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A New Microsporidian of the Genus *Nosema*, Parasite of *Chaetocnema tibialis* (Coleoptera: Chrysomelidae) from Turkey

Mustafa YAMAN[1], Renate RADEK[2] and Bhen Sikina TOGUEBAYE[3]

[1] Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, Trabzon, Turkey
[2] Institute of Biology/Zoology, Free University of Berlin, Berlin, Germany
[3] Laboratoire de Parasitologie, Département de Biologie Animale, Faculté des Sciences et Techniques Université C.A. Diop, Dakar, Sénégal

Summary. A new microsporidian parasite was discovered in *Chaetocnema tibialis* (Coleoptera: Chrysomelidae) in Turkey. The life cycle of the new species was examined by light and electron microscopy. It parasitizes the Malpighian tubules of *C. tibialis*. All development stages are in direct contact with the host cell cytoplasm and have paired nuclei (diplokaryon). Fresh spores are elongated and measure $3.82 \pm 0.3 \times 1.3 \pm 0.2$ µm. The spores possess two spherical nuclei, 8–10 coils of the polar filament and a posterior vacuole. All morphological and ultrastructural features indicate that the described microsporidium belongs to the genus *Nosema.* The microsporidian parasite presented here differs from *Nosema chaetocnemae* in spore size, number of polar filament coils, tissue specificity and locality. Therefore the parasite is considered to be a new microsporidian parasite of *C. tibialis.* This new species has been named *Nosema tokati* sp. n. after the name of its host's location.

Key words: *Chaetocnema tibialis,* microsporidian parasite, *Nosema tokati* sp. n., Chrysomelidae, Nosematidae.

INTRODUCTION

Chaetocnema tibialis (Chrysomelidae, Coleoptera) is an important pest of sugar beet in Turkey. Chemical pesticides utilized to control this pest should be limited because sugar beet leaves and pulp are used for animal nourishment; biological control agents are preferable because they have certain advantages over chemical treatments. As a group, microsporidia are the most important pathogens of insects and are the most promising microorganisms for use in microbial control (Tanada and Kaya 1993).

Address for correspondence: Mustafa Yaman, Department of Biology, Faculty of Arts and Sciences, Karadeniz Technical University, 61080 Trabzon, Turkey, E-mail: muyaman@hotmail.com

There have been relatively few studies on the parasites and pathogens of *C. tibialis,* although several microsporidia have been isolated from the members of the family Chrysomelidae (Toguebaye *et al*. 1988; Toguebaye and Marchand 1984, 1986, 1989; Toguebaye and Bouix 1989). Yaman and Radek (2003) identified the first microsporidian parasite of *C. tibialis*, *Nosema chaetocnemae*. This was the first finding of an insect-infecting microsporidium in Turkey. Later, Yaman (2004) recorded a gregarine parasite of the same beetle. During a study on the distribution of *N. chaetocnemae* in the Turkish populations of *C. tibialis* performed by Yaman (2007), a new microsporidian parasite of *C. tibialis* was observed which is presented in this study.

Acta Protozoologica 2008 48/3
Jagiellonian University Kraków, Poland

MATERIAL AND METHODS

Insect samples

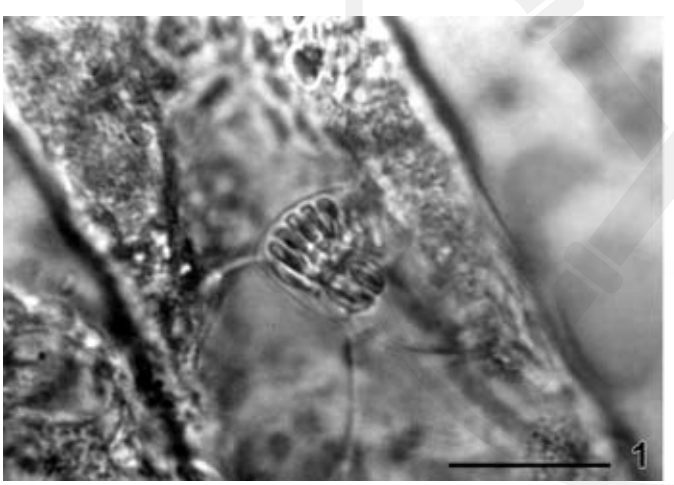
In total, 107 beetles were collected in the vicinity of Tokat (Turkey). The adults of *C. tibialis* were put into sterilized glass bottles to prevent possible contamination. They were brought to the laboratory and dissected as soon as possible.

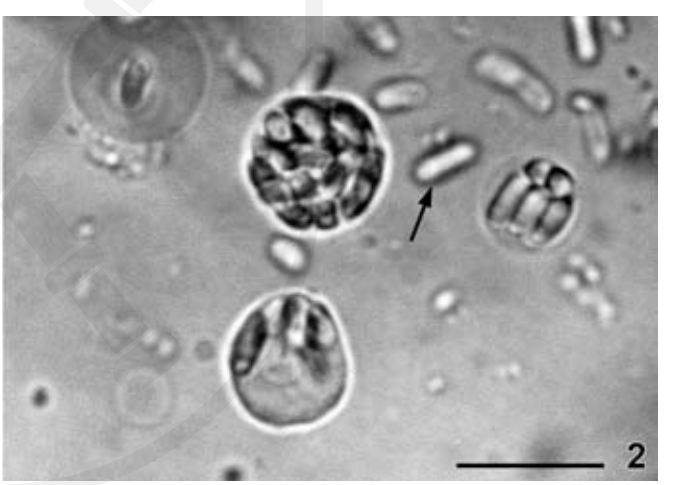
Microscopic examinations

Each beetle was dissected in insect Ringer's solution. Wet smears were examined under a light microscope at magnifications of 400x to 1000x**.** When an infection with a pathogen was observed, the slides were air-dried and fixed in methanol for 10 min. The slides were then washed with distilled water and stained for approximately 10 hours in freshly prepared 5% solution of Giemsa stain. Afterwards the slides were washed in running tap water, air-dried and re-examined under the microscope (Toguebaye *et al*. 1988, Undeen and Vavra 1997). Detected spores were measured and photographed using an Olympus BX51 microscope with a DP-25 digital camera and a DP2-BSW Soft Imaging System. Also fresh spores were measured and the combined data of fresh and stained spores were presented as the mean value ± standard deviation (SD). Parts of the infected beetles were embedded for transmission electron microscopy according to standard protocols (Yaman and Radek 2003). Ultra-thin sections were examined with a Philips 208 electron microscope.

RESULTS

A new microsporidian infection was found in adults of *C. tibialis* collected in Tokat (Turkey). Only two of the 107 dissected beetles were infected with that microsporidium. The parasites were observed exclusively in the Malpighian tubules (Figs 1, 3). Microscopic examination of parasitized individuals revealed only one type of spore and showed that all stages of the parasite developed in direct contact with the cytoplasm of the host cell. Some spores enclosed in a vesicle were also observed (Figs 1, 2). These vesicles are probably of host origin; they are not sporophorous vesicles because they contained either none (Fig. 1), one, five or more than ten spores (Figs 1, 2). Fresh spores were elongated and measured $3.82 \pm 0.3 \times 1.3 \pm 0.2\,\mu m$ (n=30). They were diplokaryotic with spherical nuclei measuring 500–600 nm in diameter (Fig. 5). The polar filament was isofilar and had 8–10 coils (Figs 5, 7). Mature coils measured 90–100 nm in diameter. The spore had a prominent posterior vacuole (Fig. 5). The well-developed polaroplast was of the lamellated type with thin



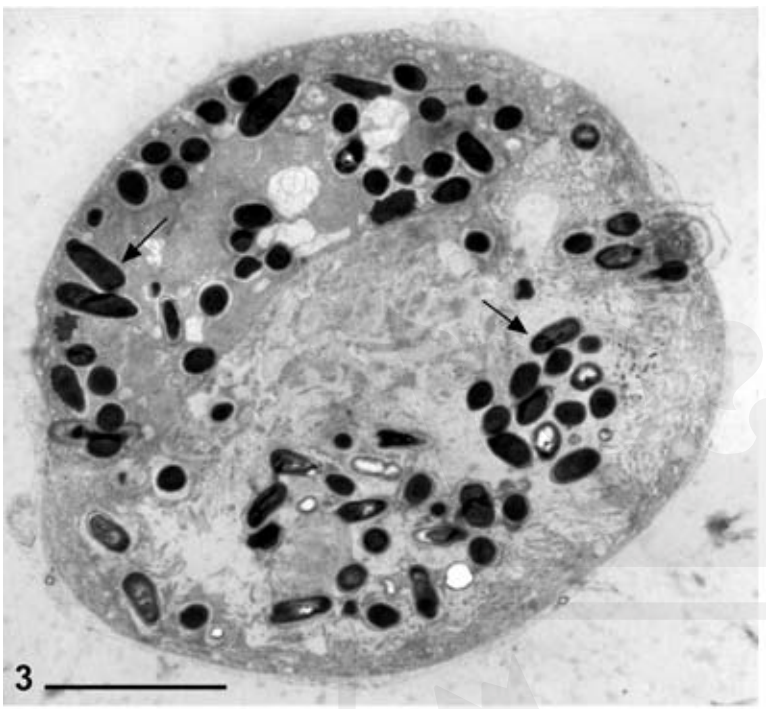


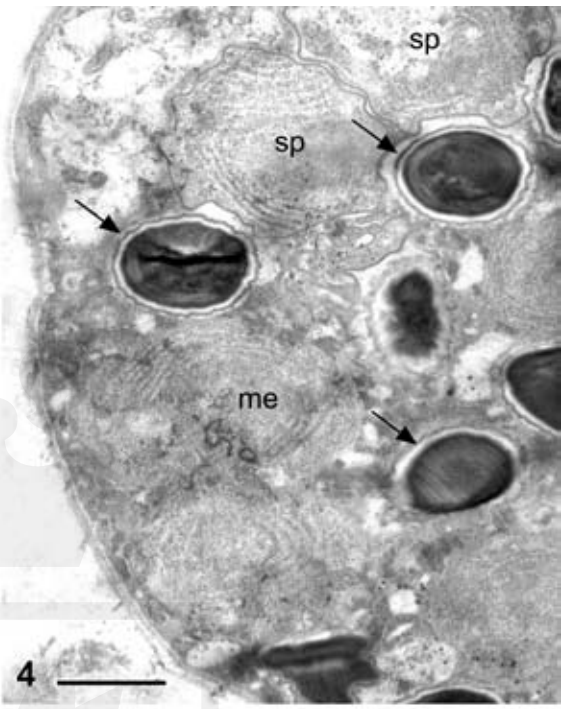
Figs 1–2. Light microscopic micrographs. **1** – spores of the microsporidian parasite within a Malpighian tubule, spores enclosed by a vesicle and an empty vesicle. Bar: 10 µm; **2** – released microsporidian spores from torn open Malpighian tubules. Spores are either free or enclosed by a vesicle. Bars: 10 µm, phase contrast.

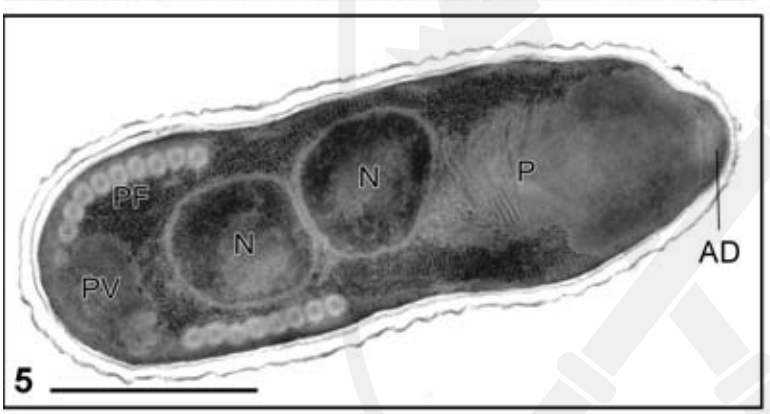
▸▸

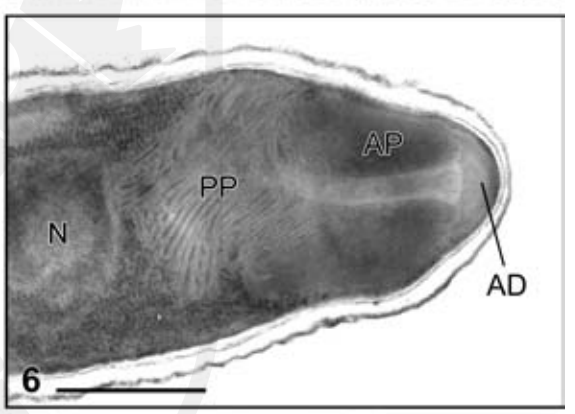
Figs 3–9. Transmission electron micrographs. **3 –** semithin cross-section of a Malpighian tubule infected with microsporidian spores lying in direct contact with the Malpighian tissue (arrows). Bar: 10 µm; **4** – cross-section of a Malpighian tubule showing different stages of microsporidia in direct contact with the Malpighian tissue; **5** – longitudinal section of a diplokaryotic microsporidian spore. Bar: 1 µm; **6** – longitudinal section of the anterior part of a microsporidian spore. Bar: 500 nm; **7** – microsporidian spore showing cross sections of the polar filament. Bar: 200 nm; **8** – diplokaryotic microsporidian meront. Bar: 1 µm; **9** – diplokaryotic microsporidian sporont with surface coat on the plasma membrane (arrow). Bar: 1 µm. AD – anchoring disc; AP – anterior part of polaroplast, N – nucleus; P – polaroplast; PF – polar filament; PP – posterior part of polaroplast; PV – posterior vacuole. Bar: 1 µm.

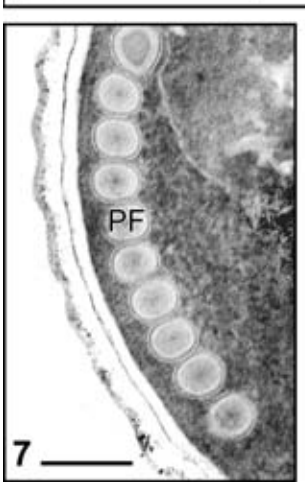
Acta Protozoologica 2008 48/3
Jagiellonian University Kraków, Poland

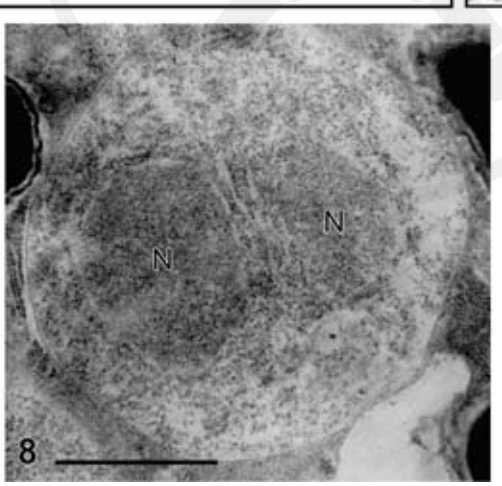


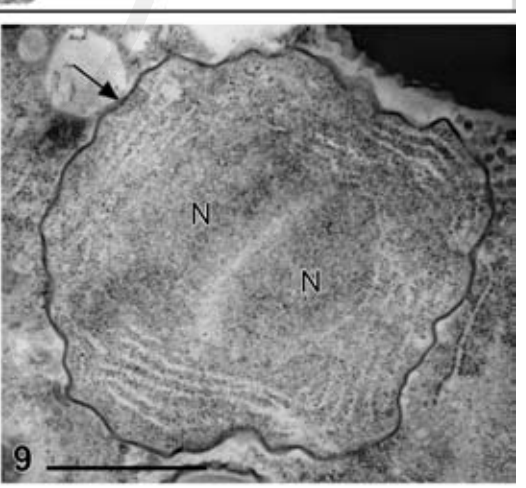












Acta Protozoologica 2008 48/3
Jagiellonian University Kraków, Poland

lamellae (anterior) and thick lamellae (posterior) (Fig. 6). An anterior anchoring disc of the polar filament could clearly be seen (Fig. 6).

Not only the spores but also meronts and sporonts were diplokaryotic (Figs 8, 9). The meronts were spherical and were identified cytologically; they had a plasma membrane without external reinforcements. The sporonts are recognizable by having an electron-dense thick envelope. In addition to the two nuclei, meronts (Figs 4, 8) and sporonts (Figs 4, 9) both contained cisterns of endoplasmic reticulum.

DISCUSSION

The parasite found in *Chaetocnema tibialis* is definitely a microsporidian. The spore ultrastructure elucidated the typical characteristics such as a polar filament with an anchoring disc, a polaroplast, a posterior vacuole, and a lack of mitochondria (Figs 5, 6, 7) (Larsson 1986, 1988; Canning and Vavra 2000). In insects, 63 different microsporidian genera had been found by 1997 (Undeen and Vávra 1997); the genera *Burenella, Canningia, Larssoniella, Nosema, Ovavesicula, Tuzetia, Unikaryon, Pleistophora* and *Endoreticulatus* have been recorded in beetles (Brooks *et al*. 1988, Undeen and Vávra 1997, Weiser *et al*. 2006). Microsporidia known to infect Chrysomelidae belong to only three genera: *Nosema*, *Unikaryon* and *Pleistophora* (Toguebaye *et al*. 1988). However, Brooks *et al*. (1988) described a new genus, *Endoreticulatus,* for the microsporidium *Pleistophora fidelis* (Hostounský and Weiser 1975) based on the ultrastructure of a microsporidium in the Colorado potato beetle, *Leptinotarsa decemlineata* (Chrysomelidae). However, of the genera infecting beetles, only *Burenella* and *Nosema* are diplokaryotic in all stages and neither possess a sporophorous vesicle; the stages are in direct contact with the host cell cytoplasm (Undeen and Vávra 1997, Toguebaye *et al*. 1988). In contrast to *Nosema*, *Burenella* has two spore types. One spore type has as many as 26, the other 57 coils of the polar filament (Canning and Vavra 2000) (8–11 coils in the new microsporidian).

Considering the ultrastructural characteristics and the developmental cycle of the new microsporidian parasite of *C. tibialis,* we conclude that it belongs to the genus *Nosema* Naegeli, 1857 (Figs 1–9). To date, the sole finding of a microsporidian parasitizing *Chaetocnema tibialis* was *Nosema chaetocnemae* Yaman and Radek 2003. Sprague *et al*. (1992) acknowledged the host species as an important taxonomic characteristic, at least in microsporidia which infect insects. Although belonging to the same genus, it is easy to distinguish the recent isolate from *N. chaetocnemae* infecting the same host (Yaman and Radek 2003). The results show that the microsporidium presented here differs from *N. chaetocnemae* in spore size and shape, number of polar coils, type of infected tissue, infection rate, and locality of the host population (Table 1).

Table 1. Characteristics of the two microsporidian parasites described from *Chaetocnema tibialis* (Coleoptera: Chrysomelidae).

		Nosema chaetocnemae Yaman and Radek, 2003	*Nosema tokati* Present study
Locality		Samsun, Turkey	Tokat, Turkey
Infection rate		max. 42%	2%
Infected organs		Gut, tracheae, muscles and Malpighian tubules	Malpighian tubules
Spore shape		Oval	Cylindrical
Spore size		3.5 × 2.1 µm	3.82 × 1.3 µm
Ultrastructural features	Spore wall thickness	176–213 nm	85–100 nm
	Polar filament	13 coils	8–10 coils
	Polar filament diameter	115 nm	90–100 nm
	Polaroplast	Relatively vesicular	Lamellar
	Nuclei	Diplokaryotic, slightly elongated	Diplokaryotic, spherical

In recent identification keys to microsporidian genera, ultrastructural characteristics (especially of the spores) are always included (Larsson 1983, 1988, 1999). The spore is the most important life cycle stage, being always present and providing abundant features to evaluate (Larsson 1999). The ultrastructure of many *Nosema* species has been described (Sato *et al*. 1982, Avery and Anthony 1983, Toguebaye and Marchand 1984, Toguebaye and Bouix 1989, Hsu *et al*. 1991, Canning and Vávra 2000, Yaman and Radek 2003, Ovcharenko and Wita 2005). These studies provide useful information for the identification of *Nosema*. The ultrastructural characteristics of *Nosema* spores were given by Sato *et al*. (1982) and Canning and Vávra (2000). In order to find out whether the *Nosema* finding from *C. tibialis* belongs to a new species, we compared its spore ultrastructure with the spores of *N. chaetocnemae* from the same host and other *Nosema* species from chrysomelids.

Acta Protozoologica 2008 48/3
Jagiellonian University Kraków, Poland

Table 2. *Nosema* species described in the family Chrysomelidae (Coleoptera).

Nosema species	Spore size	Infected organ	Host	Locality
Nosema phyllotretae Weiser, 1961	4.2 × 2–3 µm	Adipose body	*Phyllotreta atra* *Phyllotreta undulata*	England
Nosema leptinotarsae Lipa, 1968	2–5 × 1.9–3.3 µm	Haemolymph	*Leptinotarsa decemlineata*	U.S.S.R.
Nosema gastroideae Hostounský and Weiser, 1973	3–4.8 × 2.5–3 µm	Overall infestation	*Gastrophysa polygoni* and several experimental hosts	Czechoslovakia
Nosema polygrammae Hostounský and Weiser, 1975	4.8 × 2.05 µm	Gut	*Polygramma undecimlineata*	Cuba
Nosema equestris Hostounský and Weiser, 1980	4–5 × 3 µm	General infestation	*Gastrophysa viridula* *Leptinotarsa decemlineata*	Czechoslovakia
Nosema couilloudi Toguebaye and Marchand, 1984	3.4–4 × 1–1.5 µm	Gut	*Nisotra* sp.	Senegal
Nosema birgii Toguebaye and Marchand, 1986	6.2 × 3.5	Eggs and general infestation, larvae and imago	*Mesoplatys cincta*	Senegal
Nosema nisotrae Toguebaye and Marchand, 1989	5.8 × 3.1 µm	General infestation	*Nisotra* sp.	Senegal
Nosema galerucellae Toguebaye and Bouix, 1989	4.95 × 2.89 µm	Gut principally, adipose body, muscles, tracheae and Malpighian tubules	*Galerucella luteola*	France
Nosema chaetocnemae Yaman and Radek, 2003	3,52 × 2,09 µm	Gut, tracheae, muscles and Malpighian tubules	*Chaetocnema tibialis*	Samsun, Turkey
Nosema tokati sp. n. present work	3.82 × 1.3 µm	Malpighian tubules	*Chaetocnema tibialis*	Tokat, Turkey

Up to now eleven *Nosema* species have been found to parasitize the Chrysomelidae (Toguebaye and Bouix 1989, Yaman and Radek 2003), and six of them have been studied by electron microscopy. Their distinctive characteristics are shown in Table 2. Spore dimension is a good feature for comparison of the twelve *Nosema* species from chrysomelids. The new microsporidian clearly differs from *N. chaetocnemae* and other *Nosema* species in spore size (Table 1 and 2). The new microsporidian also differs from *N. chaetocnemae* in the shape of nuclei (slightly elongated in *N. chaetocnemae*), thickness of spore wall, diameter of the polar filament, and infection rate (Table 1). Furthermore, the present microsporidium infected only Malpighian tubules, whereas *N. chaetocnemae* also infects the gut, tracheae and muscles. In addition, there was no overlap in the distribution of the two microsporidians from *C. tibialis* in Turkey. Whereas the new parasite was found in Tokat (Central South Province, altitude 623 m), *N. chaetocnemae* was only observed in Samsun (Black Sea Coast, Province Samsun, altitude 57 m). Yaman's (2007) extensive study on the distribution of *Nosema chaetocnemae* in the populations of *C. tibialis* in Turkey confirms an exclusive distribution; he did not observe *N. chaetocnemae* infections in *C. tibialis* samples collected from Tokat. These regions of Turkey constitute different climatic zones and thus accommodate different groups of plants and animals (The Ministry of Environment 1992).

The new microsporidian also differs from other *Nosema* species recorded from the family Chrysomelidae in several characteristic features (Table 2). For example, the number of polar coils provides a further useful taxonomic criterion for differentiating species (Cheung and Wang 1995). The number of polar coils of the described parasite (8–11) is different from the number of coils of *N. chaetocnemae* (13) and other five chrysomelid parasites, *N. galerucellae* (7–9; Toguebaye and Bouix 1989), *N. couilloudi* (8–10; Toguebaye and Marchand 1984), *N. birgii* (12–14; Toguebaye and Marchand 1986), *N. nisotrae* (15–18; Toguebaye and Marchand 1989) and *N. phyllotretae* (13–15; Yaman *et al*. 2005a, b) (Table 1, 2).

The new microsporidium has an isofilar polar filament. Mature coils measure 90–100 nm in diameter,

Acta Protozoologica 2008 48/3
Jagiellonian University Kraków, Poland

compared to 115 nm in *N. chaetocnemae* and 100––125 nm in *N. phyllotretae* (Yaman *et al*. 2005a, b).

As seen in Table 1 and 2, in all cases at least one characteristic of the known species is different from this new chrysomelid parasite, either spore dimension, number of polar filament coils, thickness of the spore wall, host and geographic location of the host, and/or infected organs. Therefore, the described characters of the *Nosema* from *C. tibialis* seem to be sufficiently distinct to represent a new species. It was named *Nosema tokati* sp. n., after the name of the locality of the infected hosts, Tokat.

***Nosema tokati* sp. n.**

Diagnosis: Spores elongated, $3.82 \pm 0.3 \times 1.3 \pm 0.2$ µm, diplokaryotic with spherical nuclei, 500–600 nm in diameter, isofilar polar filament with 8–11 coils and 90––100 nm in diameter, well-developed lamellated type polaroplast with thin lamellae (anterior) and thick lamellae (posterior).

Type host: *Chaetocnema tibialis* (Coleoptera: Chrysomelidae).

Host locality: Sugar beet plantation, Tokat (N 40°22'23" – N40°22'29", E 36°09'46"– E 36°09'52") – Turkey.

Location in host: Malpighian tubules.

Prevalence: 2 of 107 examined beetles were infected by the parasite. Infection rate: 2%.

Type material: Giemsa-stained microscopic slides and grids for electron microscopy are stored at the Department of Biology, Karadeniz Technical University, Trabzon, Turkey.

Etymology: The name of the species refers to its infection locality Tokat in Turkey.

Our study confirms two discrete microsporidian infections in *C. tibialis* in Turkey and adds a new *Nosema* species, *Nosema tokati*, to the list of microsporidian parasites of the family Chrysomelidae. This is not the first record which reveals two *Nosema* species from the same host. *Nosema apis* was known as the sole microsporidium infecting honeybees. Recently, Higes *et al*. (2006) recorded *Nosema ceranae* as a new microsporidian parasite in honeybees (*Apis mellifera*) in Europe. Several studies have confirmed *Nosema ceranae* infections in honeybees as well (Fries *et al*. 2006, Huang *et al*. 2007, Chauzat *et al*. 2007, Klee *et al*. 2007).

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Acta Protozoologica 2008 48/3
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