of *H. ligniperda*. Based on the size and tissue localization reported by Takov *et al.* (2007), we assume that this is the same species we detected during this study. This is the first report of a microsporidium in *T. villifrons* and gregarines in *Pityogenes bistridentatus, P. conjunctus, Orthotomicus proximus, O. erosus* and *T. villifrons*.

Beauverai bassiana was recorded for the first time from *H. palliatus* by Balazy (1962), but ours is the first record of the fungus in this host in Bulgaria. Its prevalence in this study was low (3.4%). Wegensteiner (1996), Kreutz *et al.* (2004), Draganova *et al.* (2007), Sevim *et al.* (2010) and Steinwender *et al.* (2010) showed that adults of bark beetles were susceptible to entomopathogenic fungi under laboratory conditions. Bioassays with the entomopathogenic fungus *B. bassiana* showed high virulence of some isolates,

especially of that obtained in pure cultures from dead individuals of the hosts, collected from infected natural populations (Draganova *et al.* 2010). Induced mycoses with a high lethal effect could be a perspective in the use of this pathogen for control of the bark beetles.

The infection rates of nematodes were the highest and we believe that this reflects the low pathogenicity of bark beetle nematodes and the high survival rates of the infected bark beetle hosts.

The development cycle of the pathogens is often synchronized with the host cycle and this assures a successful infection and distribution (Massey 1956; Rühm 1956; Thong and Webster 1973). Pathogens are transmitted *via* spores (fungi, microsporidia), cysts (protozoa) and invasion of larvae or imago (nematodes) to the next generation. The infection takes place in the beetle galleries *via* the active penetration of the pathogens into the hosts (nematodes), *per os* infection (protozoa and microsporidia) or by external contact (fungi). The new generation of the beetles usually feeds additionally in the galleries, where contact with the pathogens occurs. The capability of the pathogens to infect the host is also species-specific. Virulent pathogens kill their host very fast after the infection. The infection has a local character and usually few hosts survive and serve to transmit the infection into new galleries. Therefore, during the investigation of a large number of live hosts, the prevalence of pathogens is low except in the cases when infection foci are found. In this case the infection rate is higher, but because of the mortality the pathogen prevalence in live beetles decreases. Gregarines are less pathogenic (Bjornson and Schütte 2003) and therefore occur more often compared with other pathogens, which is in accord with our findings.

The influence of bark beetle pathogens on the host density is still not sufficiently investigated, but existing studies show that the pathogen prevalence depends on several factors, such as pathogen characteristics, its life cycle, population host density and immunity, host development particularities and other factors (Wegensteiner 2004). Therefore, the investigations of pathogenicity and virulence should be intensified. The findings reported in this study show that bark beetles collected from different Bulgarian forests host a broad range of pathogens from different taxonomic groups, but molecular techniques, *inter alia,* are needed in order better to characterize bark beetle pathogens.

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