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IDENTIFICATION AND CHARACTERIZATION OF *ARMILLARIA TABESCENS* FROM THE TRANSCARPATHIA OF UKRAINE

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Identification and characterization of Armillaria tabescens from the Transcarpathia of Ukraine – Tsykun T., Rigling D., Nikolaychuk V.I., Prospero S. *– In the territory of the Transcarpathian region of Ukraine we first identify and characterize Armillaria tabescens (Scop.: Fr) Emel species of basidiomycete genus which known as causal agents of root rot on a wide range of woody plants through over the world. The fruiting bodies were collected in four different localities of the lowland oak-dominated forests managed by sylviculture. Significantly, basidiocarps were found on dead wood remnants and almost never on living trees. We described morphological, microscopic and molecular characteristic of collected specimens and determined phylogenetic relationships between A. tabescens from Ukraine and from other parts of the world by analyzing the genetic variation in the IGS-1 of rDNA.*

Key words*: Armillaria socialis, basidiocarps, rhizomorphs, diversity, wood-decaying fungi, managed forest.*

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Ідентифікація та характеристика Armillaria tabescens в Закарпатті (Україна). – Цикун Т., Ріглинг Д., Ніколайчук В.І., Просперо С. *– На території Закарпатської області України ми вперше ідентифікували і описали базидіальний вид Armillaria tabescens (Scop.: Fr) Emel, що належить до роду грибів, який спричиняє кореневу гниль багатьох порід дерев по всьому світу. Плодові тіла грибів були зібрані в чотирьох різних дубових господарських лісах регіону. Потрібно відмітити, що плодові тіла були знайдені лише на відмерлій деревині і майже ніколи на живих деревах. За результатами досліджень, ми описали морфологічні, мікроскопічні і молекулярні ознаки знайдених екземплярів, а також визначили філогенетичні особливості A. tabescens з України в порівнянні із світовими дослідженнями цього виду, базуючись на аналізі варіацій в IGS-1 рДНК.*

Ключові слова*: Armillaria socialis, плодові тіла, ризоморфи, біорізноманіття, дерево-руйнуючи гриби, господарські ліси.*

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Introduction

The basidiomycete genus Armillaria (Fr.: Fr.) Staude is an important natural component of the mycoflora in forest ecosystems worldwide (Shaw and Kile 1991). All Armillaria species are able to survive saprotrophically by degrading woody substrates. Moreover, some species are causal agents of root rot on a wide range of woody plants in natural ecosystems and plantations (Garraway et al. 1991, Raabe 1962, Vander Pas 1981, Legrand et al. 1996).

Five annulated (A. borealis, A. cepistipes, A. ostoyae, A. mellea, and A. gallica) and two exannulated (A. tabescens, A. ectypa) Armillaria species occur in Europe (Guillaumin et al. 1993, Zolciak et al. 1997). Armillaria ectypa (Fr.) Lamoure is a rare species confined to Sphagnum in peat bogs, whereas A. tabescens (Scop.: Fr) Emel (syn. Armillaria socialis – Antonin et al. 2006) is a common termophilic species mainly distributed in southern Europe (Guillaumin et al. 1993). Armillaria tabescens is distributed worldwide (www.cabi.org; Guillaumin et al. 1993, Keca et al. 2009, Antonin et al. 2006, Rhoads 1954, Ota et al. 1998, Schnabel et al. 2005, Qin et al. 2007, Kim et al. 2010) and its host range is also wide (Antonin et al. 2006). In Europe and central USA, this Armillaria species is usually found in oak-dominated forests (Intini 1989, Bruhn et al. 2000, Antonin et al. 2006, Keca et al. 2009, Lushaj et al. 2009) on dead oak trees (Q. conferta, Q. ilex, Q. robur, Q. suber and Q. cerris, Q. frainetto, Q.

pubescens, Q. nigra). The northern distribution limits of A. tabescens run through Central Europe from south-east of England (Rishbeth 1982) to France, southern Germany, Switzerland (Guillaumin et al. 1993), Czech Republic, and Slovakia (Antonin et al. 2006). In Eastern Europe, this species has so far been reported only on the roots of Cedrus atlantica in the Nikitskiy Botanical Garden in Crimea (Minter and Dudka 1996). Within the genus Armillaria, A. tabescens is probably the only species that could be easily identified by basidiocarp morphology. In fact, the stipes (without ring or ring zone) of individual basidiocarps are fused together at the base making dense clusters and cap's color varies from tawny to light yellow with small brownish scales at the center.

In this study we aimed to describe A. tabescens in the Transcarpathian region of Ukraine. Specifically, (1) we identified the presence and analyzed morphological characteristics of A. tabescens fruiting bodies on dead wood of lowland oak stands, and (2) we determined phylogenetic relationships between A. tabescens specimens from Ukraine and from other parts of the world by analyzing the genetic variation in the IGS-1 of rDNA.

Materials and Methods

Study site and sampling

Our study was conducted in the lowland forests of the Transcarpathian region of Ukraine. This region is situated in the south-western part of the country on the border between the Carpathian Mountains and the Middle Danube lowland. The altitude of the Transcarpathian lowland varies between 100 and 300 m a.s.l. Thanks to the protective effect of the Carpathian mountain range, this region is characterized by a warmer microclimate than other regions of Ukraine situated at the same latitude. The annual mean temperature ranges from 9 to 11 °C and the annual mean precipitation varies between 500 and 900 mm. Winters are mild (-2.8°C average temperature of January) and summers are warm (20°C average temperature of July) because of air masses coming from the Atlantic ocean (Gerenchuk 1981).

Forests of the Transcarpathian lowland are dominated by oak (Quercus robur or Q. petraea) mixed with Carpinus betulus (dry forests), Fraxinus excelsior or F. angustifolia (floodplain forests), Salix alba and S. fragilis (floodplain forests of the Tysa and Borzhava rivers), Tilia tomentosa (Mukachivskyi and Vynohradivskyi districts), and Fagus sylvatica (foothills). Locally, pure Q. robur stands as well as forest associations with Quercus cerris, Q. dalechampii, and Q. macrocarpa are also present (Gerenchuk 1981).

Sampling of the basidiocarps was conducted between August and September 2009 and 2010 (i.e. fructification period of A. tabescens in the Transcarpathia) in termophilic forests of Uzhhorodskyi, Berehovskyi, and Vynohradivskyi, Mukachevskyi districts with a predominant presence of oaks by trail method. Basidiocarps were collected along four forest trails of about 40 km length in a total. In a place where basidiocarps were found, we also observed upper layer (15 cm) of forest litter in a purpose to find rhizomorph mycelium under fruiting bodies and surrounding area (up to 0.5 m across).

Morphological study

Three basidiocarps from each cluster found in a specific locality were collected for further analyses. Morphological descriptions were conducted as indicated by Gorlenko et al. (1980). Morphological characterization was accomplished immediately after collection using fresh material. Thereafter, spore prints were obtained from the caps of the basisiocarps. Finally, herbarium specimens were prepared as described by Gorlenko et al. (1980).

Microscopic study

Microscopic features of were observed from fresh material using a 10x, 40x, and 100x microscope objective (Nikon Eclipse 100 Educational / Biological Upright Microscope MCA71201). Spores were isolated from spore suspensions diluted 10 times (see below).

Fungal isolation

Spore prints were obtained over night from cut caps on a non-absorbent surface. Single spore isolation was carried out using the modified method of Ainsworth (1995). A drop of sterile distilled water was added to the edge of a spore print. The resulting spore suspension was then dropped, by using a sterile inoculation loop, on a sterile non-absorbent surface where it was diluted 10 times by adding sterile distilled water. Thereafter, the spore suspension was spread with a sterile inoculation loop on the surface of 2% malt-extract agar plates (15 g Agar, 20 g Malt extract). This procedure was repeated 10 times for each specimen. Petri plates with spores were incubated 1–3 days at 24o C. Germinating single spores were transferred to new plates containing the same agar medium and incubated at 240C until the fluffy mycelium filled up the plate. At least 6-10 single-spore pure cultures were isolated from each basidiocarp.

Molecular analysis

Molecular analyses were conducted by sequencing the IGS-1, ITS1-5.8S-ITS2 regions of the ribosomal DNA and the encoding region of the nuclear DNA translational elongation factor 1-alpha (EF 1-alpha) region. The sequences obtained were compared with corresponding sequence data from the NCBI database (http://www.ncbi.nlm.nih.gov/genbank/).

DNA extraction. DNA was extracted from 30 mg of lyophilized mycelium obtained from three-two week old A. tabescens pure cultures. The extraction was performed using the CTAB method

described by Gardes and Bruns (1993). The DNA was resuspended in 50 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stored at 4ºC until use.

PCR amplification of the IGS-1. The IGS-1 of the ribosomal DNA weas amplified by PCR. The PCR reactions were performed in 50-µL volumes with the following final concentrations: 1 x reaction buffer (Sigma), 4 mM MgCl2, 100 µM dNTPs (Promega), 20 pmol of each primer, 2.5 U Taq DNA polymerase (Sigma), and about 50 ng of DNA template. Specific forward and reverse primers were used (i.e. LR12R and O-1, Veldman et al. 1981, Duchesne and Anderson 1990). The IGS-1 regions was amplified using a PCR program with an initial denaturation at 95 °C for 2 min, followed by 35 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 2 min, and one cycle of 72 °C for 30 min. For amplification of the EF-1 α region, the following PCR program was used: one cycle of 94 °C for 2 min, followed by 33 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min, and a final extension of 72 °C for 30 min. The PCR products were checked for successful amplification and their length was determined on 1.5% agorose gels.

Sequencing of the IGS-1. The PCR products were purified using PCR purification columns (MinElute PCR purification Kit, Qiagen) according to the manufacturer's instructions. Sequencing reactions were carried out with the Big Dye Terminator Cycle Sequencing Reaction v3.1 Kit (Applied Biosystems) following the protocol provided by the manufacturer. Post-reaction cleanup was performed using DTR Gel Filtration Cartridges (Edge BioSystems) according to the manufacturer's protocol. Each region was sequenced separately in both directions using the above mentioned specific primers. The sequences were generated using an ABI PRISM 3100 Avant automatic DNA sequencer (Applied Biosystems).

Sequence analyses. All sequences were edited manually using the software GeneStudio (TM) Professional Edition Version 2.1.2.3 and then analyzed with CLC Sequence Viewer Version: 4.6.1 (CLC bio A/S). The sequences were deposited in the NCBI database.

Phylogenetic analyses. Phylogenetic tree were constructed using the maximum parsimony method as implemented in MEGA 4 (Tamura et al. 2007). Most parsimonious (MP) trees were generated after a heuristic tree search. Additional sequences of A. tabescens specimens from different geographical localities worldwide were downloaded from the NCBI database (see Table 1).

Sequences of haploid tester strains of the five annulated European Armillaria species (A. borealis Maxmuller & Korhonen – A2; A. cepistipes (Velenovsky) – B2; A. ostoyae (Romagnesi) Herink – C5; A. mellea (Vahl:Fr.) Kummer – D4; A. gallica Marxmüller & Romagnesi – E5) were included in the analyses as outgroup. The indels (inserted/deleted sites) were included in the sequences and treated as fifth character for trees generated on the base of non coding rDNA regions. Branch swapping was based on close neighbor interchange with search level 2. Starting trees were obtained with 100 random taxon additions. Tree length distribution of 100 randomly generated trees was determined for the phylogenetic signal (Hillis and Huelsenbeck 1992). Confidence in branching points on the phylogenetic trees was determined by bootstrap analysis (1000 replicates) (Felsenstein 1985).

Results and Discussions

Ecology and distribution.

We recorded the occurrence of A. tabescens in four different districts of the Transcarpathia region of Ukraine (i.e. Uzhhorodskyi, Berehovskyi, and Vynohradivskyi, Mukachevskyi districts). Fruiting bodies were found at low elevation (up to 200 m a.s.l.) in forests dominated by Quercus robur or Quercus cerris. In contrast, no fruiting bodies were observed in coniferous forests.

In Europe, A. tabescens is mainly considered a saprophytic or weak pathogenic species on native broadleaves trees (Guillaumin et al. 1993, Rishbeth 1985, Intini et al. 1994, Keca et al., 2009, Antonin et al. 2006). However, in southern Europe this species causes significant mortality of ornamental trees and introduced Eucalyptus, and in orchards (Lushaj et al. 2009, Tsopelas 1999, Guillaumin et al. 1993). Similarly, in USA, Mexico, Japan and China A. tabescens affects exotic and fruit tree species (Rhoads 1954, Hasegawa 2005, Schnabel et al. 2005, Qin et al. 2007, Kim et al. 2010). Interestingly, in the same regions no mortality is observed in the surrounding native forests (Keane et al. 2000, Tsopelas 1999, Lushaj et al. 2009). In the literature A. tabescens is also mentioned as an important symbiont of the achlorophyllous orchid Galeola septentrionalis in Japan (Terashita and Schuman 1989).

In Ukraine, A. tabescens fruiting bodies were found on dead oak wood, i.e. butt or root collar of dead trees and stumps. Even when the clusters of fruiting bodies seemed to grow directly on the forest litter, they were always associated with woody substrates (e.g. roots, rests of stumps, branches). On apparently healthy trees no fruiting bodies were observed, except on a Carpinus betulus tree in a forest dominated by Quercus robur. In contrast to Antonin et al. (2006), in the investigated forests no rhizomorphs of A. tabescens were found in the soil. Sometimes, in our lowland forest stands A. gallica rhizomorphs were found together with A. tabescens, as previously observed in the Ozark Mountain forests in the United States (Bruhn et al. 2000).

Table 1. Characteristics of the additional *A. tabescens* isolates included in the phylogenetic analyses.

Article/ Author	Host/ origin/ source	Isolate	IGS-1
Kim *et al.* 2006/ Characterization of North American *Armillaria* species: genetic relationships determined by ribosomal DNA sequences and AFLP markers	Stipe/S.Carolina,USA/ Schnabel G	AT-MUS2	AY509190
	Basidioma/ Georgia, USA/ Schnabel G	OOI-99	AY509192
	Basidioma/ Georgia, USA/ Schnabel G	OOI-210	AY509191
Keca *et al*. 2006/ Molecular-based identification and phylogeny of *Armillaria* species from Serbia and Montenegro	Basidioma/ *Quercus robur*/ Subotica, n. Serbia	Qrob Subot	DQ115589
Shnabel *et al.* 2005/ Identification and characterization of *Armillaria tabescens* from the southeastern United States	Basidioma/ Seneca SC, USA	SC.MF-1.01	AY695414
Coetzee *et al.* 2003/ Discovery of two northern hemisphere *Armillaria* species on Proteaceae in South Africa	USA/Volk T	CMW3158	AY773966
	France/ Guillaumin JJ	CMW3165	AY773967
Keca *et al.* 2006/ Molecular-based identification and phylogeny of *Armillaria* species from Serbia and Montenegro	*Quercus robur*/ haploid culture/ France/ Chillali M	90	DQ115588
Kim *et al* 2010/ First Report of *Armillaria* Root Disease Caused by *Armillaria tabescens* on Araucaria araucana in Mexico	Mexico:Veracruz	MEX21WF-A	GQ335541
		MEX21WF-B	GQ335542
Hasegawa *et al.* 2010/ Identification of Japanese *Armillaria* species using elongation factor 1-alpha DNA sequence data	Japan	96_3_3	AB510824
		96_1_8	AB510823
		2006_20_01	AB510839
		2002_06_03	AB510844
Tsykun *et al.* 2010/ Identification and characterization of *Armillaria tabescens* from the Transcarpathia of Ukraine	*Quercus robur*/ Ukraine/ haploid culture	HAt1S5	HQ232284
	Quercus robur/ Ukraine/ haploid culture	HAt2S5	HQ232285
	Quercus robur/ Ukraine/ haploid culture	HAt5S3	HQ232286
	Betula/Finland/ haploid culture /Guillaumin JJ	A2	HQ232279
	Alnus/Finland/ haploid culture /Guillaumin JJ	B2	HQ232280
	Pinus/France/ haploid culture /Guillaumin JJ	C5	HQ232281
	France/ haploid culture/ Guillaumin JJ	D4	HQ232282
	Corylus/France/haploid culture/ Guillaumin JJ	E5	HQ232283

Typically, A. gallica rhizomorphs grew through the base of A. tabescens's caespitose basidiocarps. Molecular sequence analysis showed that rhizomorphs belonged to A. gallica (data not shown). In orchards surrounding the oak forests investigated, no A. tabescens's fruiting bodies were found. However, additional data would be necessary to exclude or confirm its presence in orchards.

The abundance of A. tabescens's fruiting bodies on dead wood suggests that this species is an important wood decomposer in the oak forests of the lowland region of Transcarpathia.

Morphological-microscopic characteristic.

We have analyzed 60 basidiocarps from 20 clusters originating from four different localities. Morphological-microscopic characteristics of the basidiocarps and their spores conformed to previously reported data (Antonin et al. 2006, Schnabel et al. 2005).

Basidiocarp's clusters. A. tabescens was always found in caespitose clusters, usually composed of 8-10 basidiocarps (sometimes 50 or more), whose characteristics are shown in Table 2, Table 3, and Fig. 1.

Table 2. Macroscopic characteristics of the basidiocarps of *A. tabescens*.

Features	Description
Spore print	Colour: white
Pileus	Diameter: 30–90 mm broad. Form: conical-hemispherical when young, later convex-conical, then applanate, can be slightly undulate when old. Surface: hygrophanous, slightly pubescent at slightly radially striate margin when old. Flesh: whitish, do not change color in fracture. Color: ochraceous yellow when young, then brown at margin and when moist, and ochraceous yellow at centre and when dried out. Scales: tomentose-fibrillose scales when young, soon glabrescent at margin but fibrillose tomentum dark brown at center.
Lamellae	Character of clamping: distant, broadly adnate to slightly decurrent when young, then slightly emarginated with decurrent tooth, rather thick when young, irregular, anastomosed/ furcated when young, later not/slightly anastomosed. Color: orange-brown when young, then brown when old, irregularly stained.
Stipe	Annulus is absent. Velar remnants indistinct, in the form of scattered fibrillose scales when young and single small fibrils when old. Size: 30-130 x 3-15 mm Form: cylindrical or slightly laterally compressed, slightly broadened at apex, with cylindrical, slightly attenuated or broadened base. Tissue: lengthways fibrillose, then almost striate, fibrillose-flocculose at apex, whitish under the cortex and colour in surface, soon create hollow inside, has bright fungoid smell. Color: whitish when young, becoming brown from the base, then red-brown, finally entirely brown-grey-black.

Table 3. Microscopic characteristics of the basidiocarps of *A. tabescens*.

Features	Size, μm	Form	Other
Basidiospores	7-10x5-7	(broadly) ellipsoid, fusoid-ellipsoid, obovoid, rarely subglobose, non-symmetric	
Basidia	35-45x8-12	clavate holobasidia	4-spored
Basidiole	14-40x4-10	clavate, cylindrical holobasidioles	
Cheilocystidia	14-40x5-8	clavate, subfusoid, subcylindrical, subutriform, regular, irregular to coralloid, one- or more-celled	
Trama's hyphae	up to 12	wide cylindrical non-symetrical	
Pileipellis' hyphae	up to 10 wide	cylindrical, radially arranged	hyaline to pale-yellowish
Pileusscales	up to 12-75x5-18	chains of ellipsoid, cylindrical, subfusoid, conical or subulate (terminale scales)	incrusted, septate and yellow-brown pigmented
Stipitipellis	6-7	cylindrical, parallel hyphae	
Caulocystidia	15-40x7-14	adpressed or erect, clavate, cylindrical, fusoid, irregular, one- or more-celled or in the form of cell's chains	
Basal mycelium's hyphae	up to 5 wide	smooth, cylindrical	
Clamp connections			hymenium and subhymenium only

Figure 1. Basidiocarps of *A. tabescens* from the Transcarpathian region of Ukraine.

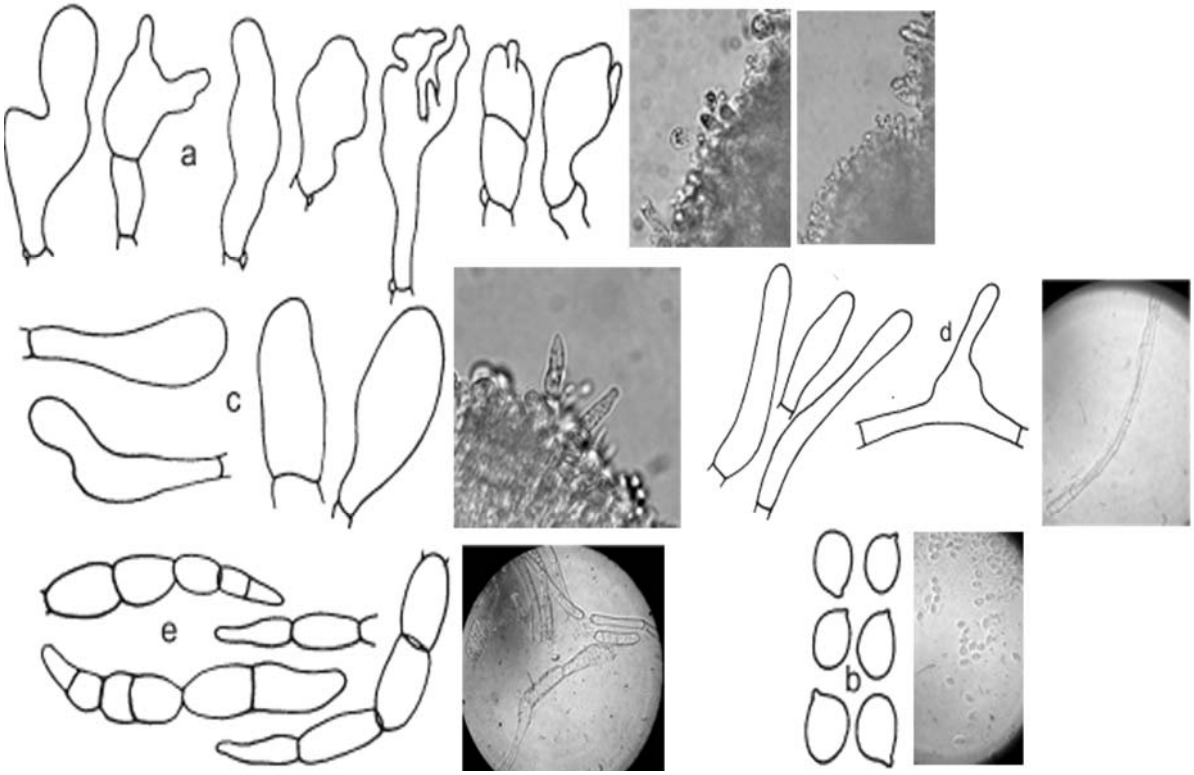

Figure 2. Microscopic characters of A. tabescens: a. cheilocystidia, b. basidiospores, c. caulocystidia, d. cells of basal mycelium, e. pileus scales (drawings: Antonin et al. 2006; pictures: T. Tsykun).

Molecular analyses

The selected DNA regions were successfully amplified by PCR (Table 1). Sizes of the PCR products (Table 4) were comparable with those previously reported by Harrington and Wingfield (1995). Comparison of the sequences obtained with sequences present in GenBank showed a high similarity for all three DNA regions considered (Table 4).

The traditional molecular method for the identification of Armillaria species developed by Harrington and Wingfield (1995) is also useful for routine discrimination of A. tabescens. This method could be particularly appropriate when the analysis of the fruiting bodies is not possible or when A. tabescens co-exist with other Armillaria species, as A. mellea or A. gallica (Bruhn et al. 2000). In these cases, DNA could be extracted from different fruiting bodies and the nuclear encoded ribosomal intergenic spacer 1 (IGS-1) region could be amplified by PCR. Finally, PCR products would be digested with the restriction enzymes AluI, HindII (or HincII), BsmI (or Mva1269I), and NdeI. Armillaria tabescens produces a unique restriction pattern with enzymes BsmI (or Mva 1269I) and HindII (or HincII) (Table 4).

Maximum parsimonious analyses showed geographical congruence for newly sequenced specimens from Ukraine in the IGS-1 region (Fig. 3, Table 4). All sequences of A. tabescens formed one, well supported, monophyletic clade. Inside this clade, three geographical groups were formed, i.e. Japan, North America and Europe. The three newly sequenced Ukrainian specimens formed a clade with A. tabescens isolates from other European localities, e.g. from France and Serbia.

Table 4. Length of the PCR amplicons, similarity with *A. tabescens* sequences from GenBank, and restriction patterns of the IGS-1 of the *A. tabescens* isolates analyzed in this study.

Length of PCR amplicons, bp	~ 845
Similarity with *A. tabescens* from GenBank (%)	96-99%
Fragment's sizes of enzyme digestion:	
HAt1S5; HAt2S5; HAt5S3	*Alu*I: 77;422;45;61;240 *Mva1269*I: 324;583 *Nde*I: 0 *Hinc*II: 368;539
A2	*Alu*I: 77;308;91;104;31;47;9;49;191 *Mva1269*I: 0 *Nde*I: 548;359 *Hinc*II: 0
B2	*Alu*I: 77;308;93;135;47;14;39;194 *Mva1269*I: 0 *Nde*I: 0 *Hinc*II: 584;323
C5	*Alu*I: 77;308;92;136;47;9;5;40;193 *Mva1269*I: 0 *Nde*I: 550;357 *Hinc*II: 0
D4	*Alu*I: 77;317;80;151;5;175;70 *Mva1269*I: 567;340 *Nde*I: 0 *Hinc*II: 0
E5	*Alu*I: 77;401;182;14;233 *Mva1269*I: 0 *Nde*I: 0 *Hinc*II: 584;323
Variable positions in aligned sequences of *A. tabescens**:	Total 21: 346; 349; 421; 434; 435; 463; 473; 500; 547; 551; 552; 571; 593; 601; 633; 634; 650; 681; 722; 755; 773
Total number of positions in the final dataset for MP tree	940
From them parsimony informative positions	142
Consistency index (CI) Retention index (RI)	0.858 0.935
Total number of equally parsimonious trees	805

*Isolates were included in alignment described in Table 1. Gaps and ambiguous positions were excluded from analysis. A position was considered as variable if variety was met more than in one aligned sequences.

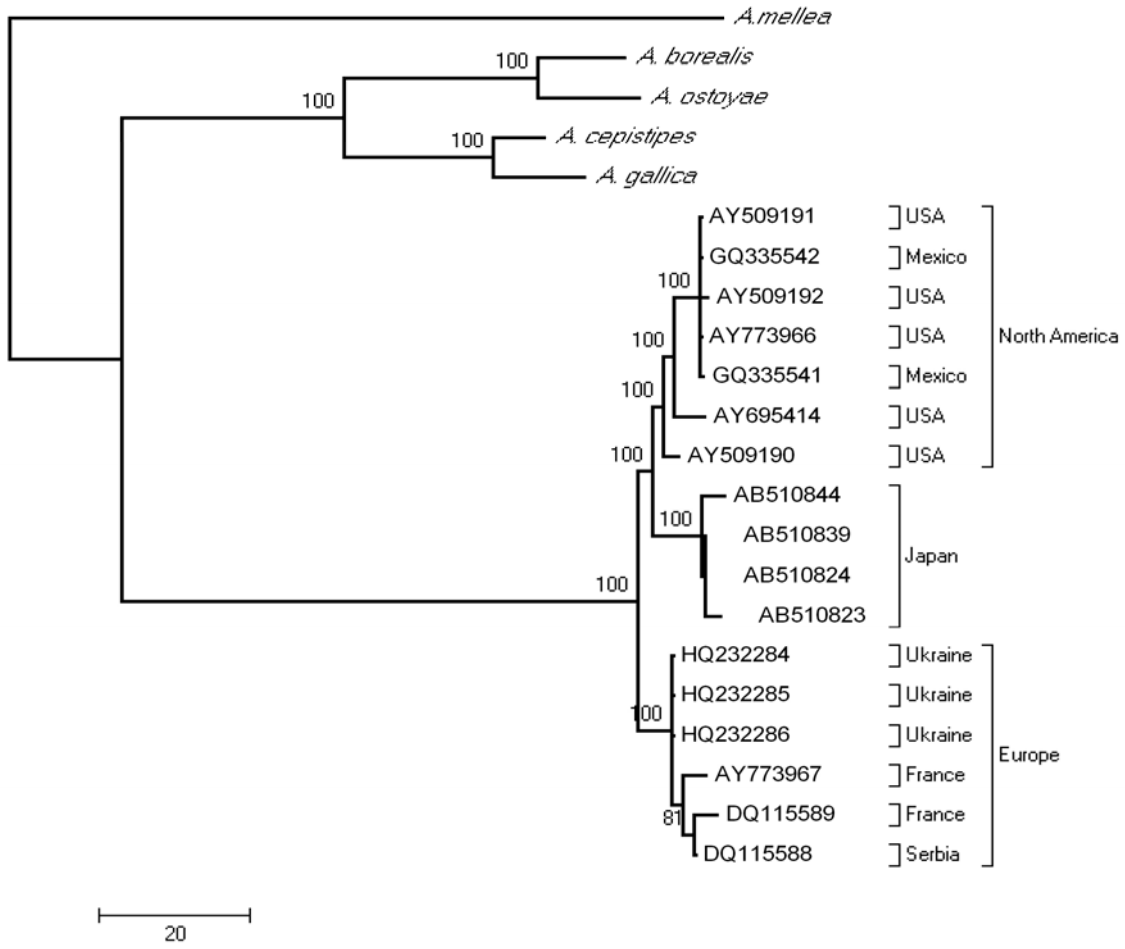

Figure 3. Phylogenetic tree based on the parsimony analysis of the nucleotide sequences of the IGS-1 region. Branch length is proportional to phylogenetic distance. Bootstrap (1000 replicates) values greater than 50 % are shown above the tree branches.

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