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IDENTIFICATION OF ECTOMYCORRHIZAL TYPES IN A WHITE POPLAR *(POPULUS ALBA* L.) PLANTATION NEAR NOvi SAD

Identifikacija tipov ektomikorize v nasadu belega topola *(Populus alba* L.) pri Novem Sadu

Abstract: Our objective was to describe and identify types of ectomycorrhiza in a white poplar (Populus alba L.) plantation in the vicinity of Novi Sad, Vojvodina. Identification was achieved by combining morphological and anatomical descriptions with molecular methods (sequencing of ITS region in ribosomal DNA). Distinct types of ectomycorrhiza were observed and they were photographed and briefly described according to morphological, anatomical and molecular characteristics. Four types of ectomycorrhiza were identified to the species level (Tuber rufum Pico, *Inocybe squamata* J.E. Lange, *Hebeloma vaccinum* Romagn, *Hymenogaster citrinus* Vittad), *six were determined to the genus level (Tomentella sp. 1 Tomentella sp. 2, Tomentella* sp. 3, *Tomentella sp. 4, Inocybe sp., Tuber sp. 1, Tuber sp. 2), additional two types of ectomycorrhiza were determined to the family level of the fungal partner (Cortinariaceae and Thelephoraceae), one type was identified to the ordo level (Agaricales) and one ectomycorrhizal fungus was only classified as belonging to Ascomycetes.*
Key words: ectomycorrhiza, white poplar, morphological-anatomical characterization, rDNA ITS region, sequencing

Izvleček: Namen raziskave je bil opisati in identificirati tipe ektomikorize v nasadu belega topola (Populus alba L.) *pri Novem Sadu v Vojvodini. Tipe smo identificirali s kombinacijo morfoloških in anatomskih opisov z molekularnimi metodami (sekveniranje ITS regije v ribosomalni DNK). Mikroskopske preparate ektomikorize smo posneli in na kratko opisali. Do vrste smo identificirali štiri tipe ektomikorize (Tuber rufum Pico, Inocybe squamata* J. E. Lange, *Hebeloma vaccinum* Romagn, *Hymenogaster citrinus* Vittad), *šest do rodu (Tomentella sp. 1 Tomentella sp. 2, Tomentella sp. 3, Tomentella sp. 4, Inocybe sp., Tuber sp. 1, Tuber sp. 2), dva tipa ektomikorize smo identificirali do nivoja družine glivnega partnerja (Cortinariaceae in Thelephoraceae), en tip je bil identificiran le to nivoja reda (Agaricales) in eden le do skupine Ascomycetes.*
Ključne besede: ektomikoriza, beli topol, morfološko-anatomska identifikacija, ITS regija rDNK, sekveniranje

1. INTRODUCTION

Establishment, growth and survival of trees in most temperate and boreal forests depend on colonization with ectomycorrhizal (ECM) fungi. Mycelium of ectomycorrhizal fungi represents the main component of forest ecosystems which link biotic with abiotic factors. ECM fungi successfully take water, organic and inorganic nutrients from soil and translocate them to fine roots of plants from which they obtain carbohydrates in return (Smith and Read, 2008).

Seedlings colonized with appropriate fungal species and strains are favored in comparison to uncolonized ones in making contacts with water and nutrients as well as with other organisms in soil (Kraigher, 1996). It has been demonstrated by several investigators (Molina et al., 1992; Smith and Read, 2008) that in addition to increased nu-

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trient uptake, which is the most significant benefit of mycorrhiza, this symbiotic relationship offers numerous other benefits: enhanced plant efficiency in absorbing water, reduced fertilization and irrigation requirements, increased drought tolerance, increased pathogen resistance, protection against damage from heavy metals and other pollutants, minimization of various plant stresses, improvement of seedling growth and survival and improvement of soil structure by the extramatrical hyphal network.

The functional compatibility and stress tolerance of ectomycorrhizal types is species specific, and therefore the information on the ectomycorrhizal community structure can provide valuable information about physiology of forest trees and functioning of forest ecosystems (Kraigher et al., 2007).

In the last decade, poplars have become one of the most interesting tree species for biotechnology. Besides their commercial importance, they combine many biotechnological advantages, such as rapid growth, simple *in vitro* propagation and the existence of genetic transformation systems (Fladung and Ahuja, 1996). Also, poplars are good candidates for use in phytoremediation because they have deep roots, cycle large amounts of water and grow rapidly (Newman et al., 1997). Poplars routinely form functional mycorrhizal associations with ectomycorrhizal (EM) fungi and arbuscular mycorrhizal (AM) fungi simultaneously (Molina et al., 1992) which can benefit them in establishment and growth in extreme conditions and makes them suitable for reforestation and reclamation purposes.

Since ectomycorrhizae are significantly beneficial for their host, the aim of this work was to describe and identify types of ectomycorrhiza in a white poplar *(Populus alba* L.) plantation in vicinity of Novi Sad, Vojvodina.

2. MATERIAL AND METHODS

1. SAMPLING

ECM roots were isolated from soil samples collected in the experimental field of the Institute of lowland forestry and environment (N 45° 17' 25, 5" E 19° 53' 38", 73 m a. s. l.) placed near village Kać, 20 km from Novi Sad, Srbija. The average annual precipitation is 597 mm and the average yearly temperature is 11℃ (Katić et al., 1979). Climate is temperately continental and the soil type is classified as fluvisol. The sampling plot is a white poplar plantation of cca twenty years old poplar trees mixed with *Vitis vinifera* L., *Robinia pseudoacacia* L., *Acer negundo* L., *Sambucus nigra* L., and *Cornussanguinea* L. The site is not flooded and the ground water level at the site oscilated below 2 m. Also, humidity of the soil surface layer was low.

Soil samples were collected in August 2007 and November 2008, at a distance of about 1 m from the tree trunk; the same five trees were included in both samplings. A soil core of 274 ml volume, 3,5 cm in diameter and 18 cm deep, was used for taking standardized samples (Kraigher, 1999). The total number of samples was ten, 5 taken in 2007 and 5 in 2008. Soil samples were stored at 4 °C for up to one month and one day prior to analysis submerged in cold water. Roots were carefully washed from soil and ECM tips were selected and separated in water under a dissecting microscope.

2. IDENTIFICATION OF ECTOMYCORRHIZAE

Identification of the fungal partner in ectomycorrhiza was achieved by combining morphological and anatomical descriptions with molecular methods. Types of ECM were analyzed and identified after morphological and anatomical characteristics with a binocular Olympus SZX 12 and microscope Olympus BX 51 (enlargement 100-2000x) according to published descriptions (Agerer, 1987-2002; Agerer et al., 2001-2006) and methodology described by Kraigher (1996). Identification with molecular methods was based on PCR amplification of ITS (Internal Transcribed Spacer) regions within nuclear ribosomal DNA. This molecular marker is considered as the best for fungus differentiation at the species level (Gardes and Bruns, 1993). After DNA extraction and amplification of the ITS region with ITS 1f and ITS 4 primer pair, excision and purification (Wizard® SV Gel and PCR Clean-up System (Promega)), sequencing was performed commercially at Macrogen Inc. (Seoul, Rep. of Korea). Species, genus or family of ectomycorrhizal fungi were determinated by comparing our sequence to the ones found in GenBank database (GenBank sequence with maximal identity to our sequence is given in Table 1). New sequences were added to and also published in GenBank. The detailed protocol for identification of ECM types with molecular methods was described by Katanić et al. (2008).

3. RESULTS AND DISCUSSION

In the white poplar plantation, fifteen ectomycorrhizal types were described and identified (a selection is presented in Figure 1). Four types of ectomycorrhiza were identified to the species level *(Tuber rufum* Pico, *Inocybe squamata* J.E. Lange, *Hebeloma vaccinum* Romagn, *Hymenogaster citrinus* Vittad), six were determined to the genus level *(Tomentella sp. 1 Tomentella sp. 2, Tomentella sp. 3. Tomentella sp. 4, Inocybe sp., Tuber sp. 1, Tuber sp.* 2), additional two types of ectomycorrhiza were determined to the family level of the fungal partner (Cortinariaceae and Thelephoraceae), one type was identified to ordo level (Agaricales) and one ectomycorrhizal fungus was only

Table 1. Descriptions of ectomycorrhizal types and molecular identification of fungal partner from the white poplar plantation

Designated identity code of ECM type	Ramification	Shape	Surface	Colour	rhizo-morphs	Mantle anatomy	Hyphae	Anastomo-ses	Cystidia	Molecular identifica-tion (GenBank code, max identity%)
MK001-Srb001-SLO10001	Irregularly pinnate., monopodial-pinnate	straght, bent	smooth	brown, golden	not observed	plectenchymatous and pseudoparen-chymatous	not observed	not obser-ved	not obser-ved	*Inocybe squamata* J.E. Lange (AM882780, 99%)
MK002-Srb002-SLO10002	simple, monopodial-pinnate	slightly bent	smooth	dark bro-wn, brown, golden-brown	not observed	pseudoparenchyma-tous -Q, between M and L type acoording to Agerer (1987-2002)	wavy, Intrahyphal hyphe	not obser-ved	not obser-ved	*Tuber rufum* Pico (EF362475, 99%)
Tip MK003-Srb003-SLO10003	simple	straight	smooth, shiny, with sand	cream	not observed	plectenchymatous and transitional - H type according to Agerer (1987-2002)	not observed	not obser-ved	not obser-ved	*Inocybe* sp. (FJ210736.1, 96%)
Tip MK004-Srb004-SLO10004	simple, mono-podial pinnate monopodial pyramidal	straight, slightly bent	grainy	golden	observed	pseudoparen-chymatous -M type according to Agerer (1987-2002), plecten-chymatous	with clamps	not obser-ved	N type according to Agerer (1987-2002)	*Tomentella* sp. based on anatomical characterisation
Tip MK005-Srb005-SLO10005	simple, monopodial-pinnate	straight, slightly bent	cottony, long spiny, with soil	dark bro-wn, mat	not observed	pseudoparen-chymatous -L type according to Agerer (1987-2002), rozet-ta like	with clamps	not obser-ved	not obser-ved	*Thelephoraceae* (DQ150115.1,96 %)
Tip MK006-Srb006-SLO10006	simple, mono-podial-pinate, monopodial-pyramidal	straight, slightly bent	smooth, grainy with sand	white with brown spots, or brown with white spots	A type according to Agerer (1987-2002)	pseudoparenchyma-tous -L, M types according to Agerer (1987-2002), plecten chymatous	with clamps	not obser-ved	not obser-ved	*Cortinariaceae* (FJ210730.1, 99 %)
MK007-Srb007-SLO10007	simple, monopodial-pinnate	straight	smooth	brown-gol-den	not observed	pseudoparenchyma-tous -Q, L types according to Agerer (1987-2002), plecten-chymatous	not observed	not obser-ved	not obser-ved	*Tomentella* sp. (EU 668207.1, 97 %)
MK008-Srb008-SLO10008	simple, monopodial-pinnate	straight, bent	smooth, short spiny	light brown	not obser-ved	pseudoparenchyma-tous -P, L types according to Agerer (1987-2002), plecten-chymatous	with clamps	not obser-ved	with clamps	*Tomentella* sp. (DQ974780.1, 96 %)
MK009-Srb009-SLO10009	simple, monopodial-pyramidal	straight, bent	woolly-cot-tony	dark brown	not obser-ved	pseudoparen-chymatous -P type according to Agerer (1987-2002)	with clamps, numerous, with ramifications	not obser-ved	not obser-ved	*Tomentella* sp. (EU668215.1, 96 %)
MK010-Srb010-SLO10010	simple, pinnate	straight, bent	smooth	light brown,	not obser-ved	pseudoparen-chymatous -M type according to Agerer (1987-2002)	not observed	not obser-ved	A type according to Agerer (1987-2002)	*Tuber* sp. (AJ879691.1, 98 %)
MK011 -Srb011 - SLO10011	simple, pinnate	straight, bent, tortuous	smooth	light bro-wn, brown, sometimes tips are lighter	not obser-ved	pseudoparen-chymatous -M type according to Agerer (1987-2002)	not observed	not obser-ved	A type according to Agerer (1987-2002)	*Tuber* sp. (AY634173.1, 99 %)
MK012-Srb012-SLO10012	simple, pinnate	straight, bent	smooth with em. hyphae or woolly-cottony	white, shiny, cre-am, white with brown spotts	not obser-ved	plectenchymatous -C type according to Agerer (1987-2002)	with clamps	not obser-ved	not obser-ved	*Agaricales* (AJ879662.1, 99 %)
MK013-Srb013-SLO10013	simple, pinan-te, pyramidal	bent, tortuous	woolly-cot-tony	white, shiny, cream	observed	plectenchymatous- B type according to Agerer (1987-2002)	with clamps and septas, ramifi-cated	observed	not obser-ved	*Hebeloma vaccinum* Romagn (AY320396.1, 99 %)
MK014-Srb014-SLO1014	simple, pinnate	bent, tortuous	smooth	brown	not observed	pseudoparenchyma-tous -M , L types according to Agerer (1987-2002)	not observed	not obser-ved	not obser-ved	*Ascomycete* (AY969730.1, 95 %)
MK015-Srb015-SLO10015	simple, pinnate	straight	smooth	brown	not observed	pseudoparenchyma-tous -M , L type according to Agerer (1987-2002)	not observed	not obser-ved	not obser-ved	*Hymenogaster citrinus* Vittad (EU784360.1, 99 %)

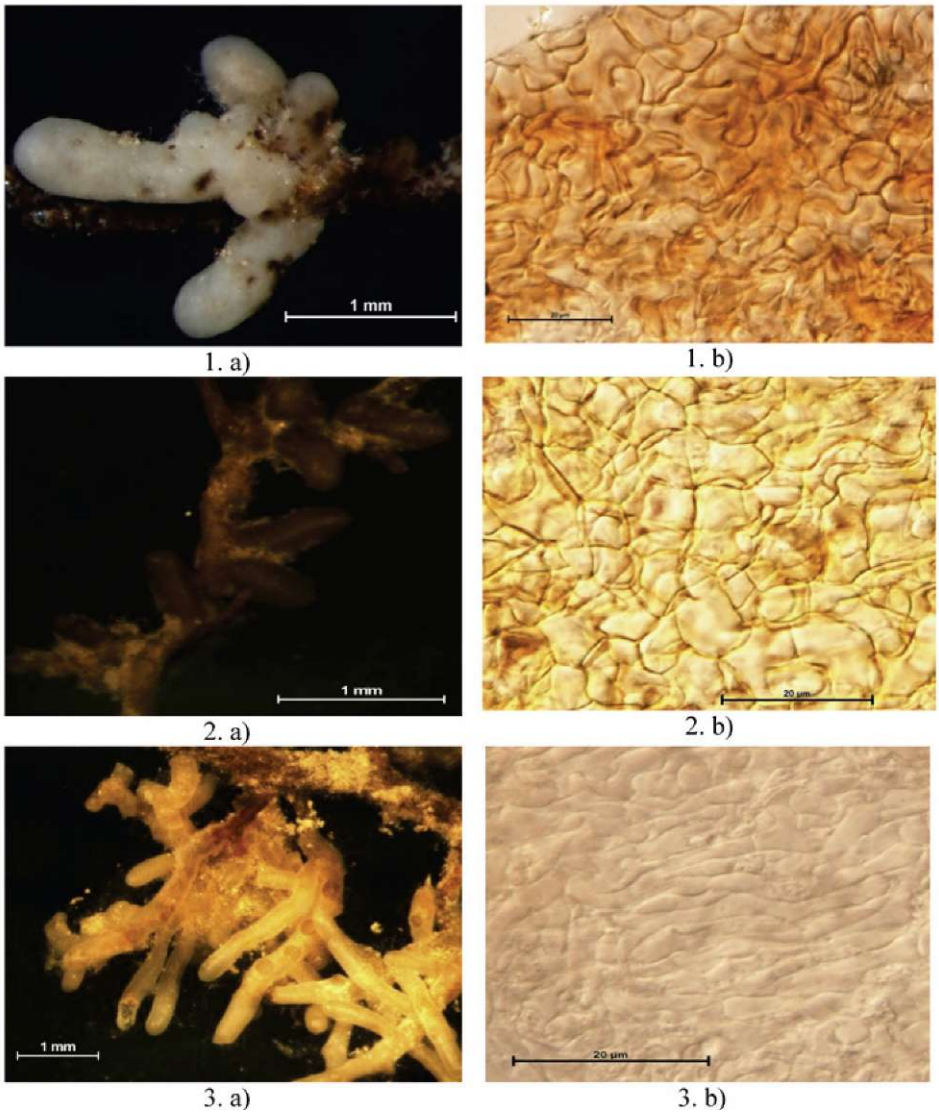

Figure 1. Selected ectomycorrhizal types from the white poplar plantation near Novi Sad 1.a) - Mycorrhizal system of ectomycorrhizal type MK006-Srb006,1.b) - plectenchymatous mantle of ectomycorrhizal type MK006-Srb006, bar represents 20 ym (photo Katanić, 2007); 2.a) Mycorrhizal system of ectomycorrhizal type MK007-Srb007,2.b) - pseudoparenchymatous mantle of ectomycorrhizal type MK007-Srb007, bar represents 50 ym (photo Katanić, 2008); 3.a) Mycorrhizal system of ectomycorrhizal type MK012-Srb012,3.b) - plectenchymatous mantle of ectomycorrhizal type MK012-Srb012, bar represents 20 ym (photo Katanić, 2008)

classified as an Ascomycete. The characterised ECM types are described in Table 1. The abundance of different ECM types was not quantified, however, *Tuber rufum* Pico and type MK006-Srb006-SLO10006 (Cortinariaceae) dominated in 2007 samples and type MK012-Srb012-SLO10012 (Agaricales) was present in most samples in 2008.

Among fifteen ectomycorrhizal types identified in the white poplar plantation, four types belonged to genus *Tomentella* and one to Thelephoraceae family, meaning that Tomentella species were very abundant at the investigated site. Members of Thelephoraceae family, especially genus *Tomentella* are known members of ectomycorrhizal community on poplars. According to Jakucs et al. (2005) tomentelloid ECM are constant, diverse and abundant members of the ECM communities in temperate continental broad-leaved forests. Species *T. pilosa* Burt and *T. subtestacea* Bourdot& Galzin are described in detail in *Colour Atlas of Ectomycorrhizae* (Agerer, 1987-2002) as exclusive poplar symbionts. Since five types of the fifteen found on the plantation belong to Thelephoraceae, this

work showed a relatively high abundance of this family in our experimental field.

Examining the ECM community of transgenic hybrid aspen Kaldorf et al. (2004) observed 23 morphotypes. However, six mycobionts dominated, forming roughly 90 % of all ectomycorrhizas: *Cenococcumgeophilum, Laccaria sp, Phialocephala fortinii,* two different members of Thelephoraceae family, and one member of the Pezizales.

Investigating ECM communities associated with aspen growing in a heavy metal contaminated site, Krpata et al. (2008) recorded 54 ECM types. The taxonomical groups with the highest belowground species richness were *Tomentella* (17 species), *Inocybe* (6), *Cortinarius* (5), *Hebeloma* and *Tuber* (each 3).

In mixed wood dominated by American aspen, Visser et al. (1998) recorded 22 types of ecomycorrhizal fungi. Dominating types, which were also most abundant in all treatments were *Cenoccocum geophilum, Cortinarius spp, Russula spp.* and *Tomentella spp.* Few years later, Cripps (2001) recorded over 54 species of ectomycorrhizal fungi occurring in symbiosys with the same species. The fungi were all Basidiomycota, primarily Agaricales, and one Aphyllophorales *(Thelephora terrestris* Fr.) distributed in seven families: Amanitaceae, Russulaceae, Tricholomataceae, Cortinariaceae, Paxillaceae, Boletaceae, and Thelephoraceae. The dark-spored Cortinariaceae was the most diverse family, with 25 species of *Cortinarius, Inocybe* and *Hebeloma* occurring in aspen stands. Members of Cortinariaceae family were also abundant in our plantaion.

Although the fungus *Cenococcum geophilum* is a generalist often found on forest trees and poplars especially in dry soil conditions (Visser et al., 1998; Kaldorf et al., 2004; Krpata et al., 2008) it was not found in our investigation. We could speculate that this is due to a relatively low number of samples or because of dominance of *Tuber* rufum and type MK006-Srb006-SL010006 (Cortinariaceae) in 2007 and type MK012-Srb012-SL010012 (Agaricales) in 2008.

Differences in composition of ECM fungi between localities depend on different plant species as a host and on different site conditions. Also, ectomycorrhizal fungi are unevenly distributed in the soil because water and nutrients in soils also have an uneven distribution. It is also known, that diversity and abundance of mycorrhizal fungi varies with changes of their habitats, and that ECM fungi can be used as an indicator of environmental pollution (Kraigher et al., 2007). In order to determine the condition of the poplar plantation and poplar trees and monitor its changes, diversity of ectomycorrhize should be monitored seasonally, which will also be our next approach. Plantation management should be adjusted to current condition of poplar trees.

4. LITERATURE

1. **Agerer R. (Ur., 1987-2002)** Colour Atlas of Ectomycorrhizae 1st-12th Edition. Einhorn-Verlag, Schwäbisch Gmünd, Germany
2. **Agerer R., Danielson R.M., Egli S.,Ingleby K., Luoma D., Treu R. (Ur., 2001- 2006)** Descriptions of ectomycorrhizae 1st - 10th Vol. Einhorn-Verlag, Schwäbisch Gmünd, Germany.
3. **Cripps C. L. (2001)** Mycorrhizal Fungi of Aspen Forests: Natural Occurrence and Potential Applications USDA Forest Service Proceedings RMRS-P-18.
4. **Fladung M., Ahuja M. R. (1996)** Gene transfer in aspen. Schmidt E. R. Hankeln T. (eds)Transgenic organisms and biosafety. Springer, Berlin Heidelberg New York
5. **Gardes M., Bruns T. D. (1993)** ITS primers with enchanced specificity for basidiomycetes- application to the identification of ectomycorrhizae and rusts. Molecular ecology, 2: 113-118
6. **Jakucs E., Kovacs G. M., Szedlay G., Eros-Honti Z. (2005)** Morphological and molecular diversity and abundance of tomentelloid ectomycorrhizae in broad-leaved forests of the Hungarian Plain. Mycorrhiza, 15: 459-470
7. **Kaldorf M., Renker C., Fladung M., Buscot F. (2004)** Characterization and spatial distribution of ectomycorrhizas colonizing aspen clones released in an experimental field. Mycorrhiza, 14: 295-306
8. **Katanić M., Grebenc T., Hrenko M., Štupar B., Galić Z., Orlović S., Kraigher H. (2008)** First identification of ectomycorrhizal types in white poplars stand *(Populusalba* L.) near Novi Sad. Topola. 181/182: 49-59 (in Serbian)
9. **Katić P., Đukanović D., Đaković P. (1979)** Climate of SAP Vojvodina, Faculty of agriculture in Novi Sad Institute of field and vegetable crops (in Serbian)
10. **Kraigher H. (1996)** Tipi mikorize: taksonomija, pomen, aplikacija. Zbornik gozdarstva in lesarstva, 49: 33-66
11. **Kraigher H. (1999)** Diversity of types of ectomycorrhizae on Norway spruce in Slovenia. Phyton, 39, 3: 199-202
12. **Kraigher H., Al Sayegh-Petkovšek S., Grebenc T., Simon P. (2007)** Types of ectomycorrhiza as pollution stress indicators: case studies in Slovenia. Environ Monit Assess, 128, 1:31-45
13. **Krpata D., Peintner U., Langer I., Walter J. F., Schweiger P. (2008)** Ectomycorrhizal communities associated with Populus tremula growing on a heavy metal contaminated site. Mycological research, 112, 9: 1069-1079
14. **Molina R., Massicotte H., Trappe J. M. (1992)** Specificity phenomena in mycorrhizal symbiosys:community-ecological consequences and practical implications. In: Allen M.F. (ed) Mycorrhizal functioning: an integrative plant-fungal process. Chapman & Hall, New York, 357-423
15. **Newman L. A., Strand S.,E, Choe N., Duffy J., Ekuan G., Ruszaj M., Shurleff B. B., Wilmoth J., Heilman P., Gordon M. P. (1997)** Uptake and Biotransformation of Trichloroethylene by Hybrid Poplars. Environ. Sci. Technol., 31: 1062-1067
16. **Smith S. E., Read D. J. (2008)** Mycorrhizal symbiosis. Academic Press, Cambridge, 815
17. **Visser S., Maynard D., Danieson R. M. (1998)** Response of ecto- and arbuscular fungi to clear-cutting and the application of chipped aspen wood in a mixedwood site in Alberta, Canada. Applied Soil Ecology, 7: 257-269