PINE WILT DISEASE:
a worldwide threat to forest ecosystems

International symposium

Program I Abstracts

10-14 July 2006 I Calouste Gulbenkian Foundation I Lisbon

www.nemalab.uevora.pt

OBJECTIVE: PROMOTE THE MEETING OF WORLD EXPERTS ON PWN, IN ORDER TO PRODUCE A CRITICAL ASSESSMENT OF THE SCIENTIFIC STATUS OF THIS FOREST PEST, AND TO DETERMINE FUTURE LINES OF RESEARCH AND INTERNATIONAL COOPERATION.

The pinewood nematode (PWN), Bursaphelenchus xylophilus, the causal agent of pine wilt disease (PWD), is a serious pest and pathogen of forest tree species, in particular among the genus Pinus. It was first reported from Japan in the beginning of the XXth century, where it became the major ecological catastrophe of pine forests, with losses reaching over 2 million m3/ year in the 1980s. It has since then spread to other Asian countries such as China, Taiwan and Korea, causing serious losses and economic damage. In 1999, the PWN was first detected in the European Union (EU), in Portugal, and immmediately prompted several government (national and EU) actions to assess the extent of the nematode's presence, and to contain B. xylophilus and its insect vector (Monochamus galloprovincialis) to an area with a 30km radius in the Setúbal Peninsula, 20 km south of Lisbon. International wood trade, with its political as well as economic ramifications, has been seriously jeopardized. The origin of the population of PWN found in Portugal remains elusive. Several hypotheses may be considered regarding pathway analysis, basically from two general origins: North America or the Far East (Japan or China). World trade of wood products such as timber, wooden crates, palettes, etc., plays an important role in the potential dissemination of the pinewood nematode. In fact, human activities involving the movement of wood products may be considered the single most important factor in spreading of the PWN. Despite the dedicated and concerted actions of government agencies, this disease continues to spread. Very recently (2006), in Portugal, forestry and phytosanitary authorities (DGRF and DGPC) have announced a new strategy for the control and ultimately the erradication of the nematode, under the coordination of the national program for the control of the pinewood nematode (PROLUNP). Research regarding the bioecology of the nematode and insect as well as new detection methods, e.g., involving real-time PCR, has progressed since 1999. International agreements (GATT, WTO) and sharing of scientific information is of paramount importance to effectively control the nematode and its vector, and thus protect our forest ecosystems and forest economy. The time has come for scientists and decision makers to meet.

SCIENTIFIC COMMITTEE:

WOLFGANG BURGERMEISTER I BBA I Germany
KAZUYOSHI FUTAI I University of Kyoto I Japan
MARC LINIT I University of Missouri I USA
MANUEL MOTA I University of Évora I Portugal
PAULO VIEIRA I University of Évora I Portugal
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SCIENTIFIC AND SOCIAL PROGRAM

10 MONDAY

9.00h-10.30h: REGISTRATION

10.30h-11.30h: OPENING SESSION

11.30h-12.00h: COFFEE BREAK

12.00h-12.30h: IUFRO WORKING GROUP WELCOME: meeting of working party 7.02.10

12.30h-14.00h: LUNCH

SESSION 1

PINE WILT DISEASE: GLOBAL ISSUES, TRADE AND ECONOMIC IMPACT

(Chairpersons: M. MOTA & J. WEBSTER)

14.00h-14.20h: ERADICATION PROGRAM FOR THE PINEWOOD NEMATODE IN PORTUGAL. J. RODRIGUES

14.20h-14.40h: A REVIEW OF HISTORICAL DATA ON ALIEN INVASIVE SPECIES IN EUROPE. T. OSZAKO

14.40h-15.00h: EU PROVISIONS AGAINST THE INTRODUCTION AND SPREAD OF *BURSAPHELENCHUS XYLOPHILUS*. H. ARIJS

15.00h-15.20h: THE PINEWOOD NEMATODE: IMPACT AND IMPLICATIONS FOR FOREST OWNERS. N. CALADO

15.20h-15.40h: COFFEE BREAK

15.40h-16.00h: INCURSION MANAGEMENT IN THE FACE OF MULTIPLE UNCERTAINTIES: A CASE STUDY OF AN UNIDENTIFIED NEMATODE ASSOCIATED WITH DYING PINES. M. HODDA

16.00h-16.20h: THE RISK OF PINEWOOD NEMATODE TO NEW ZEALAND. S. SATHYAPALA

16.20h-16.40h: ASSESSING THE SUSCEPTIBILITY OF PLANTATION PINUS SPP. IN QUEENSLAND TO PINE WILT NEMATODES. S. LAWSON I H. KOSAKA I T. AIKAWA I I. OKOCHI I G. NIKLES I R. AIGNER I J. MCDONALD

16.40h-17.00h: POTENTIAL THREAT AND PRESENT STATUS OF SURVEY OF PINE WOOD NEMATODE IN TURKEY. S. AKBULUT I P. VIEIRA I A. KETEN I M. MOTA

19.00h-20.30h: *PORTO DE HONRA*, at SOLAR DO VINHO DO PORTO

11 TUESDAY

SESSION 2

PWN: BIOLOGY AND MICROBIAL INTER-RELATIONSHIPS

(Chairpersons: F. FUTAI & M. MOTA)

9.00h-9.20h: EMBRYOLOGY OF *BURSAPHELENCHUS XYLOPHILUS*. K. HASEGAWA I K. FUTAI I J. MIWA

9.20h-9.40h: COMPARATIVE BIOLOGICAL STUDIES BETWEEN PORTUGUESE AND JAPANESE ISOLATES OF THE PINE WOOD NEMATODE *BURSAPHELENCHUS XYLOPHILUS*. M. MOTA | S. TAKEMOTO | K. HASEGAWA | Y. TAKEUCHI | N. HARA | K. FUTAI | J. MIWA

9.40h-10.00h: THE RELATIONSHIP BETWEEN PWNS AND FUNGI COHABITING IN PINE TREES INOCULATED WITH THE PWN. R. SRIWATI | S. TAKEMOTO | K. FUTAI

10.00h-10.20h: INFLUENCE OF FUNGI ON MULTIPLICATION AND DISTRIBUTION OF THE PINE WOOD NEMATODE. Y. WANG | T. YAMADA | D. SAKAUE | K. SUZUKI

10.20h-10.40h: COFFEE BREAK

10.40h-11.00h: MUTUALISTIC SYMBIOSIS BETWEEN *BURSAPHELENCHUS XYLOPHILUS* AND ITS ACCOMPANYING BACTERIA OF THE GENUS *PSEUDOMONAS*. B. ZHAO | F. LIN | X. LIU

11.00h-11.20h: PATHOGENICITY OF BACTERIA CARRIED BY THE PINE WOOD NEMATODE. Z. HAN | Y. HONG | Y. CAO | B. ZHAO

SESSION 3

PWN TAXONOMY AND DETECTION METHODS

(Chairpersons: A. RYSS & W. BURGERMEISTER)

11.30h-11.50h: TAXONOMIC DATABASES FOR *BURSAPHELENCHUS* AND OTHER APHELENCHOID NEMATODES. J. EISENBACK | P. VIEIRA | A. RYSS | M. MOTA

11.50h-12.10h: MORPHOLOGICAL DIAGNOSTICS OF THE *BURSAPHELENCHUS* SPP. WITH USE OF THE MODERN DATABASES TOOLS, AND THE SPECIES DESCRIPTION STANDARD FOR *BURSAPHELENCHUS*. A. RYSS | P. VIEIRA | M. MOTA | O. KULINICH

12.10h-12.30h: SPICULES SHAPE ANALYSES IN THE GENUS *BURSAPHELENCHUS* FUCHS, 1937: A NUMERICAL TAXONOMY APPROACH. P. SIMÕES | C. PENAS | M. BRAVO

12.30h-14.00h: LUNCH

14.00h-14.20h: THE ENLARGEMENT OF THE *XYLOPHILUS* GROUP IN THE GENUS *BURSAPHELENCHUS*. H. BRAASCH

14.20h-14.40h: SOME DATA ON THE DIAGNOSTICS ON THE JUVENILE STAGES OF THE SPECIES OF *BURSAPHELENCHUS* AND CLOSE GENERA. A. RYSS | A. CZERNECSKAJA

14.40h-15.00h: INVESTIGATIONS ON WOOD-INHABITING NEMATODES OF THE GENUS *BURSAPHELENCHUS* (NEMATODA: PARASITAPHELENCHIDAE) IN PINE FORESTS IN THE BRANDENBURG PROVINCE, GERMANY. U. SCHÖNFELD | H. BRAASCH | W. BURGERMEISTER | H. BRÖTHER

15.00h-15.20h: INTERSPECIFIC VARIATION IN ITS RDNA OF *BURSAPHELENCHUS* SPECIES (NEMATODA: PARASITAPHELENCHIDAE) OF DIFFERENT GROUPS. K. METGE | H. BRAASCH | J. GU | W. BURGERMEISTER

15.20h-15.40h: COFFEE BREAK

15.40h-16.00h: MOLECULAR CHARACTERIZATION OF ISOLATES OF THE *BURSAPHELENCHUS SEXDENTATI* GROUP USING ITS-RFLP AND RIBOSOMAL DNA SEQUENCES. C. LANGE | W. BURGERMEISTER | K. METGE | H. BRAASCH

16.00h-16.20h: ANALYSIS OF *BURSAPHELENCHUS XYLOPHILUS* (NEMATODA: PARASITAPHELENCHIDAE) PROVENANCES USING ISSR AND RAPD FINGERPRINTS. K. METGE | W. BURGERMEISTER

16.20h-16.40h: AN EFFECTIVE PCR-BASED DIAGNOSTIC METHOD FOR THE DETECTION OF *BURSAPHELENCHUS XYLOPHILUS* (NEMATODA: PARASITAPHELENCHIDAE) IN WOOD SAMPLES FROM LODGEPOLE PINE. I. LEAL

16.40h-17.00h: SATELLITE DNA AS A VERSATILE GENETIC MARKER FOR *BURSAPHELENCHUS XYLOPHILUS*. P. CASTAGNONE-SERENO I C. CASTAGNONE I C. FRANCOIS I P. ABAD

17.00h-17.20h: *PORT CHECK*: THE POTENTIAL OF REAL-TIME PCR AS A VALUABLE TOOL FOR THE DETECTION OF THE PINE WOOD NEMATODE, *BURSAPHELENCHUS XYLOPHILUS*. M. MOTA I P. VIEIRA I P. CASTAGNONE-SERENO

12 WEDNESDAY

SESSION 4

THE INSECT VECTORS: BIOLOGY AND ECOLOGY

(Chairpersons: L. LINIT & S. AKBULUT)

9.00h-9.20h: PINE WILT DISEASE IN PORTUGAL: UPDATED KNOWLEDGE ON THE VECTOR-INSECT *MONOCHAMUS GALLOPROVINCIALIS* (OLIV.). E. SOUSA I P. NAVES I L. BONIFÁCIO

9.20h-9.40h: POTENTIAL INSECT VECTORS OF *BURSAPHELENCHUS* SPP. (NEMATODA: PARASITAPHELENCHIDAE) IN SPANISH PINE FORESTS. A. GARCÍA-ÁLVAREZ I L. ROBERTSON I J. MANSILLA I A. BELLO I M. ARIAS

9.40h-10.00h: DISTRIBUTION, BIOLOGY AND POPULATION GENETIC STRUCTURE OF THE LONG HORN BEETLE *MONOCHAMUS GALLOPROVINCIALIS* (COLEOPTERA, CERAMBYCIDAE) IN FRANCE. F. KOUTROUMPA I D. ROUGON I B. VINCENT I V. ALTEMAYER I C. MARTIN I F. LIEUTIER I G. ROUX-MORABITO

10.00h-10.20h: BIOLOGY STUDIES RELEVANT TO THE VECTOR ROLE OF *MONOCHAMUS* SPECIES FOR PINE WOOD NEMATODE. C. TOMICZEK I U. HOYER-TOMICZEK

10.20h-10.40h: COFFEE BREAK

10.40h-11.00h: GENETIC STRUCTURE OF *MONOCHAMUS ALTERNATUS* IN JAPAN. E. SHODA-KAGAYA

11.00h-11.20h: OCCURRENCE OF *BURSAPHELENCHUS MUCRONATUS* IN FRANCE AND ITS ASSOCIATION WITH *MONOCHAMUS GALLOPROVINCIALIS*. B. VINCENT I V. ALTEMAYER I G. ROUX-MORABITO I F. LIEUTIER

SESSION 5

ECOLOGY AND MODELING

(Chairpersons: K. FUTAI & H. EVANS)

11.30h-11.50h: SPATIAL MODELLING OF *BURSAPHELENCHUS XYLOPHILUS* IN PORTUGAL. P. PEREIRA I P. ROQUE

11.50h-12.10h: FIELD DIAGNOSIS OF THE ASYMPTOMATIC CARRIER OF PINEWOOD NEMATODE. K. FUTAI I Y. TAKEUCHI

12.10h-12.30h: MODELLING PWN-INDUCED WILT EXPRESSION: A MECHANISTIC APPROACH. S. EVANS I H. EVANS I M. IKEGAMI

12.30h-14.00h: LUNCH

14.00h-18.30h: BUS TOUR TO SINTRA - CASCAIS

20.00h- : SOCIAL DINNER, at ADEGA DO MACHADO

13 THURSDAY

SESSION 6

THE TREE: PHYSIOLOGY, RESISTANCE AND HISTOPATHOLOGY AS A RESULT OF PWD

(Chairpersons: K. KURODA & D. BERGDAHL)

10.00h-10.20h: IMPACT OF THE PINE WOOD NEMATODE ON A MARITIME PINE FOREST IN TROÍA, PORTUGAL. E. SOUSA I L. BONIFÁCIO I P. NAVES I C. FERREIRA I M. CARAPUÇO

10.20h-10.40h: PINEWOOD NEMATODE PERSISTENCE IN ASYMPTOMATIC SCOTS PINES. D. BERGDAHL I S. HALIK

10.40h-11.00h: INOCULATION OF PINE TREES WITH AVIRULENT PINE WOOD NEMATODE UNDER EXPERIMENTAL CONDITIONS: RISK-BENEFIT ANALYSIS. H. KOSAKA

11.00h-11.20h: COFFEE BREAK

11.20h-11.40h: RAPIDITY OF DISEASE DEVELOPMENT SEEMS TO RESULT IN HIGH MORTALITY – INSIGHT FROM AN INOCULATION TEST USING HYBRIDIZED POPULATIONS BETWEEN A VIRULENT AND AN AVIRULENT ISOLATES OF *BURSAPHELENCHUS XYLOPHILUS*. S. TAKEMOTO I K. FUTAI

11.40h-12.00h: DISTRIBUTION, MIGRATION BEHAVIOR AND POPULATION DYNAMICS OF *BURSAPHELENCHUS XYLOPHILUS* (STEINER & BUHRER, 1934) NICKLE, 1970 (NEMATODA: PARASITAPHELENCHIDAE) IN YOUNG *P. SYLVESTRIS* TREES DURING EARLY WILT AT CONTROLLED OPTIMUM TEMPERATURE. M. DAUB I T. SCHRÖDER I R. SIKORA

12.00h-12.20h: PRELIMINARY EXPERIMENT ON THE PATHOGENICITY OF *BURSAPHELENCHUS MUCRONATUS* ON THREE PINE SPECIES UNDER GREENHOUSE CONDITIONS. I. BAYSAL I S. AKBULUT I B. YÜKSEL I M. SERIN I M. ERDEM

12.30h-14.00h: LUNCH

14.00h-14.20h: *BURSAPHELENCHUS* SPECIES IN DECLINING SWISS PINE FORESTS AND EFFECT OF WATERING TREATMENTS ON THEIR VIRULENCE. J. POLOMSKI I D. RIGLING

14.20h-14.40h: HISTOLOGICAL OBSERVATIONS OF *BURSAPHELENCHUS XYLOPHILUS* IN SYMPTOMATIC TISSUES OF PINE WOOD. Y. MAMIYA

14.40h-15.00h: DEFENSE SYSTEMS OF *PINUS DENSIFLORA* CULTIVARS SELECTED AS RESISTANT TO PINE WILT DISEASE. K. KURODA

15.00h-15.20h: COFFEE BREAK

SESSION 7

PWN AND INSECT VECTOR CONTROL METHODS

(Chairpersons: K. NAKAMURA & K. IKEDA)

15.20h-15.40h: NEMATOCIDAL ACTIVITY OF SKF-13 ISOLATED FROM *CINNAMOMUM CASSIA* PRESL AGAINST THE PINEWOOD NEMATODE (*BURSAPHELENCHUS XYLOPHILUS*). Z. FANG I I. KWON I C. SUNG

15.40h-16.00h: NOVEL AZAPHILONES, EPIPOLYSULFANYLDIOXOPIPERAZINES, RESORCYLIC MACROLIDES AND SHINGOLIPIDS WITH NEMATICIDAL ACTIVITY FROM CULTURES OF FRESH WATER-DERIVED FUNGI. J. DONG I K. ZHANG

16.00h-16.20h: ANTI-NEMATODAL ACTIVITIES OF THE PROTOBERBERINE DERIVATIVES AGAINST THE PINEWOOD NEMATODE, *BURSAPHELENCHUS XYLOPHILUS.* P. JEONG I W. OH I J. KIM I H. JOO I Y. MOON I S. SHIN I Y. PAIK

16.20h-16.40h: EFFECT OF LOW INOCULUM DOSES OF PINE WOOD NEMATODE ON OLEORESIN EXUDATION. L. WANG I C. PIAO I Y. LI

16.40h-17.00h: MECHANISMS INVOLVED IN NEMATODE INFECTION BY NEMATOPHAGOUS FUNGI. K. ZHANG I X. HUANG I J. YANG I J. DONG

SPECIAL SESSION

17.00h-18.00h: PHRAME (*Development of improved pest risk analysis techniques for quarantine pests, using pinewood nematode, Bursaphelenchus xylophilus, in Portugal as a model system*): GENERAL PRESENTATION AND CONCLUSION OF THE PROJECT

14 FRIDAY

SESSION 7 (CONT.)

PWN AND INSECT VECTOR CONTROL METHODS

9.00h-9.20h: USEFULNESS OF THE LIVE-CAPTURE ATTRACTION TRAP FOR MONITORING *MONOCHAMUS ALTERNATUS* ADULTS AND THEIR NEMATODE LOAD. K. NAKAMURA-MATORI

9.20h-9.40h: EFFECT OF AERIAL SPRAYING INSECTICIDE AS THE CONTROL MEASURE OF PINE WILT DISEASE. S. UGAWA I K. FUKUDA

9.40h-10.00h: PREVENTION OF PINE WILT DISEASE BY CONTROLLING THE VECTOR INSECT USING MICROBIAL AGENT. M. SHIMAZU I N. MAEHARA I H. SATO

10.00h-10.20h: STUDIES ON *SCLERODERMA GUANI* XIAO ET WU TO CONTROL THE PINEWOOD NEMATODE. X. FUYUAN I X. KEQIN I X. CHUNXIA I Z. PEI

10.20h-10.40h: COFFEE BREAK

10.40h-11.00h: THE EFFECT OF ROOTED CUTTING PROPAGATION OF NON-DAMAGED JAPANESE BLACK PINE THROUGH THE INOCULATION TEST WITH THE PINEWOOD NEMATODE ON NEMATODE-RESISTANT PLANT PRODUCTION. Y. MORI I F. MIYAHARA I S. GOTO

11.00h-11.20h: CONTROL PROGRAM OF PINE WILT DISEASE FOR LANDSCAPE CONSERVATION – IN THE CASE OF "AMANOHASIDATE" IN KYOTO, ONE OF THE MOST FAMOUS AND BEAUTIFUL SCENIC SPOTS IN JAPAN. T. IKEDA

11.20h-11.40h: HOW TO SAFELY USE PINE WOOD INFECTED BY PINEWOOD NEMATODE IN CHINA. M. LIN I P. XU I H. LIN

11.40h-12.30h: CONCLUSIONS: A) PER SESSION I B) GENERAL CONCLUSIONS

12.30h-14.00h: LUNCH

14.00h-15.00h: IUFRO BUSINESS MEETING

15.00h-16.00h: CLOSING SESSION

POSTERS SESSION[1]

INVESTIGATION OF *PINUS SYLVESTRIS* BAIT LOGS FOR HATCHING BEETLES AND WOOD-INHABITING NEMATODES OF THE GENUS *BURSAPHELENCHUS* (NEMATODA: PARASITAPHELENCHIDAE). U. SCHÖNFELD I H. BRÖTHER

MICROBIAL CONTROL OF *BURSAPHELENCHUS XYLOPHILUS* BY FUNGI. N. MAEHARA I K. FUTAI

POTENTIAL MAP FOR PINE WILT DISEASE EPIDEMIC USING A SIMPLE INDEX OF THE THERMAL CONDITION. K. NAKAMURA-MATORI

CRYOPRESERVATION OF NEMATODES: AN OPPORTUNITY FOR THE CONSTITUTION OF AN EUROPEAN GENETIC BANK. T. IRDANI I B. CARLETTI I L. AMBROGIONI I P. ROVERSI

OFFICIAL SURVEY FOR *BURSAPHELENCHUS XYLOPHILUS* CARRIED OUT ON THE TERRITORY OF THE REPUBLIC OF POLAND. W. KARNKOWSKI

COMPARISON OF SOME BIOLOGICAL CHARACTERISTICS OF *M. CAROLINENSIS* AND *M. GALLOPROVINCIALIS* (COLEOPTERA: CERAMBYCIDAE). S. AKBULUT I W. STAMPS I B. YÜKSEL I İ. BAYSAL I M. LINIT

TWENTY-FIVE YEARS OF PINE WILT RESEARCH AT THE UNIVERSITY OF MISSOURI. W. STAMPS

FIRST OCCURRENCE OF *BURSAPHELENCHUS VALLESIANUS* IN THE CZECH REPUBLIC. V. GAAR I M. ZOUHAR I O. DOUDA I M. MAREK I E. NOVAKOVA I P. RYSANEK

MODELLING PWN-INDUCED WILT EXPRESSION: VALIDATING THE MODEL THROUGH FIELD INOCULATIONS. M. IKEGAMI I S. EVANS I H. EVANS

MORPHOLOGICAL, MOLECULAR AND SEROLOGICAL CHARACTERIZATION OF *BURSAPHELENCHUS XYLOPHILUS* ISOLATES. L. FONSECA I R. CURTIS I K. HALSEY I M. SANTOS I I. M. ABRANTES I M. SANTOS

DETECTION OF LONGHORN BEETLE ACTIVITY IN LIVING TREES BY BIOACOUSTICS. M. BRANDSTETTER I C. TOMICZEK

RESEARCH TOWARDS INTEGRATED MANAGEMENT OF PINE WILT DISEASE IN KOREA. Y. CHUNG I K. CHOI I S. KOH I H. HAN I C. JUNG I S. SHIN I J. SHIN

COMPARISON OF DEVELOPMENTAL PERIODS OF CHINESE INTRODUCED AND KOREAN DOMESTIC PARASITOIDS, *SCLERODERMUS HARMANDI* AT CONSTANT TEMPERATURE. K. CHOI I W. CHOI I J. HONG I S. KOH I Y. CHUNG I I. PARK I H. HAN I C. JUNG I S. SHIN I J. SHIN

ACTIVITIES DEVELOPED BY UNAC UNDER THE NATIONAL PROGRAMME OF PINEWOOD NEMATODE CONTROL. C. VILA-VERDE

SPECIAL SESSION POSTERS (non-participants posters)

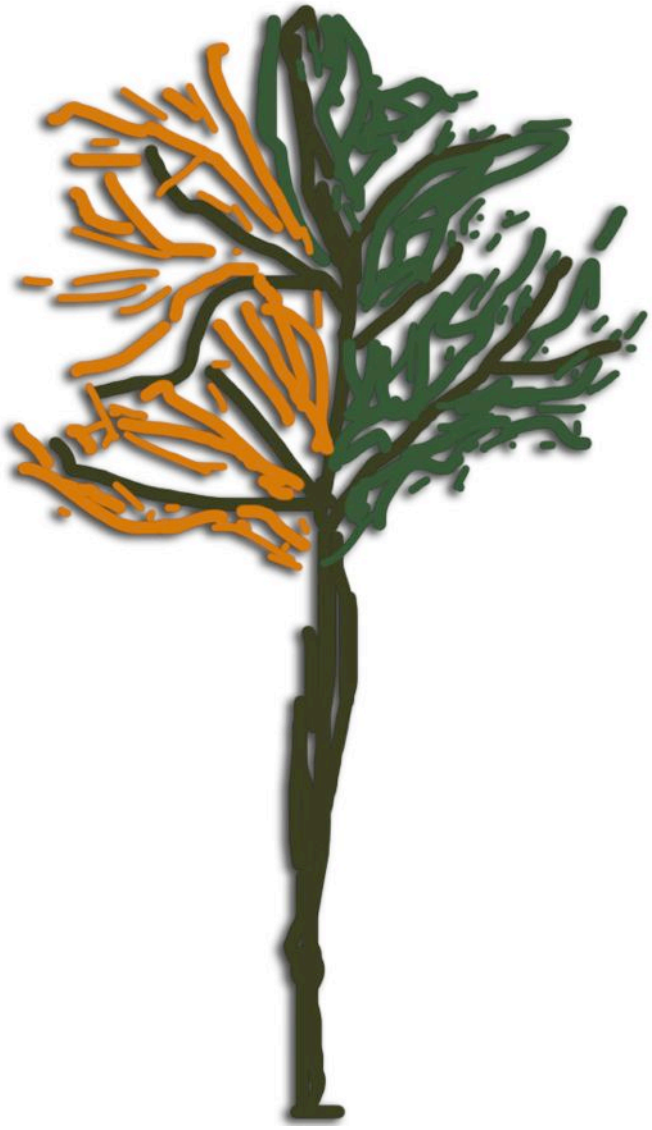
PATHOGENICITY OF *BURSAPHELENCHUS* SPP. NEMATODES TO CONIFERS IN RUSSIA. O. KULINICH

MONOCHAMUS SPECIES IN EUROPE: DISTRIBUTION AND PHYLOGENY. V. FRANCARDI I M. CESARI I O. MARESCALCHI I F. PENNACCHIO I B. MANTOVANI

PROSPECTS FOR MICROBIOLOGICAL CONTROL OF *MONOCHAMUS GALLOPROVINCIALIS* (OLIVIER). P. RUMINE I V. FRANCARDI

NEMATODES ISOLATED FROM BARK- AND WOOD-BORING BEETLES IN PINE FORESTS IN ITALY. V. FRANCARDI I L. AMBROGIONI I F. PENNACHIO I P. ROVERSI

[1] Posters will be on display in the lobby, for the duration of this symposium. There will be no specific timing for poster session.



SESSION 1

PINE WILT DISEASE: GLOBAL ISSUES, TRADE AND ECONOMIC IMPACT

(Chairpersons: M. MOTA & J. WEBSTER)

ERADICATION PROGRAM FOR THE PINEWOOD NEMATODE IN PORTUGAL

J. RODRIGUES

Direcção-Geral dos Recursos Florestais | Direcção de Serviços de Desenvolvimento Florestal
Divisão de Protecção e Conservação Florestal
Avª João Crisóstomo | 26-28 | 6º andar | 1069-040 Lisboa | Portugal
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Pinewood Nematode (PWN) was found in Portugal in May 1999 in pine forests in the Setúbal region. Given that the maritime pine (*Pinus pinaster*) is the most common tree in Portuguese forests, the Portuguese government and the economic agents of the forest sector were thus confronted by a situation that, in all likelihood, was extremely serious. Maximum priority was given to the rapid resolution of the problem, involving government entities and agents in the forestry sector who had a territorial and economic interest in this matter. Since PWN detection, actions have been taken to eradicate it as part of the National Eradication Programme for Pinewood Nematode (PROLUNP). This program became operational in November 1999 with a dedicated structure responsible for providing the means and developing the tools to control and eradicate the disease. The area affected by the pinewood nematode covers 510,000 ha on continental Portugal, surrounded by a buffer zone of approximately 500,000 ha, for safety purposes. The sum of both (1,010,000 ha) constitutes the demarcated area, which is subjected to periodic survey, eradication and insect vector control actions and where all forestry activities involving conifers are subjected to intensive control. It also occurs an annual survey in the free zone to monitor the coniferous forests in order to screen the presence of PWN. At the European level, the situation has been discussed within the Standing Committee on Plant Health. To date, Portugal has received eight missions from Food and Veterinary Office with the aim to assess the eradication programme, the state of the disease in the field and also give guidance on the application of EU deliberations in this matter. It is recognized by the Commission, the good work that Portugal has come to execute, with the goal to reach the complete eradication of the PWN in the affected zone. However, the most recent survey/eradication campaign of decline symptoms trees in the demarcated area shows a considerable increase. This increase had a larger expression in the affected zone. Considering that several samples taken from the buffer zone were tested positive for PWN in the last survey/eradication campaign, affected zone limits and demarcated area limits had to be redefined, and new actions will have to be taken. It is to relate that the area of the affected zone has varied across the years and that these trees necessarily do not correspond the trees infested with PWN, being proven by the carried through research works, that the causal agents of decline of the conifers, in the region, are sufficiently diverse. Of pines identified as showing decline symptoms in the restricted area, over 96% were found to be maritime pines. The remainder are umbrella pines. To date, all analysis of umbrella pine samples were negative for PWN presence.

A REVIEW OF HISTORICAL DATA ON ALIEN INVASIVE SPECIES IN EUROPE

T. OSZAKO

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Consideration of historical data emphasises the importance of alien invasive species causing serious problems in agriculture, horticulture and forestry. The spread of diseases in Europe, such as Dutch Elm disease (*Ophiostoma ulmi*), oak mildew (*Erysiphe alphitoides*) as early as in 1909, Scleroderris canker (*Gremmeniella abietina*), and *Sphaeropsis sapinea* has caused considerable concern among foresters. More recently *Cryphonectria parasitica* and needle cast diseases *Mycosphaerella* have threatened nursery stocks, arboreta and parks in Europe. There is a permanent threat that Sudden Oak Death (*Phytophthora ramorum*) and oak wilt disease (*Ceratocystis fagacearum*) will be accidentally imported into Europe from North America. *Phytophthora cinnamomi* appears to survive even in north-eastern Europe, where climate is unfavourable to this species. Alder decline caused by *P. alni*, and beech decline caused by *P. cambivora* and *P. citricola* are becoming more severe in many European countries. The horse chestnut leaf miner (*Cameraria orhidella*) affecting horse chestnut trees (*Aesculus hoppocastanum*) has caught the attention of citizens in many European cities. Nematodes such as the pinewood nematode *Bursaphelenchus xylophilus*, currently only found in Portugal in Europe, has seriously affected wood trade in the region. This paper focuses on the threat posed by invasive pests and pathogens to forests in Europe, supported by many examples in a historical perspective.

EU PROVISIONS AGAINST THE INTRODUCTION AND SPREAD OF *BURSAPHELENCHUS XYLOPHILUS*

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Bursaphelenchus xylophilus (Steiner et Buhrer) Nickle *et al*., the pinewood nematode –PWN, is listed in EU's basic plant health legislation Council Directive 2000/29/EC as a harmful organism. Its introduction into, and spread within all EU Member States has to be banned if it is present on plants of *Abies*, *Cedrus*, *Larix*, *Picea*, *Pinus*, *Pseudotsuga* and *Tsuga* and wood of conifers, originating in non-European countries. The entry of susceptible plant species from non-European countries is prohibited. Consignments of coniferous wood and wood products like chips and bark need to be accompanied by a phytosanitary certificate at import into the EU, confirming that the wood meets the relevant import requirements, like heat treatment or fumigation. Since 1 March 2005, also wood packaging material entering the EU has to fulfil the heat treatment or fumigation requirements of the IPPC International Standard for Phytosanitary Measures N° 15 and be properly marked. After the finding of a PWN outbreak on European territory in 1999, EU emergency measures against the dissemination of PWN

from the infested area in Portugal were taken in 2000. They have been revised several times and are currently covered by Commission Decision 2006/133/EC. The affected zone and a buffer zone of 20 km width have been demarcated in Portugal. Strict requirements for the movement of all susceptible material outside and within this demarcated area must prevent a further dispersion. Moreover, a mid-term action plan to further reduce the existing outbreak has been established by the Portuguese authorities. The correct implementation of the legal requirements in Portugal is checked yearly by the EC Food and Veterinary Office. Since 2000, the situation of PWN in the EU is closely followed by intensive annual surveys in this demarcated zone as well as on the whole EU territory. There have been no positive findings outside the demarcated area in Portugal and therefore it is concluded that, with the exception of the demarcated area in Portugal, PWN is not present on EU territory.

THE PINEWOOD NEMATODE: IMPACT AND IMPLICATIONS FOR FOREST OWNERS

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The Portuguese forest sector has a significant economic importance, has we can see by our contribution to gross domestic product (3,2%), to industrial gross domestic product (12%), to foreign trade (10%) and to national employment (5%). One of the most important productions, and industrial activity, is supported by maritime pine. The maritime pine (*Pinus pinaster*, Aiton) has several functions for the Portuguese forest. The production of wood and resin, apart from the hunting activity and the protection against sand dunes, are only some examples. In this context, the pinewood nematode represents a serious threat to the forest production and, by consequence, to the economy, especially because it can kill an adult tree in a relatively short period of time, after the infection. Since 1999, when *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, as the causal agent of pine wilt disease, was first reported in Portugal, official authorities have been conducting extensive surveys within a National Programme of Pinewood Nematode Control (PROLUNP). In 2002, UNAC – Union of the Mediterranean Forest, was first involved in these surveys. Since then, and by the establishment of several protocols, UNAC has been collaborating with PROLUNP in the annual surveys. Our interest is based on the fact that our associates are forest owners' organizations, totalising about 700.000 ha of rural land (agro-forestry), whose landowners have been severely affected by the pinewood nematode. This disease led to several constraints and restrictions to forest owners with properties included in the Restriction Zone, and with the following consequences: a) Distortion of the price system, leading to an increased trend of descending prices; b) Increase in production costs; c) Concentration of offer in specific periods of the year; d) Depreciation of wood material; e) Difficulties in wood material access into the market. These situations have caused a significant decrease in the profitability of maritime pine exploitation, with all its negative consequences, including the lack of capacity for the implementation of phytosanitary practices, although extremely necessary, due to the increased damages that the pinewood nematode causes.

THE RISK OF PINEWOOD NEMATODE (*BURSAPHELENCHUS XYLOPHILUS*) TO NEW ZEALAND

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MAF Biosecurity New Zealand has recently completed an assessment of the risk to the New Zealand environment and economy posed by the pinewood nematode, *Bursaphelenchus xylophilus* (PWN), the casual agent of pine wilt disease. The pest risk analysis assesses the risk of entry, establishment and spread in New Zealand of *B. xylophilus*, and the potential economic and environmental consequences should it become established. The risk analysis identified that New Zealand has suitable temperature and annual precipitation requirements for the establishment of PWN. The host species, mainly radiata pine and Douglas fir are distributed throughout New Zealand. The establishment of PWN in New Zealand would mainly be dependent on the availability of a suitable insect vector. Although no species of *Monochamus* beetles are currently established in New Zealand, it is not known to what extent local native and exotic insects could act as a vector for PWN. The most possible candidates are Cerambycids such as *Arhopalus ferus* (pine longhorn beetle) and *Hexatricha pulverulenta* (squeaking longhorn) which are both abundant in New Zealand. The most likely method of establishment of PWN in New Zealand is via *Monochamus* spp. carried by untreated coniferous wood packaging material. However the likelihood of this occurring is considered low. Also it has been concluded that if PWN becomes established in New Zealand it is unlikely to show extensive pine wilt symptoms under current New Zealand climatic conditions.

ASSESSING THE SUSCEPTIBILITY OF PLANTATION *PINUS* SPP. IN QUEENSLAND TO PINE WILT NEMATODES

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During 2001 and 2005, incursions of *Monochamus alternatus* (Coleoptera: Cerambycidae) were detected in Brisbane in pallets imported from China. This beetle is the primary vector of pine wood nematode (*Bursaphelenchus xylophilus*) a devastating pest of *Pinus* spp. in northeast Asia (Japan, China and Taiwan). The susceptibility of the major pine species grown in Queensland (predominantly *Pinus caribaea* var. *hondurensis* and its hybrids with *Pinus elliottii* var. *elliottii*) to this nematode are relatively unknown. To test relative susceptibilities of both the parent species and the six best performing hybrids, one-year old seedlings were sent to Japan in 2002 and 2003 and planted out in a nursery at the Forestry and Forest Products Research Institute, Tsukuba. These were later inoculated

with distilled water, *B. xylophilus* or *B. mucronatus,* as dispersal stage adults. *B. mucronatus* is endemic to Eurasia and is not pathological to *Pinus* species there, but its pathogenicity against southern USA and Caribbean pines is unknown. Additionally, stems of all eight pines were presented to captive adult *M. alternatus* beetles to test for maturation feeding acceptability. The results of this research will be commented on in relation to risk management and proactive breeding programs for exotic pest resistance in Queensland.

POTENTIAL THREAT AND PRESENT STATUS OF SURVEY OF PINE WOOD NEMATODE IN TURKEY

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Turkey is located between Europe and Asia, an important geographic transitional area. The total forest area covers about 27.22 % (21 million ha) of the country's total land but only half of that is considered to be productive. Conifer species are dominant covering over 11 million ha of total forest area without adding mixed stands with broadleaf trees. Even Turkey, with a quite large forest area, annual wood products from the General Forest Directorates, cannot compromise wood demand. There is a big deficit between wood production and demand in Turkey. To close this gap, Turkey has to import wood products from wood exporting countries. The importation of wood from different countries increases the possibilities of inadvertent introduction of exotic pests into Turkey. One of the most recent invasive species detected in conifer forests of Europe is the pinewood nematode. Because of this threat, a survey on the genus of *Bursaphelenchus* has been undertaken in different regions of Turkey since 2003. Wood samples were collected from declining conifer species in selected sites located in the North, South and West parts of Turkey. Approximately, 1200 samples have been collected from declining trees so far and nearly 350 samples were analyzed for the presence of *Bursaphelenchus* species. Three *Bursaphelenchus* species were identified.

SESSION 2

PWN: BIOLOGY AND MICROBIAL INTER-RELATIONSHIPS

(Chairpersons: K. FUTAI & M. MOTA)

EMBRYOLOGY OF *BURSAPHELENCHUS XYLOPHILUS*

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The aim of the present research was to study the embryology of the pinewood nematode (PWN) *Bursaphelenchus xylophilus* and establish molecular genetics methods that may qualify the PWN as a model organism for plant parasitic nematodes. We found that the anterior-posterior axis determination of *B. xylophilus* is opposite to that of the free-living soil nematode *Caenorhabditis elegans*, whereby the presumptive region of sperm entry into the *B. xylophilus* oocyte becomes the future anterior portion of the embryo. Otherwise, the behavior of male and female pronuclei and the cell cytoskeleton (microtubules and actin) from fertilization to two-cell stage and the cell lineage tree from the 1-cell to 46-cell stages were similar in these two nematodes. From the shape of the segregating chromosomes at the 1-cell anaphase stage, the mitotic chromosomes of *B. xylophilus* appeared to be holocentric, or at least polycentric, and it should be possible to establish a DNA transformation technique of introduction into the syncytial gonad. To understand the evolution of nematode developmental systems, we cloned the following full-length genes: *Bx-par-1* (Ser/Thr kinase), *Bx-mex-3* (RNA binding protein), *Bx-tbb-1* (beta tubulin), and *Bx-daf-21/hsp90* (heat shock protein) from *B. xylophilus* cDNA and genomic DNA. They are all the homologues of *C. elegans* genes, whose expression is necessary for early embryogenesis. We are now working to establish methods for functional analysis of *B. xylophilus* genes to understand the evolution of developmental systems by studying similarities and differences between these two nematodes.

COMPARATIVE BIOLOGICAL STUDIES BETWEEN PORTUGUESE AND JAPANESE ISOLATES OF THE PINE WOOD NEMATODE *BURSAPHELENCHUS XYLOPHILUS*

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During the summer of 2005, a series of biological studies were conducted in Japan to compare 2 isolates of the pine wood nematode (PWN) *Bursaphelenchus xylophilus* from Portugal (HF and T) and 2

isolates (C14-5 and S-10) from Japan. These studies focused on cytogenetics (chromosome structure and behavior), embryogenesis, pathogenicity (nematode propagation inside the tree, tree mortality and symptom development), sexual compatibility (mating experiments), and molecular biology (rDNA sequencing of the ITS region). Japanese black pine, *Pinus thunbergii*, was used in the pathogenicity experiments. Detailed methodologies of all observations are presented. Regarding cytogenetics and embryogenesis, the major comparative observation is that possible triploidy (3n=18) is present in one of the Portuguese isolates. Mating between Japanese and Portuguese isolates, overall, produced more progeny than mating between isolates from each country. Regarding pathogenicity, development of visible symptoms appearing in the host pine (*P. thunbergii*) was clearly faster in the Portuguese isolates than in the Japanese ones, indicating a potential threat to Japanese black pine. Sequences of the ITS region of both Portuguese isolates were identical to that of the virulent Japanese isolate S10 but differed slightly from that of another virulent isolate T4. The two Portuguese isolates may reflect some natural variability regarding pathogenicity, or they may represent 2 separate introductions into the country. The latter may be difficult to explain, however, considering the very short time span (5 to 10 years) from their probable entry.

THE RELATIONSHIP BETWEEN PWNS AND FUNGI COHABITING IN PINE TREES INOCULATED WITH THE PWN

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The relationship between PWNs and fungi cohabiting with the nematodes in 15-year-old Japanese black pine, *Pinus thunbergii*, were examined bimonthly over a year after inoculation with PWNs. The population of PWNs in the trees was high in August, but decreased slightly in December then increased again in February. From wood samples of the pine trees examined, 18 species of fungi have been isolated. Among the 18 fungi detected, *Phialophora repens*, *Sphaeropsis sapinea*, *Pestalotiopsis* sp., *Rhizoctonia* sp. were detected most frequently in every season. All of these dominant fungi had positive effects on increase in nematode population, though the population of PWNs on *Rhizoctonia* sp. was less than those on the other three dominant fungi. Under laboratory conditions, 19 species of fungi cultured on potato dextrose agar (PDA) served for PWNs as food, and the PWNs' population built up on each fungus was compared at 20ºC. PWNs dramatically increased on *Pestalotiopsis* sp. 1, *Pestalotiopsis* sp. 2, *Sphaeropsis sapinea*, *Phialophora repens*, and *Botrytis cinerea* (control), from 10 to 15 days after inoculation. From the view points of food quality and their cohabitating ability we conclude that the species of fungi that are dominant in pine trees, except *Rhizoctonia* sp., have compatible relationship with PWNs, while *Rhizoctonia* sp. and *Penicillium* sp. showned neutral, and *Trichoderma* sp. incompatible relationship to PWNs.

INFLUENCE OF FUNGI ON MULTIPLICATION AND DISTRIBUTION OF THE PINEWOOD NEMATODE

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The influence of certain fungi on the multiplication and distribution of the pine wood nematode (PWN), *Bursaphelenchus xylophilus*, was investigated in *Pinus thunbergii* cuttings. Axenized nematodes and/or one of two fungi isolated from healthy and PWN-killed *P. thunbergii* were inoculated together into autoclaved cuttings. A close relationship between the existence and distribution of fungal hyphae, and the multiplication and distribution of PWN was observed. The PWN did not multiply when only axenized nematodes were inoculated in the absence of fungi. When fungi were present, the PWN population size increased markedly. The number of nematodes was higher at sites where fungal hyphae were distributed. Restriction of a large portion of the nematode population near the inoculation site during the early stage of disease development may be closely related to the restricted distribution of fungal hyphae.

MUTUALISTIC SYMBIOSIS BETWEEN *BURSAPHELENCHUS XYLOPHILUS* AND ITS ACCOMPANYING BACTERIA OF THE GENUS *PSEUDOMONAS*

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Interactions between the pine wood nematode (PWN), *Bursaphelenchus xylophilus,* and its accompanying bacteria of the genus *Pseudomonas* were examined by cultivating axenic PWN and bacterial strains with callus of *Pinus thunbergii*. Ten bacterial strains (*Pseudomonas fluorescens*, *P. putida, P. cepacia* and *Pseudomonas spp.)* of 29 tested ones significantly facilitated the reproduction of PWN. The rest of the bacteria (19 strains of 10 species) inhibited the reproduction of PWN completely. The growth of 18 bacterial strains of the 29 tested ones, including the 10 strains promoting PWN reproduction, was significantly increased by the presence of this PWN. It indicated a mutualistic relationship between PWN and the 10 bacterial strains of the genus *Pseudomonas.* The bacterial mutualistic symbionts seem to have coevolved with PWN rather than being accidentally associated. The finding provides further evidence for our hypothesis that pine wilt disease is a complex, which is induced by both PWN and associated toxin-producing bacteria.

PATHOGENICITY OF BACTERIA CARRIED BY THE PINE WOOD NEMATODE

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The bacteria *Pseudomonas fluorescens* has been frequently isolated from the xylem of wilted black pine (*Pinus thunbergii*) trees and from the surface of pine wood nematodes (*Bursaphelenchus xylophilus*). To determine pathogenicity, callus and aseptic black pine seedlings were inoculated with only bacteria, a mixture of bacteria and the aseptic nematode *Bursaphelenchus xylophilus*, a mixture of bacteria and the aseptic nematode *B. mucronatus*, *etc*. The results showed that inoculation only with either aseptic *B. xylophilus* or *B. mucronatus* did not lead to browning of the callus and wilting of aseptic black pine seedlings, but those inoculated with a mixture of the aseptic nematodes and the bacterium showed some wilting or browning symptoms. Inoculation with a mixture of *B. mucronatus* and bacteria also caused serious wilt of pine seedlings and browning of the callus. It indicated that seedling symptoms under artificial inoculation in lab conditions were in close correlation with the bacteria carried by pine wood nematodes and poorly correlated with the species of nematode. Ten-year old black pines were inoculated with aseptic pine wood nematodes. The nematode and the bacterium were subsequently recovered from black pine. The results indicated that 5 among 8 individual pines were healthy. The aseptic pine wood nematode was isolated from the healthy black pine. This result showed that the aseptic pine wood nematode could not lead pine to wilt though the nematode could parasitize the pine xylem. In addition, bacteria were cultured in liquid media through shaking. The filtered liquid was directly used in the treatment of the callus and the ability of each kind of bacterium to produce toxins was determined. Wilt-related toxins were present in the bacterial culture fluid. Therefore, it's suggested that the disease was caused by co-infection of pine wood nematode and the bacteria.

SESSION 3

PWN TAXONOMY AND DETECTION METHODS

(Chairpersons: A. RYSS & W. BURGERMEISTER)

TAXONOMIC DATABASES FOR *BURSAPHELENCHUS* AND OTHER APHELENCHOID NEMATODES

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Undoubtedly, the most important resources for nematode taxonomy are the original species descriptions. Unfortunately, nematode descriptions have been published since the middle of the 18th century in a variety of forms including many relatively obscure journals and proceedings and sometimes in lengthy special publications. As a result, the task of collecting all of the descriptions of a single genus is daunting and has been repeated by numerous nematode taxonomists around the world. Furthermore, many of the papers were printed on acid containing paper and are rapidly deteriorating by yellowing and becoming brittle. The purpose of the present project is to collect all of the original species descriptions of the Aphelenchs including the genus *Bursaphelenchus*. The original papers are digitized with a flatbed scanner and converted to the PDF documents. The image is converted into text with optical character recognition software that is built into Adobe® Acrobat; thus each document becomes a fully searchable text that includes all of the photographs and drawings that are the same quality as the original. Theses collections are put together into monographs that are published as individual CD ROMs.

MORPHOLOGICAL DIAGNOSTICS OF THE *BURSAPHELENCHUS* SPP. WITH THE USE OF THE MODERN DATABASES TOOLS, AND THE SPECIES DESCRIPTION STANDARD FOR *BURSAPHELENCHUS*

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E-key is developed in the Pickey8 for WIN identification system (Lobanov & Dianov, ZIN RAS, 2003). It includes 78 species (complete list of the valid species of the world fauna) and 32 characters, selected from diagnoses, relationships and text keys of the most experienced genus taxonomists. Uniform classification of characters and character states was created with detailed illustrations, to avoid misunderstanding and use the same term for different structures. Quantitative characters with overlapping (between species) character state ranges were transformed into states with the discontinuous ranges (measurements and ratios). Some qualitative characters were substituted by ratios (e.g. to characterize the spicule shape). The system has the built-in algorithm ranging characters depending on their diagnostic values at each step of identification, but at any step the user may choose any available character as a filter to current set of species, thus the e-key is really polytomous and

image-operating. Matrix of species and the character states (structural part of the e-key database) has the self-sufficient scientific importance and may be easily transformed (totally or selectively for some characters or some species) using statistical packages into the dendrograms of UPGMA general phenetic similarities. The dendrogram itself may be also used as the identification module if the new population is correctly identified to genus level, the morphological data of the population are input into the database and then the database matrix is converted into the dendrogram of the similarities. The e-key may be used as the analytical tool to study the taxonomy as well as for splitting of genus to the diagnostic the species groups. The verification of the dendrogram using the information on the species links with insect vectors and their associated plants, gave an opportunity to recognize the 6 clusters (*xylophilus*, *hunti*, *aberrans*, *eidmanni*, *borealis*, *piniperdae*). Some of this groups seem to be the natural (phylogenetically based) species groups. The hypothesis about the origin and the first stages of the genus evolution was proposed. On the basis of the uniform classification of selected and illustrated species characters, the modern standard for the *Bursaphelenchus* species description has been proposed, including all spectra of the alternative character states diversity known until now.

SPICULE SHAPE ANALYSES IN THE GENUS *BURSAPHELENCHUS* FUCHS, 1937: A NUMERICAL TAXONOMY APPROACH

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The male spicule is recognized as the most relevant morphological character for species identification within the genus *Bursaphelenchus*. Twelve of the 32 diagnostic morphological characters used in the Ryss *et al.* (2005) tabular key to *Bursaphelenchus* species are associated with the male spicule and these characters are used to separate all the known valid species into six different species groups. The main objective of this study was to find out if a quantification of the outline spicule shape is sufficient to discriminate *Bursaphelenchus* species. Morphological variation of this character was studied using different methods of multivariate statistical analysis. For this study, 360 spicule outline drawings of 40 populations of 21 *Bursaphelenchus* species (*B. abietinus, B. abruptus, B. antoniae, B. borealis, B. conicaudatus, B. eggersi, B. fraudulentus, B. hellenicus, B. hofmmani, B. hylobianum, B. leoni, B. luxuriosae, B. mucronatus, B. paracorneolus, B. pinasteri, B. pinophilus, B. poligraphi, B. sexdentati, B. teratospicularis, B. tusciae* and *B. xylophilus*) from different origins were used. The outline drawings were digitized and adjusted in size and orientation. Four different methods of spicule shape quantification were used and compared: *i*) Binary grid; *ii*) 5-state grid; *iii*) Orthogonal lines and *iv*) Elliptical Fourier Analysis (EFA). All the computations were carried out using the NTSYS-pc (Numerical Taxonomy and Multivariate System package), version 2.11T (Rohlf, 2000). Clustering analysis showed that EFA was the most reliable quantification method, by allowing the delineation of more clearly and consistent delimitation of species and species groups. Using UPGMA clustering method based on distance some species were well defined and clearly separated from each other such as *abruptus,*

antoniae, hylobianum, leoni, teratospicularis and *tusciae.* On the contrary, other species were disorderly clustered together: i) *abietinus, hellenicus* and *paracorneolus*; ii) *borealis* and *poligraphi*; iii) *hofmmani* and *pinasteri;* iv) *pinophilus* and *sexdentati* and v) *conicaudatus, fraudulentus, luxuriosae, mucronatus* and *xylophilus.* The EFA method also provides the best and more congruent results using Principal Component Analysis (PCA); the projections of OTU's (Operational Taxonomical Units) onto the first three principal components corroborate the results obtained with clustering methods. The first three principal components (R's) explained 78% of the total variation in spicule shape. The plane formed by the first 2 R's clearly defined the groups of *abietinus-hellenicus-paracorneolus* and *conicaudatus-fraudulentus-luxuriosae-mucronatus-xylophilus* and the species *abruptus*, *hylobianum* and *teratospicularis*. Onto the same plane it was possible to separate a group composed by *antoniae-borealis-poligraphi-tusciae*; a second group composed by *eggersi-hofmmani-leoni-pinasteri-pinophilus;* and a third formed by *abietinus-hellenicus-paracorneolus*. Due to the great variability, *sexdentati* overlaps some species of the second and the third groups. R3 component separated *antoniae-borealis-poligraphi* from *tusciae* and *eggersi* from leoni, *sexdentati* and *pinophilus.* The quantification of the spicule shape is not sufficient to discriminate many of the *Bursaphelenchus* species, particularly those usually assigned to the *xylophilus* group, even when a more sophisticated data acquisition method like EFA was used. However, this tool could be useful in the separation of some species traditionally referred to as having similar spicule shape, such as: *abruptus* from the *xylophilus* group and *borealis* from *leoni.* Albeit being a sophisticated mathematical tool, EFA computation is quite simple and could represent an alternative to the qualitative diagnosis using the spicule shape. This study represents the first approach to describe the spicule shape using EFA and other multivariate methods in nematode taxonomy.

THE ENLARGEMENT OF THE XYLOPHILUS GROUP IN THE GENUS *BURSAPHELENCHUS*

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Members of the *xylophilus* group of the genus *Bursaphelenchus* can be clearly distinguished from other species of the genus by the presence of four lateral lines, the presence of a vulval flap in females, a characteristic shape of spicules and the arrangement of the seven caudal papillae. The identification of species within this group was previously based on the female tail shape. The description of new species in the *xylophilus* group has complicated their identification. Whereas the widely distributed species *B. xylophilus*, *B. fraudulentus*, *B. mucronatus* and also *B. kolymensis* have been known for a long time, five other species of the *xylophilus* group have been described only since 2000: *B. conicaudatus, B. luxuriosae, B. doui, B. singaporensis* and *B. baujardi.* They were mostly detected in East and Southeast Asia. This seems to indicate that this region has a rich biodiversity of species of the *xylophilus* group. *B. xylophilus, B. mucronatus, B. kolymensis, B. baujardi* and *B. doui* live in coniferous trees. *B. fraudulentus* has been found in both coniferous and deciduous trees. The other species occur in wood of

deciduous trees. The morphological characters of the species of the *xylophilus* group are presented, their differentiation is depicted and the geographic distribution is shown. The finding of a mucronate form of *B. xylophilus* in packaging wood raises uncertainty about morphological identification. *B. kolymensis* is considered to be the European type of *B. mucronatus* and probably a subspecies of *B. mucronatus*. This assumption is supported by reproductive isolation of German isolates of the European and East Asian types of *B. mucronatus*, morphological studies and previous genetic results.

SOME DATA ON THE DIAGNOSTICS ON THE JUVENILE STAGES OF THE SPECIES OF *BURSAPHELENCHUS* AND CLOSE GENERA

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Bursaphelenchus mucronatus (from Krasnoyarsk Territory of Russia), *Laimaphelenchus deconincki* (St. Petersburg region) and *Schistonchus* sp. (Kaluga region) multiplied in *Botrytis cinerea* cultures were studied to define the individual development stage and the sex of juveniles. Orsein staining were used to study the anatomy, inner morphology of the body and structure of genital primordium. Using the structure of the genital primordium, its position in the body and presence or absence of the cloacal primordium and special modified nuclei within the ventral row of nuclei, it is possible to define the stage and sex of juveniles. It was concluded that morphology of genital primordium and its position show distinct stepwise changes between juvenile stages. To define the stage number, the approach of the reverse reconstruction was used: first, changes were found between adults (A5) and pre-adult juveniles (J4), then differences between J4 and J3 and finally between J3 and J2; the latter stage was the youngest in all studied species and thus it was concluded that first moult J1/J2 occurs inside the egg shell. For amphimictic species *Bursaphelenchus mucronatus* and *Schistonchus* sp. within each stage it was possible to recognize male and female juveniles: J4 (male and female); J3 (male and female) and J2 (male and female can not be distinguished for this stage). Diagnostic characters for stage and sex of juveniles were selected and illustrated in detail. Illustrated tabular and text keys to identify the stage and sex of juveniles of amphimictic *B. mucronatus* and *Schistonchus* sp. and a stage of parthenogenetic *L. deconincki* were developed. For *L. deconincki* it is proved that J4 is the main overwintering dormant stage in NW of Russia.

INVESTIGATIONS ON WOOD–INHABITING NEMATODES OF THE GENUS *BURSAPHELENCHUS* (NEMATODA: PARASITAPHELENCHIDAE) IN PINE FORESTS IN THE BRANDENBURG PROVINCE, GERMANY

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After the first detection of the pine wood nematode (*Bursaphelenchus xylophilus*) in Europe in 1999, surveys for wood–inhabiting nematodes in pine forests have been extended to Brandenburg, Germany. Dead or dying pine trees (*Pinus sylvestris*), wood chips from local sawmills as well as beetles, particularly the black pine sawyer (*Monochamus galloprovincialis*) have been examined in recent years. Seventeen different species of the genus *Bursaphelenchus* were found. Morphological features of adult nematodes and DNA fragment patterns obtained from ITS RFLP analysis were used for species identification. The species identified were *Bursaphelenchus mucronatus* (106), *B. fraudulentus* (1), *B. sexdentati* (19), *B. vallesianus* (12), *B. poligraphi* (1), *B. pinophilus* (3), *B. borealis* (2), *B. leoni* (9), *B. silvestris* (1), *B. eggersi* (3), *B. tusciae* (3), *B.* sp. n. (2), *B. pinasteri* (2), *B. paracorneolus* (1), *B. teratospicularis* (1), *B. fungivorus* (5), *B. willibaldi* sp. n. (1). Two new *Bursaphelenchus* species are still being described. The pine wood nematode was not found in Brandenburg up to now. Bait logs cut from healthy pine trees were exposed at different locations from May to July in order to attract beetles for oviposition. Juveniles of *B. mucronatus*, a closely related species of the pine wood nematode, were found as dauerlarvae on hatching adults of the black pine sawyer. *M. galloprovincialis* and *B. mucronatus* are widespread in Brandenburg. Both have been frequently detected, following forest fires or forest cuttings in the Summer time.

INTERSPECIFIC VARIATION IN ITS RDNA OF *BURSAPHELENCHUS* SPECIES (NEMATODA: PARASITAPHELENCHIDAE) OF DIFFERENT GROUPS

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Internal transcribed spacers of rDNA were used to infer the phylogenetic relationships among *Bursaphelenchus* species, with special emphasis on members of the *xylophilus* and *fungivorus* groups. Sequence alignments and phylogenetic analysis using neighbour-joining and maximum parsimony algorithms resulted in trees with similar topologies. The 17 *Bursaphelenchus* species examined can be

separated into two main branches: the first includes all members of the *xylophilus* group, and the second includes the species of the *fungivorus* group, separated from the remaining species *B. eremus*, *B. hofmanni*, *B. rainulfi* and *B. yongensis*. Phylogenetic analysis revealed *B. abruptus* as the basal taxon of the species investigated or at least of the *xylophilus* group. The significantly supported groups are largely consistent with the morphological variation within the genus *Bursaphelenchus.* Additionally, sequence analysis revealed sequence microheterogeneity in the ITS regions of *B. mucronatus*, *B. singaporensis* and *B. yongensis*.

MOLECULAR CHARACTERISATION OF ISOLATES OF THE *BURSAPHELENCHUS SEXDENTATI* GROUP USING ITS-RFLP AND RIBOSOMAL DNA SEQUENCES

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Species of the *Bursaphelenchus sexdentati* group are characterized by four lateral lines and a typical position of caudal papillae of males. Within this group, differentiation of species is based on the shapes of the female tail and the spicules. However, observations on different isolates of the same species have revealed considerable variability of these features suggesting the existence of intraspecific genetic types. In this study, ITS1/2 sequences of 17 isolates belonging to five species of the *sexdentati* group *(B. sexdentati, B. vallesianus, B. pinophilus, B. poligraphi, B. borealis)* were obtained. In some isolates, intra-individual sequence heterogeneity at a few sites of ITS2 was detected. Cluster analyses of genetic distances between isolates were carrried out including ITS1/2 sequences of the *eggersi* and *xylophilus* groups for comparison. In the resulting phylogenetic tree, branching of clusters confirmed the affiliation of isolates to their respective species and species groups. In addition, isolates of *B. sexdentati* were separated in two subclusters suggesting the existence of two intraspecific types of this species. The information obtained from sequencing was used to select additional enzymes for extending the scope of ITS-RFLP analysis. In this way, improved distinction of species and differentiation of two intraspecific types of *B. sexdentati* was achieved.

ANALYSIS OF *BURSAPHELENCHUS XYLOPHILUS* (NEMATODA: PARASITAPHELENCHIDAE) PROVENANCES USING ISSR AND RAPD FINGERPRINTS

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Two PCR-based techniques, inter-simple sequence repeats (ISSR) and random amplified polymorphic DNA (RAPD) were used to determine genetic relationships among 30 *Bursaphelenchus xylophilus* provenances from the USA, Canada, Japan, China, South Korea and Portugal, in order to trace the origin of the recently introduced Portuguese population. Cluster analyses of genetic distances were carried out and bootstrap dendrograms were constructed. The fingerprints obtained with both methods showed reduced genetic variation for samples of introduced populations as compared to native North American populations. The results indicated that the founders of the Portuguese populations were translocated one or two times to Portugal from their colonized sites in East Asia, but not from their native habitats in North America.

AN EFFECTIVE PCR-BASED DIAGNOSTIC METHOD FOR THE DETECTION OF *BURSAPHELENCHUS XYLOPHILUS* (NEMATODA: PARASITAPHELENCHIDAE) IN WOOD SAMPLES FROM LODGEPOLE PINE

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A molecular diagnostic method has been designed for the detection and identification of *Bursaphelenchus xylophilus*. Heat shock protein 70 gene sequences from *B. xylophilus* and the closely related *B. mucronatus*, were compared and used to design primers Bx701F and Bx701R which amplify a 171 base pair fragment from *B. xylophilus* by PCR. As a control, primers Bm701F and Bm701R were designed which specifically amplify a 168 base pair fragment from *B. mucronatus*. Species-specific detection of *B. xylophilus* was carried out directly from concentrated Baermann funnel extracts using wood samples from lodgepole pine (*Pinus contorta,* Dougl. var. *latifolia*) trees from British Columbia, Canada containing an unknown nematode population, and thus bypassing the need for culturing the nematode before analysis.

SATELLITE DNA AS A VERSATILE GENETIC MARKER FOR *BURSAPHELENCHUS XYLOPHILUS*

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Bursaphelenchus xylophilus is causal agent of the pine wilt disease, one of the major conifer diseases worldwide. This nematode is a quarantine organism in the European Union, where it has recently been discovered in a restricted area in Portugal. In our laboratory, research has been developed to characterize the genetic diversity of the nematode, and to provide a more comprehensive view of the relationships between *B. xylophilus* and the non-pathogenic *Bursaphelenchus* species. For that purpose, repetitive sequences known as satellite DNA (satDNA) have been cloned and characterized from the genome of *B. xylophilus*. Its species-specific distribution and high copy number in the genome make this sequence a very promising tool for molecular identification of the nematode, as will be illustrated with results obtained in the laboratory. In particular, the recent development of a very sensitive satDNA based real-time PCR assay will be presented. Moreover, sequencing of monomers of the satDNA was performed and phylogenetic analyses were conducted to analyse the diversity and relationships i) between *B. xylophilus* isolates sampled worldwide; 2) among *B. xylophilus* populations all collected in the infested area from Portugal. Results will be discussed in the context of the origin and evolution of the pine wood nematode complex in Europe.

PORT CHECK: THE POTENTIAL OF REAL-TIME PCR AS A VALUABLE TOOL FOR THE DETECTION OF THE PINE WOOD NEMATODE, *BURSAPHELENCHUS XYLOPHILUS*

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The EU consortium project entitled “Development of generic ‘on site’ molecular diagnostics for EU quarantine pests and pathogens” (http://www.portcheck.eu.com/index.cfm), is a Specific Targeted Research Project (STREP) in support of Council Directive 2000/29/EC on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. It aims to deliver the tools and procedures to allow EU Member State Plant Health laboratories and inspection services to perform molecular diagnostic assays “on-site” and at points of entry. The project aims to develop and evaluate real-time PCR assays for a number of key quarantine organisms, including *Bursaphelenchus xylophilus* (pinewood nematode, PWN), and it will

transfer these assays to field portable real-time PCR platforms. Concerning PWN, work has been developed regarding sampling and collection of wood material from pine trees (infected and non-infected). Moreover, based on repetitive sequences previously characterized in the genome of the nematode, a Taqman PCR diagnostic assay has been set up that allow the specific identification of single *B. xylophilus* individuals. Also, and cross-linking with another EU project ("PHRAME), great effort has been placed in collecting and characterizing a representative number of isolates of *B. xylophilus* from the affected area in Portugal (Setúbal Peninsula, 30 km SE of Lisbon). So far, 24 isolates have been genetically characterized, and will be useful for the validation of the specific probes used for real-time PCR. Developments resulting from this project are presented here.

SESSION 4

THE INSECT VECTORS: BIOLOGY AND ECOLOGY

(Chairpersons: M. LINIT & S. AKBULUT)

PINE WILT DISEASE IN PORTUGAL: UPDATED KNOWLEDGE ON THE VECTOR-INSECT *MONOCHAMUS GALLOPROVINCIALIS* (OLIV.)

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In 1999 the pine wood nematode *Bursaphelenchus xylophilus* (Nematoda; Parasitaphelenchidae) was detected in dead maritime pines (*Pinus pinaster*) for the first time in Europe, near Lisbon – Portugal. This organism is the causal agent of the Pine Wilt Disease in Pines and a quarantine organism within the European Union. Thousands of insects from more than 20 species were screened for the presence of the nematode but detected only on the endemic pine sawyer *Monochamus galloprovincialis*. Before *B. xylophilus* was introduced the pine sawyer was considered a secondary xylophagous in Portugal and its biology and ecology had never been studied. After the discovery of the vector, the EFN team initiated an investigation programme with the objective of studying several aspects of the insect's biology, life-history and interaction with the nematode and hosts, of which a global synthesis of results is presented here. It was found that the pine sawyer has one generation per year in Portugal, with beetles emerging from the end of May to August. Biological parameters such as longevity, sexual maturation period fecundity and oviposition rates were studied under laboratory conditions. Also under laboratory conditions female beetles laid eggs on *P. sylvestris, P. halepensis, P. pinaster, P. radiata, P. pinea,* and *Pseudotsuga menziesii,* but insects successfully completed their life-cycle only on the first four pines. Transmission of the pine wood nematode by the adult insects peaked in the second and third weeks of adult beetle life then gradually diminished. Several traps and attractants were tested for population control purposes and the most efficient combination was the cross-vane transparent trap with turpentine and ethanol lures. The knowledge acquired during the last years has been used to define and consolidate the control and eradication procedures carried out annually by the responsible authorities in the pine wilt nematode affected zone and surrounding buffer areas, which to date have successfully prevented the spread of the disease.

POTENTIAL INSECT VECTORS OF *BURSAPHELENCHUS* SPP. (NEMATODA: PARASITAPHELENCHIDAE) IN SPANISH PINE FORESTS

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Potential insect vectors of *Bursaphelenchus xylophilus* (PWN) were studied. Pathways of introduction of PWN from Portugal to Europe, through Spain, were determined and traps were located in pine stands sites along the pathways. 19 Cerambicidae, 12 Scolytidae, 12 Buprestidae and 10 Curculionidae

species have been found. Trapped insects were examined for the presence of nematodes under their elytra. Nematodes were found on *Arhopalus ferus*, *Spondylis buprestoides, Hylastes ater, Hylurgus lingniperda*, *Orthotomicus erosus*, *Pityogenes bidentatus*, *Tomicus piniperda*, *Hylobius abietis* and *Pissodes validirrostris* specimens. The nematodes were mainly aphelenchids (*Aphelenchus* Bastian, *Cryptaphelenchus* Fuchs, *Ektaphelenchus* Fuchs*, Laimaphelenchus* Fuchs, *Paraphelenchus* Mickoletzy and *Seinura* Fuchs), but also rhabditids. *Bursaphelenchus fungivorus* Franklin and Hooper appeared associated to *Orthotomicus erosus*. *Monochamus galloprovincialis* was the most important insect species, representing a risk for the introduction of the PWN in Spanish pine forests; Cerambicidae and Curculionidae species, were taken into account because they have been reported as vectors of other *Bursaphelenchus* spp.

DISTRIBUTION, BIOLOGY AND POPULATION GENETIC STRUCTURE OF THE LONG HORN BEETLE *MONOCHAMUS GALLOPROVINCIALIS* (COLEOPTERA, CERAMBYCIDAE) IN FRANCE

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The longhorn beetle *Monochamus galloprovincialis* is known to be the main vector of the pine wood nematode *Bursaphelenchus xylophilus* (Nematoda: Aphelenchoidea), recorded from Portugal in 1999 on *Pinus pinaster*. This invasive species, considered a quarantine pest in European countries, has been responsible for severe epidemic damages on pines in Central and Southwestern Japan. Transmission of the nematode requires an insect vector with a larval developmental phase occurring in dying trees. Due to the geographical position, as well as the occurrence of three species of *Monochamus* (*M. galloprovincialis*, *M. sutor*, *M. sartor*) in France, there is a great risk of dissemination of this quarantine organism to the rest of Europe, via France. In order to study the geographic distribution and host range of *M. galloprovincialis*, we conducted a sampling in 40 localities in France on eight coniferous species using 150 flight intercept crossvane traps baited with bark beetle pheromone. *Monochamus galloprovincialis* was sampled in 20 sites in France on *Pinus sylvestris*, *P. pinaster*, *P. halepensis* mainly and on *P. nigra* and *P. uncinata*. The second objective of the study was to test the effect of host plant or geographical isolation on population genetic structure. To meet this goal, we conducted an analysis of COI-COII mitochondrial sequences on a large set of *M. galloprovincialis* populations. Results showed a low genetic variability among populations (less than 1%) and revealed no clear population differentiation patterns influenced by host plant or geographical isolation, certainly due to extensive gene flow between populations.

BIOLOGY STUDIES RELEVANT TO THE VECTOR ROLE OF *MONOCHAMUS* SPECIES FOR PINE WOOD NEMATODE

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The pine wood nematode needs a vector to be transferred to pine trees. In countries, where *Bursaphelenchus xylophilus* occurs, *Monochamus* spp. plays the most important role as vector of the nematode. Therefore specific knowledge of the distribution and biology of various *Monochamus* species is important to improve control measures and indicate risk areas for possible epidemic spread of PWN. Within the context of the EU-Project "PHRAME" *M. galloprovincialis pistor, M. sutor* and *M. sartor* were collected in different areas of Austria, cultured and reared in the lab. Lifespan and maturation feeding were studied for all three *Monochamus* species. Maturation feeding took place for the entire life span of all three *Monochamus* species with decreasing intensity. Beetles emerged from late May till September and survived up to 96 days in the lab. It shows that *Monochamus* spp. is able to vector PNW till late October. This could be one explanation, why a certain amount of infected trees in Portugal show no symptoms till the following summer. It also means that it is not enough to carry out monitoring only during a certain period of the year. Tests with *M. galloprovincialis pistor* concerning the diameter of breeding material indicated that 6 – 8 cm in diameter, followed by 9 – 12 cm in diameter was the preferred dimension. Further investigations also showed the possibility of a one or two-year-development-cycle for *M. galloprovincialis pistor*, which could have influence on future control measures of the PWN-vector.

GENETIC STRUCTURE OF *MONOCHAMUS ALTERNATUS* IN JAPAN

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Recent progress in molecular ecological methods, e.g. in the development of microsatellite markers and phylogeographical analysis using mitochondrial DNA, has enabled us to study genetic differentiation among populations. Here I report on a molecular ecological study of *Monochamus alternatus* in Japan. Firstly, I reviewed the population structure of this insect in Japan as revealed by molecular mitochondrial DNA and microsatellite markers and the process of population expansion was inferred from this genetic structure. Secondly, I showed that there was a divergence between populations in Akita and Iwate prefectures located in northeast part of Honshu, Japan, as revealed by microsatellite markers. These prefectures are at the frontier of pine wilt disease damage in Japan and such a strong population structure is thought to have formed by different dispersal routes of the pine sawyer into these frontier populations. Other aspects of the sawyers' geographical variation are also discussed.

OCCURRENCE OF *BURSAPHELENCHUS MUCRONATUS* IN FRANCE AND ITS ASSOCIATION WITH *MONOCHAMUS GALLOPROVINCIALIS*

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As a consequence of the recent introduction of the pine wood nematode, *Bursaphelenchus xylophilus*, in Portugal, nematodes of the genus *Bursaphelenchus* and the insect vector were studied in French pine forests to analyse the risk of propagation and the routes of dissemination of the nematode in case of its introduction in France. Our hypothesis was that the existence of non-pathogenic species of *Bursaphelenchus* could impede the spread of *B. xylophilus* by occupying the same ecological niche. A survey of *Bursaphelenchus* species in France was carried out on trap trees, in 12 localities distributed in various pine forests of the country. The contamination of *M. galloprovincialis*, the main vector of *B. xylophilus*, was also studied in these localities. A total of 201 logs were analysed. *B. hellenicus*, *B. leoni*, *B. mucronatus* and *B. sexdentati* were isolated, but not *B. xylophilus*. The morphometric separation of the different species was determined by discriminant function analysis. The presence of *B. mucronatus* (and the absence of *B. xylophilus*) was confirmed by molecular analysis. *B. mucronatus* was isolated from several regions. The infestation of *M. galloprovincialis* by *B. mucronatus* varied from 0% to 44%.

SESSION 5

ECOLOGY AND MODELING

(Chairpersons: K. FUTAI & H. EVANS)

SPATIAL MODELLING OF *BURSAPHELENCHUS XILOPHILUS* IN PORTUGAL

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Bursaphelenchus xylophilus, the causal agent of pine wilt disease (also refered as "pine wood nematode", PWN) is native to the United States, where it was first described in 1934; since then it was also identified in Japan (1972), where to it was introduced through timber trade, and from where it spread into China, Taiwan and Korea. This nematode species, of minute dimensions, may kill a mature tree within a short time after infection. It has been classified as a quarantine organism in the European Union (Directive 77/93 EEC). In 1999 it was identified in Portugal, vectored by a cerambycid wood-boring beetle, *Monochamus galloprovincialis;*.it seems to affect maritime pine (*Pinus pinaster*) only. Due to the economic importance of pine wood in Portugal and the risk of the nematode spreading to other European countries, an international research project (PHRAME) was set to investigate the PWN; the UMC team's task was to model the spatial distribution of PWN in Portugal. Diverse information was collected: climatological data, landscape metrics, land use types distribution, remote sense land classifications, fire outbreak distribution and fire risk, and data on the presence/absence, both of the nematode and of its vector. All this information was gathered in a Geographic Information System. Research in Japan has shown some dependency of the PWN on climatic variables. Yet the data on temperature, precipitation and evapotraspiration available for Portugal was inappropriate to look for an eco-climatic optimum for *B. xylophilus.* Co-kriging was thus preformed, using 30-years data series of monthly climatological data and landscape metrics. The first models of the potential distribution of the nematode in Portugal were based upon the PROLUNP inventories. Yet because the presence of the nematode is so far restricted to a small area (2580 km^2 within the Peninsula of Setubal, PS), straight extrapolations for the whole of Portugal were not satisfactory. A second generation of models considered instead the probability of a tree being killed by the PWN, ignoring the nematode's inventories. This probability was estimated considering that the death of trees infected by the PWN occurs when trees are only in poor condition. Thus, we used growth curves for *Pinus pinaster* to spatialise growth potential over the entire area of Portugal. These models assume that if water stress reduces tree resistance to infection, trees located in areas of high water stress will be more prone to die from PWD; in contrast, trees that grow on sites of high suitability are expected to resist infection. Three scenarios were considered for these models: (1) the first scenario ignores data from the PS and concentrates on tree condition only; according to this scenario the nematode will reach any point in the country, irrespective of distance; (2) in the second scenario, the nematode is expected to spread from the first focus of infection, PS, at a speed estimated from the four-years inventory series; in this scenario, the nematode may still reach any point in the country, depending on time; (3) the last scenario considered dispersal routes, assuming that the nematode and its vector need pine trees corridors to move throughout; under this scenario, some parts of the country may always be nematode free. Each scenario produced a different outcome that ought to be explored together.

FIELD DIAGNOSIS OF THE ASYMPTOMATIC CARRIER OF PINEWOOD NEMATODE

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To prevent pine wilt disease (PWD) from spreading over pine forests, elimination of pine trees killed by the pinewood nematode (PWN), *Bursaphelenchus xylophilus* is desirable, though this method is very laborious and time-consuming. If such dead trees are left in the field, pathogenic nematodes and their vector, *Monochamus* beetles, could spread from tree to tree without any difficulty. In our university arboretum, where many precious foreign pine species are planted in the field, all pine trees killed by PWD have been eradicated thoroughly before the next pine wilt season. Despite intensive efforts in removing dead trees from the stands, new dead trees tend to appear in the vicinity of the stumps of trees killed in the previous year, and wilting recurs in the same pine stand every year. Why does PWD recur at the same stand even after thorough eradication of dead pine trees? One of the possible reasons is asymptomatic carrier.s I have warned against spreading of PWD due to leaving such asymptomatic carrier in the field. In north Vermont, USA, where temperature ranges from –12 °C to 24 °C (http://www.travel-vermont.com/weather/index.asp), the trees of Scots pine (*Pinus sylvestris*) were found to contain the pinewood nematode 2 to 11 years after inoculation and the majority of these infected trees remained healthy and asymptomatic for 7 to 11 years. If such PWD-infected pine trees delayed symptom appearance and overlapped with the following season of the beetles' activity, such trees could play a role as huge attractants to the vector beetles and pose a danger to pine stands. To examine nematode survivability in asymptomatic trees, wood samples must be incubated for a long time (30 days ore more) under warm conditions and thereby facilitate nematode propagation, because nematode population in asymptomatic trees is usually too low to be detected. To exterminate the PWN, a rapid and accurate diagnostic method is needed. To detect PWNs from pine trees in two natural stands (Japanese black pine and a Japanese red pine) we applied a new diagnostic method based on a simple DNA extraction and nested-PCR, and found that many trees of either pine species contained pinewood nematode though some of them displayed no external and/or internal symptoms. Some trees of Japanese black pine survived for one or more year after PWNs infection without any symptoms, suggesting that they should be overlooked in eradication.

MODELLING PWN-INDUCED WILT EXPRESSION: A MECHANISTIC APPROACH

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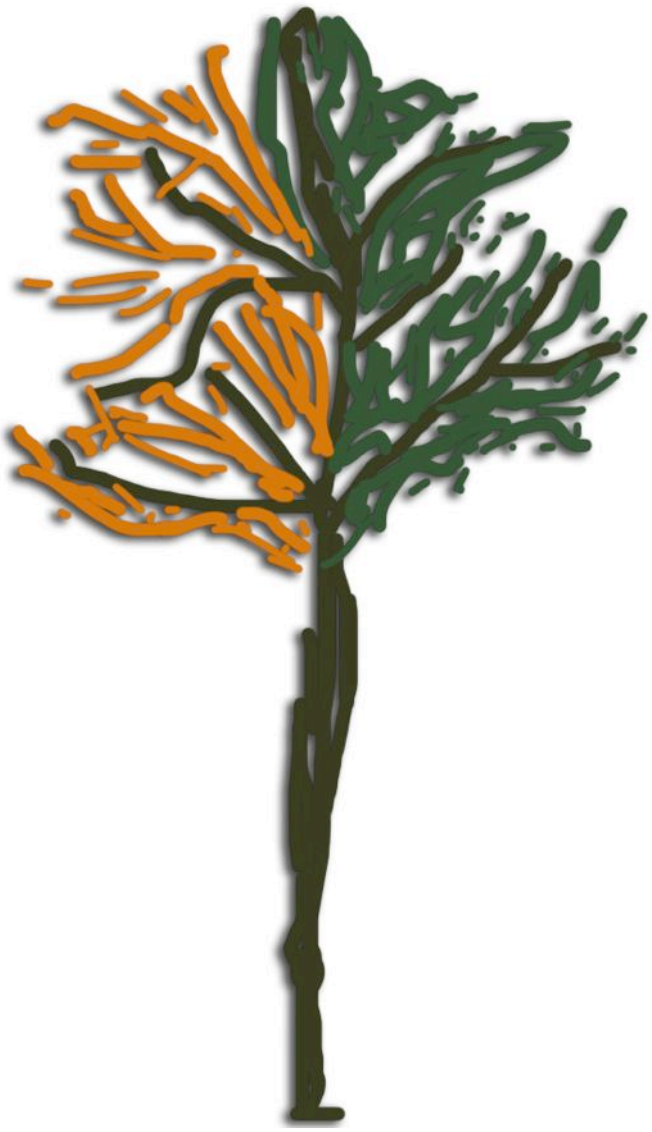
Many pine species in Europe are susceptible to Pine Wilt Disease (PWD), with certain species (e.g. *Pinus pinaster*) expressing significant susceptibility that often results in tree death. To evaluate the interactions between nematode (*Bursaphelenchus xylophilus)* population dynamics and its tree host, a

mechanistic model describing tree water-carbon relations and interactions between nematodes has been developed. A fully coupled, point scale and daily time step soil-vegetation-atmosphere transfer (SVAT) mechanistic model has been developed and forms the core of this new modelling approach. The model predicts vertical and lateral water movement through the soil-plant-atmosphere continuum and gross primary productivity (GPP) by simulating relevant terrestrial hydrology processes (interception, vertical and lateral soil water movement, runoff, soil and canopy evaporation, and N-sensitive photosynthesis-coupled transpiration) for a forest stand of known structure, growing in locally determined soil and climate. It has been adapted to describe the interactions between tree physiology and a dynamic population of pinewood nematode by simulating the likelihood of wilt expression in trees where the nematode has been introduced through maturation feeding by vector insects in the genus *Monochamus*. Central modelling assumptions are: (a) that under normal conditions and in the absence of PWN, with rising plant water demand and a constant water supply, the xylem pressure decreases, increasing the risk of cavitation. Trees will usually reduce the deficit by minimising transpiration through stomatal closure during peak demand when the plant is actively photosynthesising. Recovery, even from quite severe reductions in xylem pressure and potential cavitation, usually takes place during the hours of darkness. (b) The presence of PWN, either causing local defence reactions in the tree and by population build up leading to blockage of xylem vessels, results in increased vulnerability to cavitation that compromises the host's ability to recover from episodes of high-negative xylem pressure. Preliminary simulations indicate that high ambient temperatures or low soil water availability can result in onset of irreversible cavitation and wilt expression, describing well the situation observed under normal field conditions. The presence of an increasing PWN population, either in its own right or, particularly in combination with extreme environmental conditions of drought and/or air temperature, causes cavitation to occur more sharply, resulting in rapid death of the host tree. By comparison, under well watered conditions trees can survive, as can certain trees when late season periods of rainfall alleviate summer drought conditions. The population size of PWN in a tree also appears to be significant: late and/or small initial numbers of nematodes and low temperatures limit the population size of PWN at the end of the growing season. Combined with a compromised xylem, this may result in tree death in the year following infestation. We postulate this may be linked to the likelihood of trees not being able to accumulate sufficient non-structural reserve carbon to allow full re-flushing in the following spring. To verify modelling assumptions, and to test the model's predictive ability, a field trial is being conducted in Portugal during the 2006 summer, and is described elsewhere. Finally, it is proposed that mechanistic models such as this can make a significant contribution towards improving the evaluation of potential risk of the spread of this disease to other parts of Europe, as the basis for decision-making on appropriate phytosanitary measures at local and regional scales.



SESSION 6

THE TREE: PHYSIOLOGY, RESISTANCE AND HISTOPATHOLOGY AS A RESULT OF PWD

(Chairpersons: K. KURODA & D. BERGDAHL)

IMPACT OF THE PINE WOOD NEMATODE ON A MARITIME PINE FOREST IN TROÍA, PORTUGAL

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The pine wood nematode (PWN) *Bursaphelenchus xylophilus* was detected for the first time in Europe in 1999 in Setúbal peninsula, Portugal. The nematode is restricted to the "affected zone", a heterogeneous region which comprises urban and rural landscapes, where the majority of the forests are composed of maritime pine (*Pinus pinaster*), along with stone pine (*P. pinea*) and, less frequently, Aleppo pine *P. halepensis*. We studied the impact of the PWN on a maritime pine stand of approximately 400ha on Tróia peninsula, inside the nematode affected zone. The results of a five-year study showed that strict and rigorous eradication procedures and vector control measures carried out yearly have diminished significantly the impact of the disease on the peninsula. We found that *B. xylophilus* is an important mortality factor for the dominant, older and bigger *P. pinaster* trees, while the smaller trees (with diameters below 15cm) are usually not affected by the nematode and are affected by other biotic (pine processionary moth, scolitids) and abiotic (hidric stress) factors and agents. Overall, the presence of the PWN is gradually changing the structure and composition of the pine stands which are becoming less dense with lower age classes. *P. pinea* and *Juniperus* spp. natural regeneration is protected and thus being favored.

PINEWOOD NEMATODE PERSISTENCE IN ASYMPTOMATIC SCOTS PINES

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The pinewood nematode (PWN, *Bursaphelenchus xylophilus*) is present in many pine growing areas of North America but rarely causes pine wilt disease (PWD), especially, in cooler climates. In 1987 and 1993, we inoculated Scots pine (*Pinus sylvestris*) trees with the PWN and used sterile water for controls. All trees have been periodically observed and sampled to determine how long the PWN could survive in living trees. For trees inoculated in 1993, slightly over 50% were found to still harbor a PWN population in 1998. However, for both 1987 and 1993 inoculations, fewer asymptomatic, living trees were found to maintain a PWN population in 2001 and 2003. Some mortality has been observed in both PWN- and control-inoculated trees, but the PWN was never recovered from control trees and not from all dead or living PWN-inoculated trees. Final sampling of living, inoculated trees was completed on May 30, 2006 (results still pending). We believe the PWN can persist in asymptomatic, living trees, for long periods of

time without causing PWD. Therefore, these infested trees may hamper sanitation and other PWN management efforts, especially, in regions currently experiencing PWD. We also believe the harvesting of healthy-appearing trees may not be adequate to prevent movement of the PWN in wood products without additional treatment.

INOCULATION OF PINE TREES WITH AVIRULENT PINE WOOD NEMATODE UNDER EXPERIMENTAL CONDITIONS: RISK-BENEFIT ANALYSIS

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Virulence of the pine wood nematode, *Bursaphelenchus xylophilus*, varies widely from highly virulent to avirulent, or less pathogenic. The inoculation of pine trees with avirulent *B. xylophilus* induces the resistance of trees to subsequent inoculation with virulent *B. xylophilus*. This induced resistance may constitute a measure to develop a biological control strategy for pine wilt disease. The characteristics of induced resistance have been identified by the experiments completed within a year. This time, the long-term and years of repeated experiments were conducted to further understand the nature of induced resistance and the potential for control measures. Induced resistance was re-confirmed though the effects for control measures were not as effective as trunk injection with nematicides. The avirulent *B. xylophilus* was surely avirulent through the longitudinal and repeated observations though the avirulent *B. xylophilus* rarely caused tree mortality. In conclusion, the benefit, i.e., chance of tree survival, outweighs the risk of tree mortality by avirulent *B. xylophilus* itself, when pine trees were to be inoculated with avirulent *B. xylophilus* at least in the experimental conditions where pine trees are subsequently inoculated with virulent *B. xylophilus*. The explorative application of this resistance-inducing method by avirulent *B. xylophilus* might be possible in areas where pine wilt disease occurs naturally.

RAPIDITY OF DISEASE DEVELOPMENT SEEMS TO RESULT IN HIGH MORTALITY – INSIGHT FROM AN INOCULATION TEST USING HYBRIDIZED POPULATIONS BETWEEN A VIRULENT AND AN AVIRULENT ISOLATES OF *BURSAPHELENCHUS XYLOPHILUS* –

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Mortality caused by the pinewood nematode (PWN), *Bursaphelenchus xylophilus*, still remains insufficiently characterized. One of the reasons is that only a few isolates have been used in previous

comparative studies that attempted to describe biological characteristics of the PWN related to its pathogenicity. In this study we prepared 27 hybridized populations with a variety of pathogenicity by mixing 2 isolates, S10 (virulent) and C14-5 (avirulent), at various proportions (S10 proportion in each population was 100, 99, 90, 70, 50, 30, 10, 1 or 0%) to characterize the progress pattern of seedling death. One- and two-year-old seedlings of Japanese black pine, Pinus thunbergii, served the inoculation test. Numbers of the dead seedlings were recorded every 2 days for 60 days after inoculation. Using these records, we calculated 2 indices, mortality velocity and tolerance limit of the seedlings following Asai and Futai (2005), who pointed out that the mortality velocity reflects rapidity of disease progress and that tolerance limit reflects the critical value of nematode load necessary to kill a seedling. Mortality of seedlings at 60 days after inoculation (eventual mortality) varied from 20% to 100% for one-year-old seedlings and from 6% to 100% for 2-year-old ones showing positive correlation to the S10 proportion in each of the inocula. There was no correlation between the tolerance limit and the eventual mortality. This may indicate that the tolerance limit reflects physiological conditions of host rather than characteristics of inocula. On the other hand, the eventual mortality correlated closely with the mortality velocity. We also found that seedling death was rather durable when it progressed slowly. On the basis of these analyses, we concluded that the mortality caused by the PWN would eventually be higher when the disease development is faster.

DISTRIBUTION, MIGRATION BEHAVIOR AND POPULATION DYNAMICS OF *BURSAPHELENCHUS XYLOPHILUS* (STEINER & BUHRER, 1934) NICKLE 1970 (NEMATODA: PARASITAPHELENCHIDAE) IN YOUNG *P. SYLVESTRIS* TREES DURING EARLY WILT AT CONTROLLED OPTIMUM TEMPERATURE

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Pathogenicity of *B. xylophilus* represents the ability to break through the tree's defense system, to invade the host and finally affect its water supply by means of blockage of the water uptake. Population density of the pine wood nematode (PWN) is a crucial factor enhancing this process. Population growth and migration behavior of nematodes inside host trees were studied by an inoculation experiment with three to four year old *P. sylvestris* seedlings at 25 °C in a climate chamber. Altogether, 120 pines were inoculated with 4000 nematodes per tree of a *B. xylophilus* population (adults and larvae) originally isolated in Portugal from *P. pinaster* in 2003 and reared on *Botrytis cinerea*. Migration and population dynamics of PWN was examined during a 27 day period within which all trees died. PWN were extracted from 10 trees at each of the 9 sampling dates respectively. To better track the migration of nematodes, trees were divided in 17 segments according to morphological features of the trees including the root-

system. The composite nematode population in all segments together reached 19 794[1] Individuals per trees (1820[1] nematodes/ g dry weight) 12 days after inoculation, whereas 370[1] individuals per tree initially were reisolated after one day. PWN could be isolated from all tree segments (except roots) as early as 4 days after inoculation. A concentration of *B. xylophilus* in roots in comparison to rising population densities in above ground plant parts was observed when relative water content of wood started to decrease. Results indicate that *B. xylophilus* migrate rapidly throughout its host before building up high population levels. There is evidence that *P. sylvestris* of the observed age class can retain high population densities of PWN in roots, while densities in wood parts might be relatively low.

PRELIMINARY EXPERIMENT ON THE PATHOGENICITY OF *BURSAPHELENCHUS MUCRONATUS* ON THREE PINE SPECIES UNDER GREENHOUSE CONDITIONS

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In this study, the pathogenecity of B. *mucronatus* was tested under greenhouse conditions. Three native pine species of Turkey, *Pinus sylvestris*, *P. nigra* and *P. Brutia*, were selected for nematode inoculation. Three year old seedlings were used. A certain number of nematodes were inoculated into 15 seedling of each tree species and 10 seedlings of each were served as controls. The experiment ended 90 days after inoculation. Nearly half of the seedlings of both *P. sylvestris* and *P. nigra*, wilted as a result of nematode infection. Only one seedling of *P. brutia* wilted. New experiments should be carried out with larger numbers and different age classes of pine species under field conditions and using different densities of *B. mucronatus*.

BURSAPHELENCHUS SPECIES IN DECLINING SWISS PINE FORESTS AND EFFECT OF WATERING TREATMENTS ON THEIR VIRULENCE

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The most frequent *Bursaphelenchus* species found in declining Scots pine stands (*Pinus sylvestris*) in a dry Alpine valley in Canton Valais in Switzerland were *B. vallesianus* (75%) and *B. mucronatus* (20%). Pathological potential of the both species in relation to soil water regime were investigated by inoculation experiment with 4-years old pine seedlings (*P. sylvestris*) in greenhouse in 2005. *B. vallesianus* and *B. mucronatus* were highly pathogenic under the experimental conditions, caused mortality of 86% and 84%, respectively. However, under limited soil water conditions the mortality rate increased to 100% and

[1] Median

disease onset was earlier. *B. mucronatus* multiplied more intensively in all treatments compared to *B. vallesianus* and reached significantly higher population density (1202 and 520 nematodes per g FW, respectively). Nematodes were distributed throughout the whole plant and penetrated also roots. However, they were detected most frequently in the lower part of the stem, below the inoculation site.

HISTOLOGICAL OBSERVATIONS OF *BURSAPHELENCHUS XYLOPHILUS* IN SYMPTOMATIC TISSUES OF PINE WOOD

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The objective of the present study is to document the histopathological damage and its relationship to the progressive pathology in pine trees after inoculation with the pinewood nematode, *Bursaphelenchus xylophilus*. Seedlings 6-months old and 3-5 years old of *Pinus densiflora* and *P. thunbergii* were used to examine histopathological damage of wood tissues and symptomatic progress of seedlings at intervals after nematode inoculation, especially during early stage of pathogenesis. At the inoculation site, nematodes entered most axial resin canals of cortex and xylem and destroyed parenchyma cells. Beyond the inoculation site only a few nematodes were found in resin canals of cortex. Cell death recognized by granulation of the cytoplasm and brown cell contents were sporadically observed among axial and radial parenchyma cells of xylem throughout the stem as early as 3 days after inoculation. The 6^{th} day after inoculation, death of axial and ray parenchyma cells of xylem of the seedlings which showed the typical disease symptom, marked reduction of oleoresin exudation, was widely distributed in the stem. At this stage, no population growth of nematodes was observed throughout the seedling and no destruction of parenchyma cells occurred necessarily in wood tissues. After complete stop of oleoresin exudation on the seedling, destruction of wood tissues such as parenchyma cells of axial and radial resin canals, ray, cambium and phloem became more advanced as nematode populations grew rapidly in wood of the seedling. Cell death occurring at initial stages of pathogenesis was indicated as one of the most remarkable pathological progress of nematode inoculated seedlings. Destruction of wood tissues was resulted from nematode feeding on parenchyma cells as disease progress.

DEFENSE SYSTEMS OF *PINUS DENSIFLORA* CULTIVARS SELECTED AS RESISTANT TO PINE WILT DISEASE

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In the stems of pine species resistant to pine wilt disease, such as *Pinus taeda* and *P. strobus* growing in North America, migration and propagation of the pine wood nematode (PWN) are suppressed and the nematodes disappear from pine tissue, in contrast to the highly susceptible Japanese pine species, *P. thunbergii* and *P. densiflora*. Resistant cultivars (families) of these susceptible species have been found in heavily damaged forests. Although they are potential saviors of pine forests in Japan, certain proportions of seedlings obtained from those cultivars are susceptible and are killed after infection. To obtain reliable seedlings with stable resistance, it is important to find some criteria that can be used to select truly resistant trees for seed orchards. In the tissue of resistant cultivars, there must be systems that prevent nematode activities even if the effect is weaker than those in *P. taeda*. The initial migration of PWN in the shoots was investigated on the cuttings of non-resistant and resistant cultivars of *P. densiflora* and compared with that in *P. taeda*. PWN was inoculated on the apices of 20-cm long cuttings. Every day or two, cuttings of each cultivar were sectioned into short segments (less than 5cm). Nematodes were extracted from each segment and were counted. PWN in the cortex and xylem tissue was counted separately for the cuttings of *P. densiflora*. The anatomical characteristics were then investigated on seedlings inoculated with PWN. In *P. taeda* cuttings, PWN distribution was restricted to the inoculated area during 4 days from inoculation. On the other hand, suppression of nematode migration was not detected in resistant cultivars of *P. densiflora* judging from the PWN numbers in each stem segment. When PWN population in xylem tissue was compared, a tendency was detected: in resistant cultivars, PWN populations during 5 days from inoculation were smaller in the area more than 5cm below from inoculated sites. In contrast, PWN population in cortex indicates no specific tendency in resistant cultivars. These results suggest that xylem tissue contributes to the defense system in the early period of infection although it is not yet clear whether the structural barrier is effective or toxic substances exist in xylem.

SESSION 7

PWN AND INSECT VECTOR CONTROL METHODS

(Chairpersons: K. NAKAMURA & K. IKEDA)

CONTROL PROGRAM OF PINE WILT DISEASE FOR LANDSCAPE CONSERVATION - IN THE CASE OF "AMANOHASIDATE" IN KYOTO, ONE OF THE MOST FAMOUS AND BEAUTIFUL SCENIC SPOTS IN JAPAN -

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Amanohasidate in Kyoto has been given many titles, and is well known as one of the three most beautiful scenic spots in Japan. The others being *Matsushima* and *Miyajima*. From a long time ago, "Amanohasidate" has been best known for its coastline of white sand and green pines on a sandspit spreading 3.2 km long by 40 to 170 m wide. The splendid cluster of pine trees growing there, *Amanohasidate*, has been the subject matter of books and theatre over time. *Amanohasidate* is located in the innermost part of Wakasa-bay and is surrounded by mountains that play an important role as background landscape and add spice to *Amanohasidate* itself. Therefore, it is very important to preserve the whole area, including *Amanohasidate* and the surrounding mountainous area, from any damage to the pine forest. It is important that we leave the legacy of *Amanohasidate* in good condition for the next generation. There are more than 5,000 pine trees of over 10 cm diameter at *Amanohasidate*. Recently, the number of pines dying of pine wilt disease has ranged from 10 to 30 per year. The trend of pine death has begun to increase. The disease doubled in frequency after 2000, and more than 100 pine trees died in 2002. Nevertheless, the number of dead pines in 2002 is not very serious when compared with that occurring in other pine forests. However, as *Amanohasidate* is ranked as one of the most wonderfully scenic places in Japan and is very important economically for tourism. Even one pine death is not allowed. Consequently, various measures have been instigated to prevent pine wilt death from spreading. These measures have been successful, and are described in this presentation.

USEFULNESS OF THE LIVE-CAPTURE ATTRACTION TRAP FOR MONITORING *MONOCHAMUS ALTERNATUS* ADULTS AND THEIR NEMATODE LOAD

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Monitoring of the prevalence of *Monochamus alternatus* adults and its nematode load is useful for understanding the local epidemic pattern of pine wilt disease (PWD). Nakamura et al. (1999) devised a live capturing trap for M. alternatus adults by modifying a commercially available attraction trap provided by a Japanese company, Sankei Chemical Co., originally used for eradicating *M. alternatus* adults. The number of captured adults using this trap roughly reflects the population size of the adults flying around the area, although it cannot provide an accurate estimation of the field density of the adults because of its susceptibility to the site conditions such as topography and influence of the dead pine trees as a

superior competitor in attracting *M. alternatus* adults. Nematode load of the captured adults was significantly lower when compared to those of the newly emerged adults. However, the nematode load of the flying adults may constitute important information for explaining the local PWD epidemic. The live-capturing attraction trap is a useful tool for monitoring the activity of *M. alternatus* as the vector of PWD.

EFFECT OF AERIAL SPRAYING INSECTICIDE AS THE CONTROL MEASURE OF PINE WILT DISEASE

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Asian and European pine species are susceptible to the pine wood nematode, *Bursaphelenchus xylophilus*. In Japan, pine forests have been heavily infected, and the damaged volume in a year reached approximately one million m^3 since 1973. After *Monochamus alternatus* was shown as the main vector of this disease in 1971, aerial spraying of insecticide has been conducted for pest control. In this study, to clearly assess its effect, we surveyed the damage in *Pinus densiflora* stands in relation to aerial spraying for six years. Eleven quadrats (each 30m long and 20m wide) were set in *Pinus densiflora* stands around Mt. Tsukuba (877m) in central Japan. These quadrats were divided into 3 groups by the years of aerial spraying of insecticide during 1996-2004: spraying all year (P1-5), no spraying (C1-5), spraying only in 1996 (M1). The cumulative mortalities of *Pinus densiflora* of P1-5, C1-5 and M1 were 42.63±6.98%, 78.35±12.21% and 47.46%, respectively. The annual mortalities of P1-5, C1-5 and M1 were 2.61±0.88%, 7.52±1.90%, 2.54%, respectively. These average values of P1-5 were significantly smaller than those of C1-5 on both the accumulative ($p < 0.001$) and the annual mortality ($p < 0.001$). Moreover, the "Disease Progress Index (DPI)," which represents the potential progress speed of damage, was calculated from the successive change of accumulative mortality on the approximation of the logistic function. The cumulative mortalities except M1 were approximated with a logistic curve ($r > 0.91$). The DPI of P1-5, C1-5 and M1 were 0.14±0.05, 0.56±0.10, 0.11, respectively, and its average of P1-5 was significantly smaller than that of C1-5 ($p = 0.003$). The high correlation coefficient suggests the change of cumulative mortality can approximate with the logistic curve in the quadrat size of the warm temperate zone. The aerial spraying with insecticide was capable of controlling the progress speed of the damage of pine wilt disease. This resulted in the difference of the cumulative and the annual mortality between with insect pest control and without it.

PREVENTION OF PINE WILT DISEASE BY CONTROLLING THE VECTOR INSECT USING MICROBIAL AGENT

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Studies on microbial control of *Monochamus alternatus*, vector of the pinewood nematode in Japan, have been carried out. Through the survey of its pathogens, an isolate of an entomopathogenic fungus, *Beauveria bassiana* F-263 was found to be the strongest and was selected as a control agent of this insect. To control larvae of *M. alternatus* in damaged pine logs, application of nonwoven fabric strips with conidia of this fungus onto the bark caused sufficiently high mortality of the larvae. Use of nonwoven fabric strip formulation in the Summer to kill larvae under the bark was thought to be the most practical among the present application methods of *B. bassiana* conidia. Adults of *M. alternatus* were less susceptible to this fungus, but direct contact with conidia on nonwoven fabric strips produced high mortality of the adults within a short period. This method cannot be used on healthy trees as prevention application, but can be used to kill emerging adults from the nematode-infested trees. The adults infected with *B. bassiana* reduced the amount of maturation feeding, and thus, transmission of the nematode to healthy pine twigs was almost completely inhibited. This formulation of *B. bassiana* on nonwoven fabric strips is now being applied for the registration as a commercial microbial insecticide in Japan. Conidia of *B. bassiana* dispersed from a nonwoven fabric strip in the field were detected 50 to 100 m apart from the strip, however, their density was far below the infectious level on a mulberry leaf to the silkworm. The fungus had almost no infectivity to 2 coleopteran predators, *Dastarcus helophoroides* and *Harmonia axyridis*, weak virulence to the predatory bug, *Orius sauteri*, and moderate virulence to the honey bee, *Apis mellifera*. The fungus did not affect species of plants examined. The fungus is also thought to have no effect on soil microorganisms based on the experiments of mixing the conidia in forest soil.

STUDIES ON *SCLERODERMA GUANI* XIAO ET WU TO CONTROL THE PINEWOOD NEMATODE

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After clear-cutting dead pine trees caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus* (Sterner et Buhrer) Nickle, some dead branches and stumps remained which were bored by the larvae of the pine sawyer beetle (PSB), *Monochamus alternatus* (Hope). Several natural enemies for controlling PSB were found in Dongshanqiao district near Nanjing. *S. guani* is one of the most successful natural enemies that parasitize the larvae of PSB and it is easy to reproduce by the larvae of

PSB. Three hundred thousand larvae of PSB were collected annually in the forest for the purpose of massive rearing of *S. guani* in the lab. The studies of the biology, reproductive methods, release techniques and control effect to the larvae of PSB by *S. guani* have been concluded. The massive rearing of *S. guani* in the lab raised 13 million adult females of *S. guani* by 0.3 million of the larvae of PSB annually from 2004-2006. After that we released *S. guani* to control the larvae of PSB in the same and other PWN infected areas. Both collecting the larvae of PSB and releasing the adults of *S. guani* were an effective and ecological way to control PSB and PWN in the large area because of its low risk to humans and to the environment. Dead pine trees were significantly reduced in the test area.

THE EFFECT OF ROOTED CUTTING PROPAGATION OF NON-DAMAGED JAPANESE BLACK PINE THROUGH THE INOCULATION TEST WITH THE PINEWOOD NEMATODE ON NEMATODE-RESISTANT PLANT PRODUCTION

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Japanese black pine (*Pinus thunbergii*) forests have been seriously damaged during last five decades by pine wilt disease, caused by the pine wood nematode (PWN), *Bursaphelenchus xylophilus*. A breeding project of Japanese black pine resistant against PWN began in 1978, with 16 resistant plus trees selected from 14,620 candidates through inoculation testing with a moderately virulent PWN isolate “Shimabara”. Several clonal seed orchards have been established in southwestern Japan by grafting of these resistant trees. In Kyushu district, resistant plants of Japanese black pine have been produced through the inoculation test with “Shimabara” to open-pollinated seedlings obtained from the seed orchard. However, inoculation is a hard and costly work for nurserymen, therefore the price of a resistant plant is extremely expensive (e.g. 650 Japanese yen or 4.6 €). The purpose of this study was to establish a new method for producing resistant plants without the inoculation test. We performed rooted-cutting from 15 non-damaged individuals through the inoculation test with “Shimabara” to 38 open-pollinated seedlings from the seed orchard. Ramets have been propagated by rooted-cutting for three successive years (2000, 2001 and 2002). These ramets have been inoculated with “Shimabara” in the corresponding years, 2002, 2003 and 2004, respectively. The proportion of non-damaged ramets ranged from 61 to 71% in the three years, higher than those of the seedlings produced from the orchard (33-55%), or those from non-orchard sources (3-25%). To produce resistant plants of Japanese black pine more efficiently; we selected more resistant seedlings by successive inoculation tests with more virulent isolates. First, we selected 15 non-damaged individuals by inoculating with a virulent isolate “Ka-4” to 18 open-pollinated seedlings. Secondly, we selected 9 non-damaged individuals through additional inoculation testing with an another virulent isolate “Karatsu 3”, out of them as ortets for rooted-cutting. When the ramets propagated from 9 ortets were inoculated with “Karatsu 3”, the proportion of non-damaged plants was extremely high (97.7%). These results suggest that the cutting-propagation of

seedlings selected by using virulent isolates offers an effective method for the practical production of resistant plants against PWN.

MECHANISMS INVOLVED IN NEMATODE INFECTION BY NEMATOPHAGOUS FUNGI

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Plant-parasitic nematodes have been reported to cause serious losses throughout the world. In recent years, nematophagous fungi, one of the natural enemies of nematodes, have been employed in biological control because of their unique ability to capture and infect nematodes. To explore the mechanisims involved in the infection by nematophagous fungi, researches in the areas of mechanical activities, nematicidal metabolites, and virulence enzymes have been conducted in our lab. Efficient killing of nematodes was observed both with *Stropharia rugosoannulate* Farlow ex Murrill and a newly identified *Stropharia* sp.. These fungi were confirmed to have the ability to immobilize the free-living nematode *Panagrellus redivivus* in minutes and the pine wood nematode *Bursaphelechus xylophilus* within hours, on agar plate. The *Stropharia* cultures studied, share the characteristic of abundantly producing cells with finger-like projections called acanthocytes. We showed that the nematode-attacking and paralyzing activities were carried out by these spiny acanthocytes, which were believed to have a similar role with the hyphae appendages found in other nematode-trapping fungi. Additionally, we also performed scanning electron microscopy and transmission electron microscopy to elucidate the structural details of the acanthocytes. Nematode-trapping fungi, an important kind of nematophagous fungi, can capture, infect and digest their prey using predacious structures (trapping devices). Thus to clone the genes related to the trapping-device and conidia formation will provide insight into the infection process. In our study, the nematode-trapping fungus, *Monacrosporium sphaeroides*, was transformed with a plasmid harboring the hygromycin B phosphotransferase gene, via restriction enzyme-mediated integration (REMI). After screening the transformants, five mutants with typical trapping devices were obtained and Southern blot analysis revealed that the foreign plasmid DNA had integrated into the genome. Based on the standard method, plasmid rescue was also performed to clone the corresponding genes. But because of the rearrangement, deletion mutation and limitation of the technology, the cloning of the genes was not successful. Now, the construction of genome library is being carried out and the next step is to get the genes by screening the library. Except for the mechanical activities, nematicidal metabolites are another important pathogenic factor involved in the infection. In the survey for the metabolites against *B. xylophilus,* four hundred fungi strains were assayed, and the further phytochemical studies undertaken have resulted in the isolation of various diketopiperazines, terpenoids, peptides as well as polyketides. For example, a strain of *C. rosea*, a fungus commonly used as biocontrol agent, showed significant ability to kill nematodes, and then five new verticillin-type epipolysulfanyldioxopiperazine were isolated from wheat solid substrate fermentation, along with four known compounds. In vitro immersion tests showed that all nine

compounds exhibited antinematodal activity. In the molecular mechanisms of nematophagous fungi infecting their hosts, it has been suggested that hydrolytic enzymes participate in several steps of host infection. Thus we designed to clone and characterize the virulence enzymes in the representative fungi of nematode-trapping fungi as well as endo-parasitic fungi. Seventeen genes encoding cuticle-degrading enzymes including serine proteases and chitinases were cloned. Moreover, the bioassay experiments showed that these enzymes were involved in the process to penetrate the cuticle and eventually digest them.

NEMATOCIDAL ACTIVITY OF SKF-13 ISOLATED FROM *CINNAMOMUM CASSIA* PRESL AGAINST THE PINEWOOD NEMATODE (*BURSAPHELENCHUS XYLOPHILUS*)

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250 medicinal plants were tested for nematicidal activity against the pine wood nematode, *Bursaphelenchus xylophilus* using a 96-well plate bioassay. Responses were varied with plant material and its concentration, especially extracts of *Cinnamomum cassia* (Lauraceae) showing the strongest nematicidal activity against adults and juvenile nematodes of *B. xylophilus* at 2 000µg/ml. The extract was chromatographically fractioned and the active compound was identified. The compound SKF-13 caused nearly 100% mortality at 100µg/mL. In greenhouse and field experiments, SK-13 had 90% protective efficacy against the pine wood nematode, and appeared to be one of the most useful nematicides reported in controlling pine wilt disease caused by *Bursaphelenchus xylophilus*.

NOVEL AZAPHILONES, EPIPOLYSULFANYLDIOXOPIPERAZINES, RESORCYLIC MACROLIDES AND SPHINGOLIPIDS WITH NEMATICIDAL ACTIVITY FROM CULTURES OF FRESH WATER-DERIVED FUNGI

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The ability of one hundred and thirty strains of fresh water-derived fungi to produce nematicidal metabolites against the pine wood nematode, *Bursaphelenchus xylophilus*, which were isolated from the submerged woody substrates in Lake Fuxian, Yunnan, China, were investigated. Interestingly, it was found that cultures of many fungal strains were pathogenic to the tested nematodes. Subsequently, several novel fungal antibiotics, with structures belong to azaphilones, sphingolipids, 15-member

resorcylic macrolides or epipolysulfanyldioxopiperazines, were isolated from cultures of *Caryospora carllicarpa* YMF1.01026, *Gliocladium roseum* YMF1.00133, *Paraniesslia* sp. YMF1.01400 and *Pseudohalonectria adversaria* YMF1.01019. Their structures were elucidated by detailed NMR spectroscopic analysis. All the relative stereochemistry for the chiral centers was designated on the basis of ^{1}H-^{1}H coupling constants and NOESY correlations. All the compounds were assessed for nematicidal activity against *B. xylophilus*.

ANTI-NEMATODAL ACTIVITIES OF PROTOBERBERINE DERIVATIVES AGAINST THE PINEWOOD NEMATODE, *BRUSAPHELENCHUS XYLOPHILUS*

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To screen a potential antinematodal agent for the pine wood nematode (PWN), *Brusaphelenchus xylophilus*, we have developed an *in vitro* assay system for the protoberberine derivatives and tested them for their inhibitory activity as an effective trunk-injection agent. From the primary screening works on 25 available compounds, two compounds, HWY-4213 and 5038, were found to exhibit potent nematicidal activity against *B. xylophilus in vitro* with IC50 of 0.08 and 0.52 mM, respectively. In particular, the inhibitory activity of HWY-4213 *in vitro* was higher than those commercially available compounds such as abamectin when tested for 6 h period. Although the exact mechanism of action of these compounds remains to be determined, we postulate that these compounds might have blocked sterol biosynthesis in PWN. A field test for HWY-4213 and its derivatives is now underway in order to evaluate their application as trunk-injection agents against PWN. [Supported by the Pine Wilt Nematode Project of the Korea Forest Research Institute (to YKP)].

EFFECT OF LOW INOCULUM DOSES OF PINE WOOD NEMATODE ON OLEORESIN EXUDATION

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The interruption of oleoresin exudation inside trees is a major symptom of pine wilt disease, caused by pinewood nematode *Bursaphelenchus xylophilus*. Based on this, an oleoresin exudation method was used as early diagnosis for pine wilt and observed at regular intervals. The results showed that the less the inoculum doses were, the later the abnormal oleoresin exudation of pines appeared. On June

12,2002, different low doses with 25,50,500 nematodes (female: male= 1:1)/tree of pine wood nematode were used to inoculate 15 thirty-year-old Japanese black pines(Pinus thunbergii)and 15 thirty-year-old Masson pines (*P. massoniana*) in Nanjing, Jiangsu Province. The oleoresin exudation of Japanese black pines was 10-30 days earlier than that of Masson pines inoculated with nematodes at the same dose. All the tested Japanese black pines died during those days but six; three of the Masson pines inoculated with 25,50 nematodes respectively did not die until the following summer. This result suggested that it is difficult to early diagnose the infested Masson pines, with low dose nematodes, by the oleoresin exudation method.

HOW TO SAFELY USE PINE WOOD INFECTED BY PINEWOOD NEMATODE IN CHINA

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Pine wood nematode is the most serious pest of forest trees in China. Since the early 1980s, infested areas have progressively spread throughout Nanjing and along the coast of southeast China, which is a warm temperate zone. In 2004, infested areas totaled 79 000 ha. Eradication of dead trees by means of felling and burning was basically adopted as the control method at that time. The packaging wood material was kiln-dried with moist heat 70℃ (centre temperature) for 10 h and relative humidity (RH) 80% by piping steamed heat. There were no pests after heat treatment. The moisture contends was kept under 20% after treatment, in accordance with current international wood packaging standards. The network and video administration were applied by temperature control call register and humidity control instrument. A special mark has been awarded this certificate for compliance with the standard CN-001 HT-32. On the other hand, gum and medium or high-density fiberboards (MDF or HDF) were made with the disease wood.

POSTERS SESSION

INVESTIGATION OF *PINUS SYLVESTRIS* BAIT LOGS FOR HATCHING BEETLES AND WOOD-INHABITING NEMATODES OF THE GENUS *BURSAPHELENCHUS* (NEMATODA: PARASITAPHELENCHIDAE)

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In connection with EC monitoring surveys for the pine wood nematode (*Bursaphelenchus xylophilus*) (Decision 218/2001/EC and following versions) wood material and beetles, especially the black pine sawyer (*Monochamus galloprivincialis*), were investigated for wood-inhabiting nematodes in Brandenburg, Germany. Healthy pines (*Pinus sylvestris*) cut down between the months of May and July at 45 locations attracted the sawyers for oviposition. Five beetle species hatched from the bait logs: *M. galloprovincialis* (143), *Acanthocinus griseus* (28), *Hylobius abietis* (1), *Pissodes notatus* (14) and *Phaenops cyanea* (2). Propagating juveniles were found in beetles of the black pine sawyer only. *M. galloprovincialis* developed in the bait logs from 16 locations. It was possible to detect the nematode species *Bursaphelenchus mucronatus* in beetles of *M. galloprovincialis* and wood of the bait logs. Although no nematodes were found in wood in time of cutting the trees, *B. mucronatus* developed sometimes to high degrees up until Autumn. Especially in the warm and dry year of 2003, up to 6 000 nematodes per 100 g wood were detected. Single beetles took up to 40 000 juveniles. *B. xylophilus* was found neither in wood nor in the beetles. Exposing segments of bait logs at room temperature and diping them into a water bath weekly allowed the beetles developing several month earlier than in the open air. Investigation of bait logs and catching beetles for nematodes is a useful method for early detection of contamination with nematodes of the genus *Bursaphelenchus* passing by *M. galloprovincialis*.

MICROBIAL CONTROL OF *BURSAPHELENCHUS XYLOPHILUS* BY FUNGI

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An important factor which affects the number of pinewood nematodes (*Bursaphelenchus xylophilus*) carried by the Japanese pine sawyer (*Monochamus alternatus*) is the fungal flora in wilt-killed pine trees. *In vitro* studies showed that *Monochamus* beetles carried a greater number of nematodes when the blue-stain fungus *Ophiostoma minus* was dominant in wood around artificial pupal chambers of the beetles, while the numbers of nematodes decreased when *Trichoderma* spp. or *Verticillium* sp. was prevalent. The former fungus is suitable and the latter two fungi are unsuitable for nematode

propagation. In field surveys, intense blue-stain on the pupal chamber walls of the beetles increased the number of nematodes aggregating around such chambers and the number carried by the beetles that emerged from the chambers. To reduce the number of nematodes carried by the beetles, we attempted to change the mycoflora and also to prevent blue-stain fungi from spreading throughout pine wilt-killed Japanese red pine (*Pinus densiflora*) wood by inoculating other fungi into the dead logs. *Trichoderma* sp. 3 and *Verticillium* sp. inoculation treatments tended to decrease the number of the nematodes carried by the beetles.

POTENTIAL MAP FOR PINE WILT DISEASE EPIDEMIC USING A SIMPLE INDEX OF THE THERMAL CONDITION

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A potential map for pine wilt disease (PWD) epidemic was made referring to the MB index, a type of thermal index originally proposed by Taketani et al. (1975). The MB index is calculated as a yearly total of residual monthly average temperature below 15 C when the average temperature is grater than 15 C. Based on field observations from the five locations in the different districts in Japan where decline of PWD occurrence in *Pinus densiflora* forest(s) along the elevation was observed, I determined the MB value for dividing the naturally controlled and un-controlled area of PWD epidemic as being 19 - 22. The MB value was calculated for each 1km-mesh using mesh climate data provided by the Japanese Meteorological Agency (2001) and mapped by GIS software. The meshes were afterwards classified into three categories according to the MB values: less than 19 (naturally controlled area); equal to or greater than 19 and less than 22 (boundary area); equal to or greater than 22 (uncontrolled area). The map of the classified area successfully represented the actual situation of PWD epidemic in many cases. In some *P. thunbergii* stands, however, severely damaged area was estimated as naturally controlled area, possibly because of the difference in susceptibility to PWD from *P. densiflora*.

CRYOPRESERVATION OF NEMATODES: AN OPPORTUNITY FOR THE CONSTITUTION OF AN EUROPEAN GENETIC BANK

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The maintenance of a nematodes collection on *Botrytis cinerea* plates is not only labour-intensive for the necessity of repeated sub-culturing, but sometimes unwanted biotypes or loss of pathogenicity and viability can arise due to selection pressure under laboratory conditions. Moreover, this practice does

not always assure from the risk of substrate contaminations or human errors. Temperatures below –130°C are known to assure long-term and possibly indefinitive preservation of certain biological specimens since under these conditions, biochemical and most physical processes are completely arrested. Moreover, it allows us to dispose at any time of a great number of living nematodes for planning experiments. Hence, cryopreservation at ultra-low temperature (-196°C, i.e. the temperature of liquid nitrogen or -140°C, the temperature of the mechanical freezer) is a sound alternative for the long-term conservation of animal genetic resources. Recently, our study has realized the application of a cryo-preservation method to several nematode species and this has opened the possibility to a Nematodes European Cryo-bank (NEC) constitution. This project is actually much more realistic and it would ensure not only the preservation of genetic diversity but, it could establish a cryo-storage facility for all the European user community.

OFFICIAL SURVEY FOR *BURSAPHELENCHUS XYLOPHILUS* CARRIED OUT ON THE TERRITORY OF THE REPUBLIC OF POLAND

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The pinewood nematode (*Bursaphelenchus xylophilus*) is a EU quarantine pest in accordance with Council Directive 2000/29/EC, of 8 May 2000, on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. Each Member State is obliged to conduct on its territory surveillance for the presence of this nematode, following the E.C. Pinewood Nematode Survey Protocol, 2000. In Poland survey for the presence of *B. xylophilus* has been conducted since 2003 by inspectors of the State Plant Protection and Seed Inspection Service in cooperation with the Governmental Forests. Previously, samples of imported wood and wood products had only been examined. During surveillance, inspectors follow up the instruction manual produced by the Central Laboratory of the State Plant Protection and Seed Inspection Service based upon the EU Protocol. The survey is performed in forests; saw mills, and places where imported wood and wood products are stored (including pallets, packaging material, etc.). It should be mentioned that the predominant tree species in Polish forests (about 70% of the entire forest area), is Scots pine (*Pinus sylvestris*), which is highly susceptible to *B. xylophilus*. Samples of wood, packaging material, etc. are taken with an axe or drill. Additionally, samples of wood chips, wood particles, sawdust, scrap etc. are also collected. From each site, a sample weighing at least 300 g is collected. The samples are packed in polyethylene bags and sent to Laboratories of the Inspection (Voivodeship Laboratories or the Central Laboratory) for examination. Before extraction of nematodes, samples are subjected to incubation for 10 days at 25°C. Then, the collected material is cut into small fragments, then placed in a 1-2 l capacity beaker, which is filled with water for three days. Afterwards, water with debris (and possibly nematodes) is poured onto a nematological sieve with a proper

diameter, placed in a Baermann funnel. The nematodes are extracted from the debris using the Baermann funnel method for one day. Nematodes (if any) found in sample are identified firstly under a light (compound) microscope. If they are identified as belonging to the PWNSC group (Pinewood Nematode Species Complex), detailed identification to species with PCR-RFLP test (Dutch method) of each one is performed. The only laboratory performing the PCR test is the Central Laboratory in Toruń. In the period between 2003-2005 the laboratories of the State Plant Protection and Seed Inspection Service examined 5.454 samples of wood, chips, sawdust, etc. In 11 samples, nematodes belonging to the PWNC group were found. In all cases these nematodes were identified with PCR-RFLP as *Bursaphelenchus mucronatus* Mamiya & Enda. Additionally, two samples contained nematodes not belonging to this group. They were identified; based on morphological features, as belonging to species *Bursaphelenchus glochis* Baujard and *Bursaphelenchus sexdentati* (Rühm) Hunt. Based on the survey, the status of *Bursaphelenchus xylophilus* in Poland may be recognized as "Absent – confirmed by survey". Further surveys confirming this status will be continued within the next years.

COMPARISON OF SOME BIOLOGICAL CHARACTERISTICS OF *M. CAROLINENSIS* AND *M. GALLOPROVINCIALIS* (COLEOPTERA: CERAMBYCIDAE)

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The study of biological characteristics of the pine sawyer beetles (*Monochamus spp*.) are of interest because the beetles are vectors for the pinewood nematode, the causal agent of pine wilt disease. In this paper, some biological characteristics of laboratory colonies of *M. carolinensis*, the North American vector beetle and *M. galloprovincialis* from Turkey reared on *Pinus sylvestris* were compared. Comparisons were made between the number of eggs laid, the number of larval entry holes, the number of adults emerging, generation survivorship, survivorship from egg to larva and survivorship from larva to adult. The number of eggs laid and the number of larval entry holes differed significantly. Apparent survivorships from egg to larva and from larva to adult were also different but the number of adults emerged and generation survivorship did not differ significantly. There are remarkable similarities in some biological characteristics of both vectors which suggest that *M. galloprovincialis* would have a similar vector pattern as *M. carolinensis*.

TWENTY-FIVE YEARS OF PINE WILT RESEARCH AT THE UNIVERSITY OF MISSOURI

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The causal agent of pine wilt disease, the nematode *Bursaphelenchus xylophilus*, was first identified in the United States at the University of Missouri in 1979. Since that time, the Forest Insects Lab at the University of Missouri has been active in pine wilt research on a variety of fronts. We have examined the ecology, physiology, and behavior of the nematode and its beetle vectors. This poster briefly outlines some of the research that has been conducted over the past 25 years at the University of Missouri. We describe techniques that have been developed to study the beetle and the nematode, results of population dynamics studies of laboratory colonies, flight behavior of the beetle vector, and chemotactic response and regulation of nematodes to vectors and host plants. We have also collaborated with other researchers from around the world.

FIRST OCCURRENCE OF *BURSAPHELENCHUS VALLESIANUS* IN THE CZECH REPUBLIC

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Monitoring, together with accurate diagnosis of quarantine nematodes, are essential steps for effective plant protection. In recent years, monitoring of the pine wood nematode was conducted in the Czech Republic. *B. xylophilus* has not been found so far. Many pine trees with specific symptoms of pine wilt diseases were sampled. *B. vallesianus* has been found in wood samples of dying pine trees with cammock necrosis symptoms. The locality is the south-eastern part of the Czech Republic. Larvae and adult beetles of *Monochamus galloprovincialis* have been found in this locality as well. Nematodes were transferred from wood chips to *Botrytis* culture for rapid multiplication. Light microscopy and restriction digestion of rDNA for diagnosis were used. Fragment of rDNA was cloned into the plasmid PTZ57RT and sequenced. In spring 2006, a second locality with occurrence of *B. vallesianus* has been found in the western Bohemia. This work was supported by the Ministry of Agriculture of the Czech Republic, project number QF 4156.

MODELLING PWN-INDUCED WILT EXPRESSION: VALIDATING THE MODEL THROUGH FIELD INOCULATIONS

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Many pine species in Europe are susceptible to Pine Wilt Disease (PWD), with certain species (e.g. *Pinus pinaster*) expressing significant susceptibility that often results in tree death. Evaluation of potential risk of the spread of this disease to other parts of Europe is essential as the basis for decision-making on appropriate protection schemes. To evaluate the interactions between nematode population dynamics and its tree host, a mechanistic model describing tree water-carbon relations and interactions between nematodes has been developed. The model is described elsewhere (Evans et al., this conference); initial results indicate that temperature is the most controlling factor rather than dryness, with disease development potentially delayed in dryer regions due to lower transpiration during the growing season. Simulations also indicate that tree death can also occur in the growing season following infection due to reduced carbon shortage, negatively affecting re-growth. To validate the model under field conditions, an inoculation experiment is currently taking place in Portugal, during the 2006 growing season. Measurement of a number of relevant eco-physiological (transpiration, respiration, photosynthesis, sap-flow and water potential) and eco-climatic (soil water potential, relative humidity, temperature and photosynthetically active radiation) parameters is taking place in inoculated and control trees. Destructive samples will also be taken to measure xylematic water conductance, changes in wood carbon storage and xylem vulnerability. Together these data will be used to both calibrate and validate the mechanistic model. Field data will also be used to describe in detail symptom developments of PWD in dry regions, as the basis for developing early detection methods for this disease.

MORPHOLOGICAL, MOLECULAR AND SEROLOGICAL CHARACTERIZATION OF *BURSAPHELENCHUS XYLOPHILUS* ISOLATES

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In collaboration with the Direcção Geral de Recursos Florestais wood samples from areas infected with *Bursaphelenchus xylophilus* were collected for analysis. Thirteen isolates of *B. xylophilus* were cultured on malt extract agar with *Botrytis cinerea*. Light microscope observations showed a wide variation in the female tail, some with round and some with a mucron type tail. Morphological characterization was confirmed by ITS-RFLP analysis using the restriction enzymes *Afa*I, *Alu*I, *Hae*III, *Hin*fI, and *Msp*I. In order to develop a serological method for the identification and quantification of PWN from wood sections, monoclonal phage antibodies (phMAbs) were produced using a M13 bacteriophage library

which displays specific single-chain antibody fragments (ScFv). The phage library was exposed to a homogenate of mixed stages of a Portuguese isolate of *B. xylophilus*. Screening of the phage library was accomplished by a procedure known as “panning” which selects the phage that display ScFv with high affinity for the nematode antigens. After four rounds of “panning” four phMAbs were selected. Their differential reactivity was demonstrated by Phage ELISA against different isolates of *Bursaphelenchus*.

DETECTION OF LONGHORN BEETLE ACTIVITY IN LIVING TREES BY BIOACOUSTICS

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In Portugal the pine wood nematode (PWN) is transmitted from tree to tree by the longhorn beetle *Monochamus galloprovincialis*. From what is known so far, the nematode cannot infect a new tree without the help of the vector. The early detection and felling of *Monochamus* infested pine trees would be an important step forward to prevent spread of PWN. It is well known from the scientific literature, that all kind of beetle larvae make noises when they feed in wood or under the bark. Within the context of the EU-Project "PHRAME" investigations with bioacoustic tools were carried out in the lab and under outdoor conditions. At the beginning sound measurements in *Monochamus* infested logs and bark beetle infested logs were carried out in the lab. With a special bioacoustics software sound-waves were recorded and analysed. It was possible to define certain frequencies typical for longhorn beetles and for bark beetles. The sonogram for *Monochamus galloprovincialis* showed only one crack and an extremely short term click and differed from sonograms of bark beetles, which had a much lower amplitude and different frequencies. It was possible to distinguish between longhorn and bark beetles and to differentiate sequences inside a longhorn beetle-file and between different sonograms of bark beetles. Field experiments carried out within the effected zone in Portugal did not produce the same good results as in the lab. Ambient sounds competed with the sounds of the insects to such an extent that the differentiation of sonograms was not possible.

RESEARCH TOWARDS INTEGRATED MANAGEMENT OF PINE WILT DISEASE IN KOREA

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The pinewood nematode, *Bursaphelenchus xylophilus*, is the causal agent of pine wilt disease (PWD). It was first found in Busan-city in 1988. After this first detection, the Korean government has made many efforts such as direct control by felling and burning of wilted trees, fumigation of logs by insecticide

(metham-sodium), and preventive control by aerial spray with an insecticide (MEP) to immediately eradicate pinewood nematode. But these efforts seemed to have failed, since PWD has gradually spread and is now distributed in several sites of southern Korea. Pinewood nematode is now considered the most serious pest of pine because it causes rapid and extensive tree mortality and results in significant economic, aesthetic and cultural loss. Although this nematode is distributed within a limited area, it has great potential to threaten our forests beacuse 1) susceptible hosts, *Pinus densiflora* and *P. thunbergii* are abundant. *P. densiflora* is widely distributed throughout Korea and *P. thunbergii* is abundant near the coast. 2) effective insect vector, *Monochamus alternatus* is widely distributed and seems to rapidly increase its population according to the enlargement of damage. The Korean Forest Research Institute (KFRI) established a "Pine Wilt Disease Research Center" to enforce the research related to pine wilt disease in December 9, 2005. Current research projects in KFRI consist of several research fields, such as biology and pathogenicity of the pinewood nematode, systematic and diagnostic methods for the pinewood nematode, systematics, biology and ecology of *Monochamus* insect vectors, biological and chemical control of pinewood nematode and Japanese pine sawyer, silvicultural practices, development of new pesticides, etc. The general research projects have close cooperation with universities, provincial forest research institutes, and related companies. In Korea we concentrate to develop a better management strategy of pine wilt disease, and try to put together all research capacity in our country and also make progress in research cooperation with China and Japan.

COMPARISON OF DEVELOPMENTAL PERIODS OF CHINESE INTRODUCED AND KOREAN DOMESTIC PARASITOIDS, *SCLERODERMUS HARMANDI* AT CONSTANT TEMPERATURE

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Pine wilt disease caused by the pinewood nematode, *Bursaphelenchus xylophilus* has been a serious problem in pine stands in Korea. The first occurrence of pine wilt disease in Korea was in 1988 and it was accidentally introduced from Japan. This damage eventually spread to the southern provinces and eastern coastal region of Korea. In China, *Sclerodermus harmandi* is the one of the most successful natural enemies parasitizing the larvae of the pine sawyer beetle, *Monochamus alternatus*. *S. harmandi* was introduced from China in July and September, 2005. Six thousand individuals parasitoids were inoculated on the larvae of the pine sawyer beetle, *M. alternatus* and on *Psacothea hilaris*. *S. harmandi* is easy to reproduce on the larvae of two hosts. The total developmental period of introduced *S. harmandi* from China was 29.33 days on *M. alternatus* and 25.95 days on *P. hilaris*, respectively. The periods of egg, larva and pupa of their parasitoids were 1.73±0.23, 13.80±5.57 and 13.80±1.60 days on the full grown larvae of pine sawyer beetle, *M. alternatus* and 1.40±0.53, 10.44±0.53 and 14.11±2.15 days on the full grown larvae of *P. hilaris*, respectively. The total developmental period of domestic *S. harmandi* collected in Powang, Korea was 27.0 days on *M. alternatus* and 28.75 days on *P. hilaris*. The periods of egg, larva and pupa of their parasitoids were 1.33±0.52, 11.67±0.52 and 14.00±0.84 days on

the full grown larvae of pine sawyer beetle, *M. alternatus* and 1.50±0.57, 8.50±3.11 and 18.75±0.50 days on the 5th larvae of *P. hilaris*, respectively. This population is in its 7th generation up until now. All experiments were conducted at 25±1°C, 60-70%RH and 16L: 8D photoperiod.

ACTIVITIES DEVELOPED BY UNAC UNDER THE NATIONAL PROGRAM OF PINEWOOD NEMATODE CONTROL (PROLUNP)

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The pinewood nematode, *Bursaphelenchus xylophilus* (Steiner & Buhrer) Nickle, is the causal agent of pine wilt disease. In 1999, this nematode was reported for the first time in Europe near Setúbal (40 km southeast of Lisbon), associated with maritime pine (*Pinus pinaster*, Aiton). As a result, official authorities implemented an extensive survey within a National Programme of Pinewood Nematode Control (PROLUNP). This nematode was confirmed as being restricted to a precisely delimited area. Since then, annual surveys have been conducted, not only in this area but also in the remaining territory, in order to monitor the pinewood nematode occurrence and readjust the limits of the infected area. The Union of the Mediterranean Forest (UNAC), represents actually 7 forest owners organisations, established in different zones, mainly in the South of Portugal, totalising about 700.000 ha of rural land (agro-forestry) and 16.000 landowners. Among the activities developed, since 2002, UNAC has established several protocols with the official authorities within the annual surveys for the pinewood nematode control. Our collaboration is extended to field and laboratory activities. In the lab, live nematodes were extracted by the Baermann funnel technique, from samples collected from symptomatic trees, and identified, by microscope, according to their morphologic features. Although the work conducted by UNAC has not a research purpose, some general aspects observed along the surveys may be interesting. In this context its worth to point out that in most of the populations obtained there are numerous nematodes species, besides the pinewood nematode species complex, such as *Bursaphelenchus* spp., *Aphelenchoides* spp. and *Laimaphelenchus* spp. Probably the most interesting aspect relies in the presence of mucronated forms among the populations observed. The number of these populations seems to be increasing along the surveys performed and in many populations females with and without mucron are present simultaneously. The establishment of a PCR protocol, as a complementary method for the surveys, would allow to determinate if we are in the presence of populations of *B. mucronatus* and *B. xylophilus* simultaneously or populations of *B. xylophilus* with and without mucron. This protocol would also allow overcoming other limitations of the morphological identification.

SPECIAL SESSION POSTERS (non-participants posters)

PATHOGENICITY OF *BURSAPHELENCHUS* SPP. NEMATODES TO CONIFERS IN RUSSIA

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The pinewood nematode, *Bursaphelenchus xylophilus*, causes serious wilt disease in native pines in Japan and in China. This nematode has not been found in Russia, but a closely-related nematode, *Bursaphelenchus mucronatus*, is widespread in Asian and European Russia and can easily mate with *B. xylophilus*. Pathogenic studies with various isolates of *B. mucronatus* and *B. xylophilus* to Russian conifers have been completed. Two field experiments were made to determine the pathogenicity and survivability of various isolates of *B. xylophilus, B. mucronatus* and their hybrids on Scots pine, *Pinus sylvestris*. Two-year old seedlings growing in the Moscow region were inoculated with one of two *B. mucronatus* isolates from Russia and one from China. No seedlings died by 14 months after inoculation and only nematodes of the Russian Far East isolate were extracted from seedling stem samples. In another field experiment, 5 year-old Scots pine seedlings were inoculated with isolates of *B. xylophilus* from Canada, *B. mucronatus* from Russia and France and their hybrids. Nearly all of these isolates survived natural climatic conditions of Central Russia one year after inoculation, but no seedlings died. Pathogenicity tests have also been carried out with *B. mucronatus* isolates from dead pines *(P. sylvestris* and *P. koraiensis)* in the Russian Far East. Further experiments with these isolates have shown strong pathogenicity to local conifers *(Pinus koraiensis* and *Larix olgen;* all seedlings died*)* and a moderate effect on *P. sylvestris* and *P. densiflora*. By analyzing the results of the survey and pathogenicity experiments, we have attempted to determine why various isolates of both nematode species show differences in pathogenicity and what is the cause of pathogenicity.

MONOCHAMUS SPECIES IN EUROPE: DISTRIBUTION AND PHYLOGENY

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Five species of *Monochamus* occur in Europe: *M. sutor* (Linnaeus 1758), *M. sartor* (Fabricius, 1787), *M. galloprovincialis* (Olivier, 1795), *M. urussovi* (Fischer, 1806) and *M. saltuarius* (Gebler 1830). *M. sutor*, a Euro-Siberian species, prefers *Picea abies* (L), but also lives on *Pinus sylvestris* L., *P. mugo* Turra and *P. nigra* Arnold, and secondarily on *Abies alba* Miller and *Larix* sp. *M. sartor,* distributed in central-

northern Europe and the Balkan Peninsula, lives on *Picea abies*, *Pinus sylvestris, P. cembra* L. and *P. mugo*, but can also be found on *Abies alba* and *Larix* sp. Both of these species are present in the Italian Alps. *M. galloprovincialis*, widely distributed in Mediterranean, mountainous and continental habitats throughout Europe as far as Siberia, lives on various *Pinus* species. It includes two subspecies: *M. g. galloprovincialis* and *M. g. pistor* (Germar, 1818). The former, occurring in south-western Europe, is found in Italy, mainly on *P. pinaster, P. nigra* and *P. halepensis*; *M. g. pistor* (Germar) occurs in central-eastern Europe and lives on *P. sylvestris*, *P. nigra austriaca* Höss and *Picea abies*. In Italy, it has been recorded exclusively in alpine habitats. *M. urussovi*, a Siberian-north-eastern European species, lives on *Picea abies*, *Abies sibirica* Ledeb. and *Abies alba*. It is considered vicariant with respect to *M. sartor* and is absent from Italy. *M. saltuarius*, a Euro-Siberian species, prefers spruce forests, but can also attack various *Pinus* species, including *P. sylvestris* and *P. nigra*, and secondarily *Larix* and *Abies*. In Italy, it has only been recorded in Alto Adige and Veneto. Morphological analyses can distinguish the European species on the basis of several characters which, however, show a certain degree of variability, especially in *M. galloprovincialis*. To clarify the taxonomic and phyletic aspects of the five European species, we analysed the sequences of the complete mitochondrial cytochrome oxidase I gene and a fragment of the small RNA subunit (1536 base pairs). Both sequences show complete identity between the two subspecies of *M. galloprovincialis*, as well as strong affinity between *M. sartor* and *M. urussovi*. Hence, the morphological subdivision of *M. galloprovincialis* into two subspecies and the separation of *M. sartor* and *M. urussovi* into two species are not supported by the molecular data. Chromosome analyses of Italian species show that the diploid autosomal complement ranges from 18 in *M. saltuarius* and *M. sutor* to 20 in *M. galloprovincialis*, and 22 in *M. sartor*, with a XX–Xyp sex determining system shared by all analysed taxa. *M. saltuarius* karyotype appears as the most primitive from which the others may be derived through Robertsonian fissions and pericentric inversions. Furthermore, karyotype analyses substantiate molecular conclusions about the identity between *M. galloprovincialis galloprovincialis* and *M. galloprovincialis pistor*.

PROSPECTS FOR MICROBIOLOGICAL CONTROL OF *MONOCHAMUS GALLOPROVINCIALIS* (OLIVIER)

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The control of cerambicid coleopteran populations of the genus *Monochamus*, potential vectors of the phytoparasitic nematode *Bursaphelenchus xylophilus*, faces particularly difficult problems. The attention of researchers dealing with methods and strategies for the control of these xylophages is focused on the possible use of microorganisms with entomopathogenic characteristics, such as the fungus *Beauveria bassiana* (Bals.) Vuill. In studies on the presence of entomopathogenic fungi in central Italian pine forests, we isolated numerous strains of *B. bassiana* from soil, bark and adults of various xylophages. The isolates were tested in the laboratory to assess their entomopathogenic effects on *M.*

galloprovincialis, a species widely distributed in Italy. The trials demonstrated the efficacy of three strains (Bba1, Bba6 and Bba8), which induced almost 100% mortality of the xylophage in the laboratory in a week. The three isolates are preserved in the "Collection of Entomopathogenic Microorganisms" of the ISZA of Florence. The results provide interesting prospects for the use of these *B. bassiana* isolates in the control of *M. galloprovincialis* populations in forests. Diffusion of the entomopathogen on bait-piles, using "non-woven fabric" strips impregnated with fungal suspensions or wheat grains or alginate pellets infected with the fungal mycelium, could kill male and female adults of the cerambicid without strongly interfering with other components of the pine forest ecosystem.

NEMATODES ISOLATED FROM BARK- AND WOOD-BORING BEETLES IN PINE FORESTS IN ITALY

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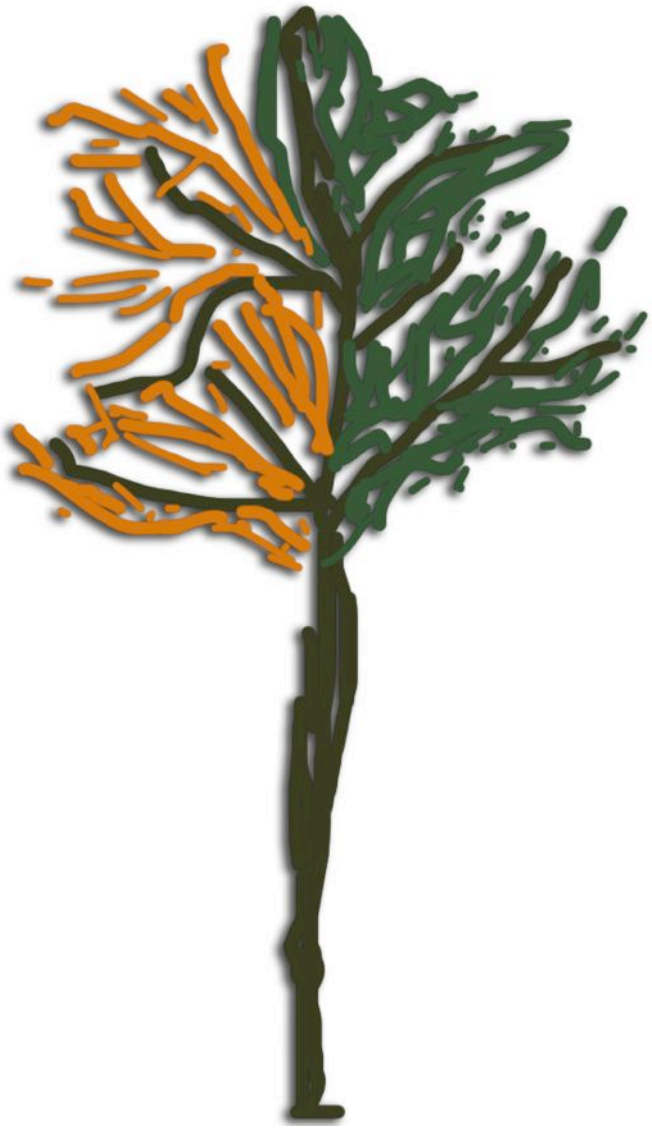
In forest ecosystems, various categories of xylophagous insects (especially scolytids and cerambicids) have obligate and facultative relationships with phytopathogenic organisms. Particulary interesting in this regard is the nematode *Bursaphelenchus xylophilus*, a North American species that has been very important in forestry since the last century. It causes damage to *Pinus* in various areas of the Oriental Region, as well as in Portugal where the nematode was recorded for the first time in 1999. Since 1993, the species has been included in the A1 list of organisms to be quarantined (EEC directive 77/93). In view of the current state of alarm by Italian phytosanitary services concerning the danger of introduction of *B. xylophilus* to Italy, experimentation was initiated in Tuscany in 2003 to devise standardized traps to catch cerambicid and scolytid coleopterans for subsequent laboratory investigations aimed at identifying the presence of phytoparasitic nematodes. The captured specimens belonged to the cerambicid species, *Monochamus galloprovincialis* (Olivier), *Arhopalus syriacus* (Reitter), *A. ferus* (Mulsant) and *Acanthocinus griseus* (Fabricius), and to the scolytid species, *Ips sexdentatus* Borner and *Hylurgus ligniperda* Fabricius. Nematodes were found in 94.4% of the specimens, with a prevalence of species belonging to the genus *Deladem*us (Tylenchida, Neotylenchidae), found in association only with *M. galloprovincialis* and *Arhopalus* spp. The genus *Parasitaphelenchus* (Aphelenchida, Parasitaphelenchidae), second in order of capture frequency, was found in association with *I. sexdentatus* and sometimes also with *H. ligniperda* and *A. griseus*. Other nematodes belonged to Rhabditida and Diplogasterida. The specimens obtained with traps set in the central Italian pine forests did not contain any dispersal juveniles of the genus *Bursaphelenchus*. In contrast phoretic juveniles of *Bursaphelenchus mucronatus* Mamiya & Enda were recorded in *Monochamus galloprovincialis* individuals that emerged from maritime pine specimens collected in Liguria.



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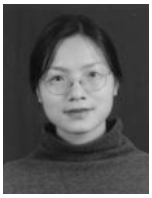

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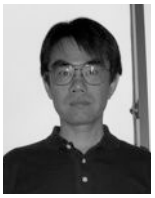

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