Journal of Helminthology (2004) **78,** 231–236 DOI: 10.1079/JOH2004245

Evolutionary relationships within '*pygmaeus*' group microphallids using genetic analysis and scanning electron microscopy

K.V. Galaktionov[1], S.A. Bulat[2], I.A. Alekhina[2], D.H. Saville[3], S.M. Fitzpatrick[3] and S.W.B. Irwin[3]*

[1]Zoological Institute of the Russian Academy of Sciences, White Sea Biological Station, Universitetskaja Naberezhnaja 1, 199053 St Petersburg, Russia: [2]Eukaryote Genetics Laboratory, Petersburg Nuclear Physics Institute, Gatchina, 188350, Russia: [3]School of Environmental Sciences, University of Ulster, Shore Road, Newtownabbey, Co. Antrim, BT37 0QB, UK

Abstract

There are four species of '*pygmaeus*' microphallids, namely *Microphallus pygmaeus*, *M. piriformes*, *M. pseudopygmaeus* and *M. triangulatus* (Trematoda: Microphallidae) which are parasites of marine birds and their sporocysts give rise to transmissible metacercariae inside littoral gastropods (mostly littorines). Universally primed polymerase chain reaction (UP-PCR) showed no apparent pattern between genetic diversity of the metacercariae as estimated by genomic banding profiles and their geographic region or molluscan host species. At the same time UP-PCR product cross-hybridization showed that *M. pseudopygmaeus* and *M. triangulatus* are genetically very similar, indicating that these taxa represent one species complex. In contrast, *M. pygmaeus* and *M. piriformes* are genetically well separated from each other and also from the *pseudopygmaeus–triangulatus* complex. Scanning electron microscopy of ventral spines, and analyses of spine angles and the number of teeth per spine, showed that all species differed significantly from one another. It was concluded that *M. piriformes* represents the original western member of the '*pygmaeus*' group. *Microphallus pygmaeus* probably diverged from *M. piriformes* as it progressively specialized for sea duck final hosts. *Microphallus pseudopygmaeus* and *M. triangulatus* diverged from each other and the *piriformes–pygmaeus* ancestral line relatively recently. *Microphallus pseudopygmaeus* specialized for adoption of a wide range of gastropod host species and *M. triangulatus* developed morpho-functional specialization associated with final host exploitation.

Introduction

The '*pygmaeus*' group of microphallids is composed of species in which metacercariae develop inside daughter sporocysts without encystment. Four species occur on the shores of the North Atlantic: *Microphallus pygmaeus* (Levinsen, 1881); *M. piriformes* (Odhner, 1905) Galaktionov, 1983; *Microphallus* sp. 1 (*M. pseudopygmaeus*) Galaktionov, 1980; *Microphallus triangulatus* (Galaktionov, 1984). All four species are parasites of marine and coastal birds and their daughter sporocysts occur in littoral gastropod molluscs, mostly members of the genus *Littorina*. As metacercariae develop to a transmissible stage inside their sporocysts, the life cycle of these

*Author for correspondence
Fax: +02890 366028
E-mail: swb.irwin@ulster.ac.uk

parasites is completed when infected molluscs are ingested by seabirds. The four species can be distinguished by the gross morphology of adults and metacercariae (Galaktionov, 1980, 1983, 1984) and the life cycles also display significant differences. For example, experimental infections of *M. piriformes* develop more successfully in gulls than in ducks (Galaktionov, 1993). Adults of the other three species are found in ducks, especially eiders, although Galaktionov (1993) showed that under experimental conditions, 50% of *M. pygmaeus*, 20% of *M. triangulatus* and no *M. pseudopygmaeus* metacercariae developed in gulls. The larval stages of three of the species have only been found in gastropods of the genus *Littorina* but, significantly, *M. pseudopygmaeus* makes use of additional gastropod genera such as *Hydrobia*, *Epheria*, *Margarites*, *Onoba*, *Solariella* and *Cryptonatica* (Galaktionov, 1986, 1993).

Because the '*pygmaeus*' microphallids are similar in terms of their anatomy and life cycles, confusion between species has occurred in the past. For example, Irwin & Saville (1988) referred to *M. pygmaeus* when they were studying metacercariae of *M. piriformes* and the *M. pygmaeus* Form A described by James (1968) was in fact *M. piriformes*. Saville *et al.* (1997) resolved the confusion between *M. pygmaeus* and *M. piriformes* and the present study represents a continuation of that work, to establish if the four '*pygmaeus*' microphallids are different species and, if possible, to shed light on their evolutionary relationship within the '*pygmaeus*' group. Genetic (genome fingerprinting) analyses are presented of '*pygmaeus*' group specimens from differing geographical regions and, in the case of *M. pseudopygmaeus*, from differing molluscan hosts. Universally primed polymerase chain reaction (UP-PCR) was adopted rather than random amplification of polymorphic DNA (RAPD) (Williams *et al.*, 1990) because it consistently generates multibanded highly reproducible profiles regardless of the organism being studied (Bulat *et al.*, 1998, 2000; Lübeck *et al.*, 1999). Scanning electron microscopy (SEM) of metacercariae of the four species was confined to the anterior ventral spine structure. In adult worms, these spines maintain an intimate contact with the host's tissues and Davies (1979) suggested that, in the microphallid *M. similis*, they probably aid in maintaining worm attachment and have an irritating abrasive effect on the host's intestinal mucosa. Interspecies differences in the shape of these spines might therefore represent evolutionary modifications associated with final host preferences, and they were studied here as potentially phylogenetically informative characters.

Materials and methods

Fully-formed transmissible metacercariae were collected from littoral and upper-sublittoral molluscs, sampled in 1999 from the White Sea (Chupa inlet of the Kandaksha Bay, 66°20′ N, 33°40′E), the Barents Sea (Yarnyshnaya and Dalnezelenetskaya bays near Dalnie Zelentzy, 69°07′N, 36°05′E) and the Norwegian Sea (Balsfjord near Tromsø, 69°49′N, 18°50′E). The species of metacercariae used for genetic analysis, their molluscan hosts, the sample sites and identification labels attributed to each are listed in table 1.

Table 1. Molluscan host and source location of metacercariae of *Microphallus pseudopygmaeus*, *M. pygmaeus*, *M. piriformes* and *M. triangulatus*.

Species of metacercariae	Molluscan host species	Source location	Identity label
M. pseudopygmaeus	*Margarites helicinus*	Barents Sea	A-1
	Onoba aculeus	Barents Sea	A-2
	Littorina saxatilis	Norwegian Sea	A-3
	L. obtusata	Norwegian Sea	A-4
M. pygmaeus	*L. saxatilis*	Barents Sea	B-1
	L. saxatilis	White Sea	B-2
	L. saxatilis	Norwegian Sea	B-3
	L. obtusata	Norwegian Sea	B-4
M. piriformes	*L. saxatilis*	Barents Sea	C-1
	L. saxatilis	White Sea	C-2
	L. saxatilis	Norwegian Sea	C-3
M. triangulatus	*L. saxatilis*	White Sea	D-1
	L. saxatilis	Norwegian Sea	D-2
	L. obtusata	Norwegian Sea	D-3

To ensure purity of the sampled metacercariae, they were recovered from living sporocysts and washed thoroughly in bacteria-free seawater that had been passed through a 0.22 μm Millipore filter. Fully-formed specimens were selected and carefully separated from sporocyst remains before being washed in a further ten changes of the filtered seawater. They were then fixed in 96% ethanol for genetic analysis or 3% (v/v) aqueous glutaraldehyde for SEM studies.

The genetic analysis included UP-PCR (Bulat & Mironenko, 1990; Bulat *et al.*, 1992, 1998) which enables amplification of DNA from any organism without previous knowledge of its DNA sequences by generating multiband profiles (fingerprints) by gel electrophoresis. Universal primers (UP) used have the following sequences: AA2 (16 mer): 5′-CTGCGACCCAGAGCGG-3′ (Mironenko *et al.*, 2000) and L15/AS19 (15mer): 5′-GAGGGTGGCGGCTAG-3′ (Lübeck *et al.*, 1998). Several DNA concentrations were tested in PCR in order to ensure the reliability of electrophoretic banding profiles. Negative PCR reactions (having no DNA template) were always run alongside tests and they never produced any banding profiles. Bands were scored across species as strongly present, weakly present, or absent, and MacClade 3.04 was used to search for the most parsimonious phylogenetic tree. Universally primed polymerase chain reaction product cross hybridization assay (an advanced variant of the UP-PCR technique by Bulat *et al.* (1998)) facilitated the investigation of overall sequence similarity (homology) of UP-PCR products. Cross-hybridization of UP-PCR products was applied in an attempt to ascertain if all four taxa represent separate species. This technique had already been successfully applied to fungi (Bulat *et al.*, 1998; Lübeck *et al.*, 1999) and trypanosomatids (Bulat *et al.*, 1999). In the present study, the isolates tested were those having UP-PCR profiles that differed in some respect.

The SEM technique used by Saville *et al.* (1997) was adopted to provide details of ventral spine structure for

https://doi.org/10.1079/JOH2004245 Published online by Cambridge University Press

each of the species. Micrographs which were taken mid-way between the oral and ventral suckers, were used to measure the angle of the spine teeth (see fig. 1) and also to count the number of teeth per spine, all spines being assessed for both measures. Differences between species were analysed by one-way ANOVAs and Tukey-Kramer post-hoc comparisons. All specimens observed by SEM were taken from *Littorina saxatilis.*

Results

Universally primed polymerase chain reaction profiles generated with the two UP primers are shown in fig. 2. Within each species there is a good similarity amongst isolates tested while each species is featured by a distinct genomic structure. *Microphallus pseudopygmaeus* is visibly similar to *M. triangulatus*, a relationship found in three of five equally parsimonious trees identified by MacClade. In the consensus tree, *M. pseudopygmaeus* and *M. triangulatus* were linked as sister taxa, but the relationship between this group, *M. pygmaeus*, and *M. piriformes* was represented as an unresolved polytomy.

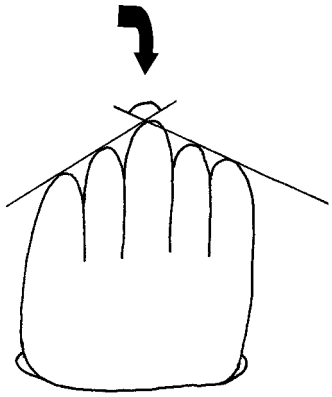

Fig. 1. An illustration of the angle (arrowed) measured for each ventral spine, located midway between the oral and ventral suckers in metacercariae of *Microphallus pygmaeus*, *M. pseudopygmaeus*, *M. piriformes* and *M. triangulatus*.

No apparent pattern could be found between the genetic diversity of metacercariae and their source (geographic region, host species). For example, A2 and A4 isolates of *M. pseudopygmaeus* gave almost identical UP-PCR profiles for both primers even though they were sourced from different locations and hosts (fig. 2). Nevertheless some genetic differences were revealed between isolates of *M. pseudopygmaeus* from *L. saxatilis* (A3) and *L. obtusata* (A4) collected from the same locality of the Norwegian Sea. Isolates of *M. piriformes* taken from *L. saxatilis* from the Barents Sea (C1) and the White Sea (C2) produced different profiles (primer L15/AS19) whereas the C2 isolate did not differ from the C3 originating from Norway (table 1). *Microphallus pseudopygmaeus* and *M. triangulatus* isolates from different geographical regions and different molluscan hosts displayed no significant genetic diversity.

The UP-PCR cross-hybridization results (fig. 3) showed that *M. pseudopygmaeus* (A) and *M. triangulatus* (D) are genetically very similar, thus, these taxa represent one species complex (the AD complex). In contrast, the *M. pygmaeus* (B) and *M. piriformes* (C) genetically are well separated from each other and also

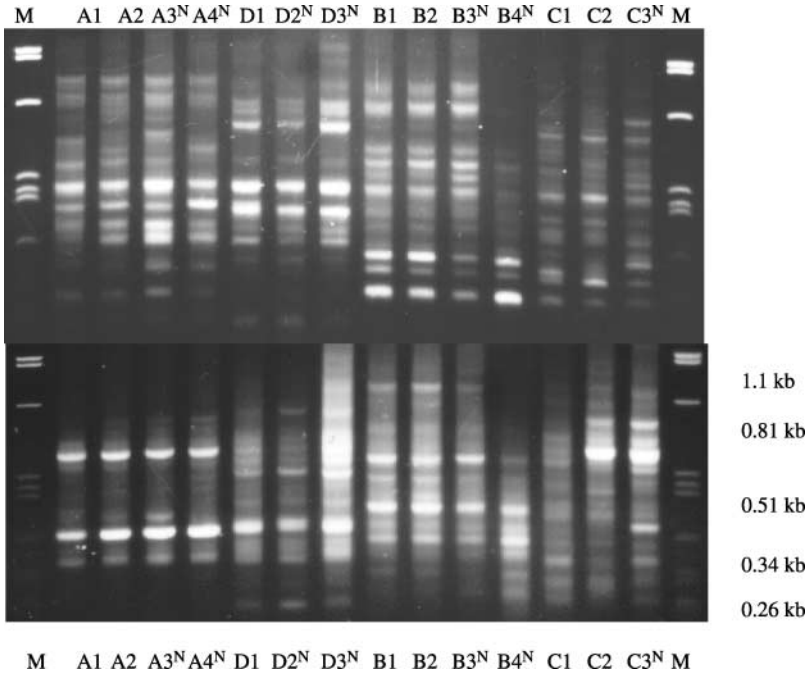

Fig. 2. Universally primed polymerase chain reaction banding profiles for '*pygmaeus*' metacercariae from various sources. Top – results with AA2 UP primer. Bottom – results with L15/AS19 UP primer. Lanes are in the following order (from left to right): A1–A4 *Microphallus pseudopygmaeus*; D1–D3 *M. triangulatus*; B1–B4 *M. pygmaeus*; C1–C3 *M. piriformes* (for full explanation see table 1). N, Norwegian strains; M, molecular weights markers (lambda phage DNA digested with Pst1) (some markers are shown).

https://doi.org/10.1079/JOH2004245 Published online by Cambridge University Press

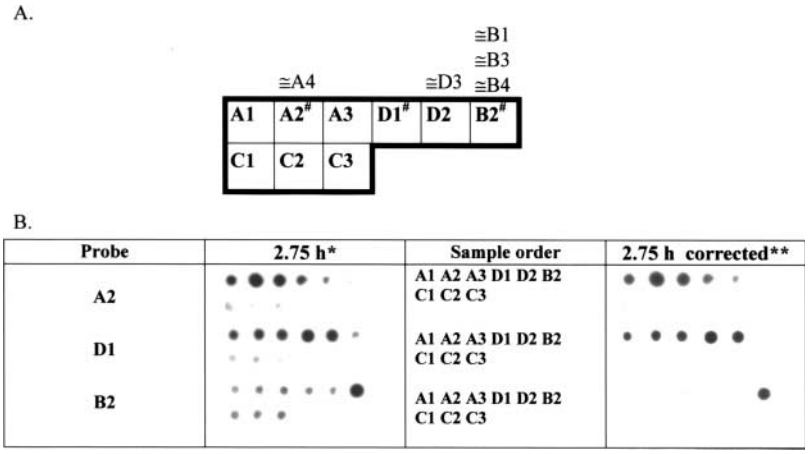

Fig. 3. Universally primed polymerase chain reaction product dot blot hybridization for '*pygmaeus*' metacercariae. A – dot blot scheme: UP-PCR products generated with AA2 primer. Strains with similar UP-PCR profiles are shown above the scheme. B – results of dot blot hybridization. #Strains that had their UP-PCR products used as labels. *Film exposure (in hours). **Image processed by software. Strain designation: A1–A4 *Microphallus pseudopygmaeus*; D1–D3 *M. triangulatus*; B1–B4 *M. pygmaeus*; C1–C3 *M. piriformes*.

distinct from the AD complex (fig. 3). Bearing these differences in mind, it is still apparent that all three UP-PCR hybridization groups (AD, B and C) generate weak overlapping hybridization signals (fig. 3). Thus, in terms of genome structure and evolution, the three groups are obviously closely related.

The SEM study of ventral spines demonstrates differences in the shape and number of teeth per spine (fig. 4 A–D). The mean angle of the spines and the number of teeth per spine are presented in table 2. The spine angles differ significantly ($P < 0.0001$) between species, with the highest angle occurring in *M. piriformes*. Similarly, the number of teeth per spine differs significantly ($P < 0.0001$) between species with *M. piriformes* having the highest number of teeth. For both measures, post hoc tests showed that all species differ significantly from each other.

Discussion

The results of the genetic (genomic fingerprinting) analysis presented here showed that two of the four pygmaeus microphallids have their own distinct genomic structure and it therefore confirms the validity of species status for *M. piriformes* and *M. pygmaeus* despite the confusion in the literature. *Microphallus pseudopygmaeus* and *M. triangulatus* are also shown to be the most closely related species and can be considered as sister taxa in the process of speciation, with morphological differences more noticeable than genetic differences. A comparison of the anatomy of these two species would not immediately indicate this close relationship, but both species are of a similar size, with a distinct similarity in the shape and arrangement of the reproductive structures. The obvious diagnostic difference in overall body shape is largely due to one feature, i.e. the very well developed

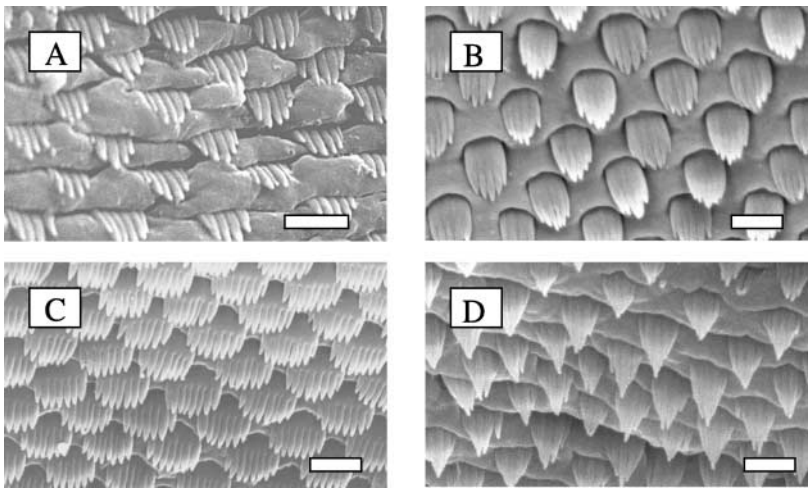

Fig. 4. Scanning electron micrographs of ventral spines located midway between the oral and ventral suckers of metacercariae. A, *Microphallus pygmaeus*; B, *M. pseudopygmaeus*; C, *M. piriformes*; D, *M. triangulatus*. Scale bar = 1 μm.

https://doi.org/10.1079/JOH2004245 Published online by Cambridge University Press

Table 2. Table of spine angles and number of teeth per spine for each of the species of *Microphallus*.

	n	Spine angle (mean ± SE)	*n*	Teeth number (mean ± SE)
M. piriformes	19	146.16 ± 2.63	22	7.55 ± 0.13
M. pseudopygmaeus	25	96.88 ± 2.29	55	5.62 ± 0.08
M. pygmaeus	85	117.58 ± 1.24	87	4.45 ± 0.06
M. triangulatus	43	69.98 ± 1.75	40	4.85 ± 0.09
Tukey-Kramer test		All species significantly different		All species significantly different
ANOVA		$P < 0.0001$		$P < 0.0001$

posterio-lateral glands in *M. triangulatus*. The unusual ability of *M. pseudopygmaeus* to infect a wide variety of gastropod genera as intermediate hosts may be important in the speciation process.

The fact that *M. piriformes* has a distinctly larger spine angle and more teeth per spine than the other species may be important. The spines of adult microphallids are in intimate contact with their host's mucosa, and the anatomy of the mucosa probably differs slightly from bird species to species. The suitability of the shape and structure of the spines to carry out their function in their chosen host might well represent a factor that would be selected for. *Microphallus piriformes* is the only one of the four '*pygmaeus*' species that has been found in gulls and oystercatchers (*Haematopus ostralegus*) in the Barents Sea region and Galaktionov (1993) and Galaktionov *et al.* (1997) demonstrated that, although it occurs in common eiders at the White Sea, in experimental conditions development is less successful in eiders than in gulls. The other three species in the '*pygmaeus*' group showed a distinct preference for development in ducks and perhaps their spines, with smaller angles and fewer teeth, have evolved to suit these hosts.

According to Belopolskaya (1963, 1983), the first members of the family Microphallidae appeared in the Northern Hemisphere on the eastern coast of Asia not later than the Pleistocene. In the post-glacial period microphallids, which were spread from there by migrating birds, invaded the Atlantic coast of North America and then were spread from there, again by migrating birds, to the coast of Western Europe. If that were the case, in order to fully investigate the '*pygmaeus*' group phylogeny we might require additional specimens from other geographical regions such as north-east Asia. *Microphallus calidris* is a species with the same type of life cycle as North Atlantic '*pygmaeus*' microphallids and it is widely distributed on the Pacific coast of north-east Asia (Tsimbaljuk *et al.*, 1968, 1978). It seems possible that this species could be an ancestor (or closely related to an ancestor) of the '*pygmaeus*' group, as the morphology of *M. calidris* is very close to that of *M. piriformes* (Galaktionov, 1983). Like *M. piriformes*, *M. calidris* makes use of littorines (*Littorina kurila* and *L. sitchana* according to Tsimbaljuk *et al.* (1968)) as first intermediate hosts and adult worms of *M. calidris* have only been recorded in waders and gulls (Belopolskaya, 1963; Tsimbaljuk *et al.*, 1968).

If the above theory is correct, *M. piriformes* may represent the original member of the North Atlantic '*pygmaeus*' group. The other members of the group show a distinct host preference for ducks. Bustnes & Galaktionov (1999) showed that the high prevalence of *M. piriformes* in periwinkles coincided with concentrations of gulls on northern sea coasts. This did not apply to *M. pygmaeus* which, although developing in both gulls and eiders under experimental conditions, is only found in common eiders and some other sea ducks in the natural environment (Galaktionov, 1993; Galaktionov *et al.*, 1997). It seems reasonable to suggest that the divergence between *M. piriformes* and *M. pygmaeus* probably arose as a specialization associated with the progressive involvement of sea ducks (especially common eiders) as final hosts. Common eiders eat many more periwinkles and other gastropods than other seabirds (Belopolski, 1971). As '*pygmaeus*' group trematodes have an abridged life cycle in which metacercarial development is completed in these molluscs, common eiders must represent extremely numerous and available hosts for exploitation.

Microphallus pseudopygmaeus and *M. triangulatus* probably utilize mainly common eiders, or possibly other sea ducks, in their life cycles (Galaktionov, 1984, 1993, 1996). The present study has shown that these two very closely related species differ significantly in spine characteristics and each species possesses a feature that differs distinctly from the other three species. *Microphallus triangulatus* has a very distinct shape, due to the presence of very well developed posterio-lateral glands, and *M. pseudopygmaeus* is the only species which can infect numerous genera of gastropods as molluscan hosts (Galaktionov, 1986, 1993). The present results suggest a recent divergence of these two species from each other and the *piriformes–pygmaeus* ancestral line. Their unique characteristics may represent key innovations in that the evolution of *M. pseudopygmaeus* is strongly influenced by its ability to adopt a wide range of species as first intermediate hosts. This would surely provide *M. pseudopygmaeus* with an evolutionary opportunity and, not surprisingly, this species is one of the commonest and most widely distributed in the Arctic (Galaktionov & Bustnes, 1999; Galaktionov & Skirnisson, 2000). In *M. triangulatus*, perhaps the morpho-functional specialization associated with the huge development of posterio-lateral glands has provided a similar, though less obvious, evolutionary opportunity. Geographical isolation from the other species can be ruled out as an influencing factor as the ranges of these species overlap, and it is likely that genetic diversity in these microphallids does not reflect the geographic origin of the specimens analysed.

Acknowledgements

This research was supported by the International Association for the promotion of co-operation with scientists from the New Independent States of the former Soviet Union (INTAS) grant 01-0210.

References

Belopolskaya, M.M. (1963) Trematodes of the family Microphallidae Travassos, 1920. pp. 259–502 *in*

https://doi.org/10.1079/JOH2004245 Published online by Cambridge University Press

Skriabin, K.I. (*Ed.*) *Trematodes of humans and animals*. Vol. **21** (in Russian).

Belopolskaya, M.M. (1983) Ecology and geographical distribution of helminths of migrating birds. pp. 174–189 *in* Polianski, Y.I. (*Ed.*) *Free-living and parasitic invertebrates (morphology, biology, evolution)*. Leningrad, Leningrad State University Press (in Russian).

Belopolski, L.O. (1971) The food composition of the seabirds of the Barents Sea. *Uchenie Zapiski Kaliningradskogo gosudarstvennogo universiteta (Scientific notes of Kaliningrad State University)* **6**, 41–67 (in Russian).

Bulat, S.A. & Mironenko, N.V. (1990) Species identity of the phytopathogenic fungi *Pyrenophora teres* Dreschler and *P. graminea* Ito & Kuribayashi. *Mikologia i Phytopatologia* **24**, 435–441 (in Russian).

Bulat, S.A., Kaboev, O.K., Mironenko, N.V., Ibatullin, P.M., Luchkina, L.A. & Suslov, A.V. (1992) Polymerase chain reaction with universal primers for study of genomes. *Soviet Genetics* **28**, 549–557 (in Russian).

Bulat, S.A., Lübeck, M., Mironenko, N., Jensen, D.F. & Lübeck, P. (1998) UP-PCR analysis and ITS1 ribotyping of *Trichoderma* and *Gliocladium* fungi. *Mycological Research* **102**, 933–943.

Bulat, S.A., Mokrousov, I.V. & Podlipaev, S.A. (1999) Classification of trypanosomatids from insects and plants by the UP-PCR (universally primed PCR) technique and cross dot blot hybridization of PCR products. *European Journal of Protistology* **35**, 319–326.

Bulat, S.A., Lübeck, M., Alekhina, I.A., Jensen, D.F., Knudsen, I.M.B. & Lübeck, P.S. (2000) Identification of an UP-PCR derived SCAR marker for an antagonistic strain of *Clonostachys rosea* and development of a strain-specific PCR detection assay. *Applied and Environmental Microbiology* **66**, 4758–4763.

Bustnes, J.O. & Galaktionov, K.V. (1999) Anthropogenic influences on the infestation of intertidal gastropods by seabird trematode larvae on the southern Barents Sea coast. *Marine Biology* **133**, 449–453.

Davies, C. (1979) The forebody glands and surface features of the metacercariae and adults of *Microphallus similis*. *International Journal for Parasitology* **9**, 553–564.

Galaktionov, K.V. (1980) Four types of metacercariae species in molluscs, *Littorina saxatilis* and *L. obtusata* from the Barents and White Seas. *Vestnik Leningradskogo Universiteta* **3**, 21–28 (in Russian).

Galaktionov, K.V. (1983) Microphallids of the *"pygmaeus"* group. I. Description of species *Microphallus pygmaeus* (Levinsen, 1881) nec Odhner, 1905 and *M. piriformes* (Odhner, 1905) nom. nov. (Trematoda: Microphallidae). *Vestnik Leningradskogo Universiteta* **15**, 20–30 (in Russian).

Galaktionov, K.V. (1984) Microphallids of the *"pygmaeus"* group. II. *Microphallus triangulatus* sp. nov. (Trematoda: Microphallidae). *Vestnik Leningradskogo Universiteta* **3**, 5–11 (in Russian).

Galaktionov, K.V. (1986) Metacercariae of the family Microphallidae Travassos, 1920 from benthic molluscs of the south-eastern part of the Barents Sea. *Parazitologia* **20**, 389–396 (in Russian).

Galaktionov, K.V. (1993) *Trematodes life cycles as components of ecosystems*. Apatity, Kola Scientific Centre Press, 190 pp. (in Russian).

Galaktionov, K.V. (1996) Life cycles and distribution of seabird helminths in Arctic and subarctic regions. *Bulletin of the Scandinavian Society for Parasitology* **6**, 31–49.

Galaktionov, K.V. & Bustnes, J.O. (1999) Distribution patterns of marine bird digenean larvae in periwinkles along the southern Barents Sea coast. *Diseases of Aquatic Organisms* **37**, 221–230.

Galaktionov, K.V. & Skirnisson, K. (2000) Digeneans from intertidal molluscs of SW Iceland. *Systematic Parasitology* **47**, 87–101.

Galaktionov, K.V., Saville, D.H., Irwin, S.W.B. & Malkova, I.I. (1997) A comparison of *Microphallus pygmaeus* (from northern Russia) and *M. piriformes* (from northern Russia and Northern Ireland) cultivated *in vivo* and *in ovo*. *BSP Spring Meeting, Abstracts*, 82 Manchester.

Irwin, S.W.B. & Saville, D.H. (1988) Cultivation and development of *Microphallus pygmaeus* (Trematoda: Microphallidae) in fertile chick eggs. *Parasitology Research* **74**, 396–398.

James, B.L. (1968) Studies on the life-cycle of *Microphallus pygmaeus* (Levinsen, 1881) (Trematoda: Microphallidae). *Journal of Natural History* **2**, 155–172.

Lübeck, P.S., Alekhina, I.A., Lübeck, M. & Bulat, S.A. (1998) UP-PCR genotyping and rDNA analysis of *Ascochyta pisi* Lib. *Journal of Phytopathology* **146**, 51–55.

Lübeck, M., Alekhina, I., Lübeck, P.S., Jensen, D.F. & Bulat, S.A. (1999) Delineation of *Trichoderma harzianum* Rifai into two different genotypic groups by a highly robust fingerprinting method, UP-PCR, and UP-PCR product cross-hybridisation. *Mycological Research* **103**, 289–298.

Mironenko, N.V., Alekhina, I.A., Zhdanova, N.N. & Bulat, S.A. (2000) Intraspecific variation in gamma-radiation resistance and genomic structure in the filamentous fungus *Alternaria alternata*: a case study of strains inhabiting Chernobyl Reactor No. 4. *Ecotoxicology and Environmental Safety* **45**, 177–187.

Saville, D.H., Galaktionov, K.V., Irwin, S.W.B. & Malkova, I.I. (1997) Morphological comparison and identification of metacercariae in the "*pygmaeus*" group of microphallids, parasites of seabirds in western Palearctic regions. *Journal of Helminthology* **71**, 167–174.

Tsimbaljuk, A.K., Kulikov, V.V. & Baranova, T.I. (1968) Biology of *Microphallus calidris* Belopolskaia et Ryjkov, 1963 (Trematoda: Microphallidae). pp. 125–283 *in* Skriabin, K.I. & Mamaev, Y.L. (*Eds*) *Helminths of the Pacific Ocean animals*. Moscow, Nauka Press.

Tsimbaljuk, A. K., Kulikov, V. V. & Baranova, T. I. (1978) Helminths of invertebrates from the intertidal zone of the Iturup island. pp. 69–126 *in* Kusakin, O.G. (*Ed.*) *Fauna and vegetation of the shelf of the Kurile islands*. Moscow, Nauka Press.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A. & Tingey, S.V. (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research* **18**, 6531–6535.

(Accepted 12 March 2004)