Biosystematics of *Agrocybe molesta* and sibling species allied to *Agrocybe praecox* in North America and Europe

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Mating compatibility experiments and comparative morphology were used to investigate the *Agrocybe praecox* species complex in North America and Europe. This species complex was found to consist of four morphologically indistinguishable biological species plus an easily recognizable taxon, *Agrocybe molesta*. The traditionally used characteristics, such as spore dimensions and macroscopic morphology, are not diagnostic for species recognition. However, ecological characteristics such as habitat, substrate, and geographical origin, are more useful for recognizing taxa within the *A. praecox* species complex.

Key words: *Agrocybe*, Mating compatibility, Biological species, Sibling species.

Species complexes are commonly encountered in the Agaricales (Basidiomycetes). These groups of phenotypically similar species may result from either recent or slow morphological evolution. *Agrocybe praecox* (Pers.: Fr.) Fayod is the central taxon in a species complex which includes several allied taxa and numerous varieties (Watling, 1982; Watling & Gregory, 1981). Some of the allied taxa include *A. howeana* (Peck) Singer, *A. acericola* (Peck) Singer, *A. temnophylla* (Peck) Singer, *A. alachuana* (Murr.) Singer, *A. sphaleromorpha* (Bull.: Fr.) Fayod, *A. praecox* var. *cutifracta* (J. Lange) Singer, *A. praecox* var. *britzelmayrii* (S. Schulzer) Singer, *A. paludosa* (J. Lange) Kühner & Romagn., *A. praecox* var. *sylvestris*, and *A. gibberosa* (Fr.) Fayod. *Agrocybe molesta* (Lasch) Singer, another member of section Agrocybe, is clearly a distinct species but has been historically confused with *A. praecox* (Horak, 1968).

The taxa listed above were described because they were perceived to be discrete morphological entities. However, when a large number of these taxa are encountered and compared, the variation appears continuous. This variation makes species boundaries unclear, but provides an opportunity to investigate the biosystematic nature of a basidiomycete species complex. The continuous intergradation of several morphologically related taxa provides two hypotheses, either *A. praecox* is a single phenotypically variable taxon, or it is a group of several discrete taxa. If the latter is true, can diagnostic features be found to distinguish one species from another?

A better understanding of fungal species and evolution in fungi has been increased by mating compatibility studies (Burnett, 1983). The genetic structure of Basidiomycete populations is regulated by at least two incompatibility systems, homogenic and heterogenic. Homogenic incompatibility reduces inbreeding and promotes outcrossing (Koltin *et al.*, 1972), which reduces the loss of alleles through genetic drift. Heterogenic incompatibility (Hoffmann & Esser, 1978) is one of several probable mechanisms that establish genetic isolation by preventing either the coexistence of unlike nuclei in a common cytoplasm, or karyogamy in the basidium. Although its prevalence and molecular basis is not well known, heterogenic incompatibility establishes pre-zygotic isolation barriers.

Mating compatibility has been widely studied in members of the Agaricales (Lange, 1952; Farr *et al.*, 1977; Vilgalys & Miller, 1983; Kemp, 1975; Korhonen, 1978*a*), the Aphyllophorales (Macrae, 1967; Korhonen, 1978*b*; Chase & Ullrich, 1983; Parmasto, 1985; Boidin, 1986; Lemke, 1969; Ullrich, 1973), and Heterobasidiomycetes (Wong *et al.*, 1985; Wells & Wong, 1985). These studies have revealed many biological species which fall into three classes. The species may be (1) morphologically recognizable taxa; (2) morphologically similar but ecologically distinct; (3) both morphologically and ecologically indistinguishable.

The usefulness of mating compatibility is strengthened by the lack of documented interspecific hybridization and antagonistic homokaryotic interactions demonstrated by the dark barrage zones of *Hirschioporus* Donk (Macrae, 1967), and the lethal interactions of species related to *Coprinus congregatus* (Bull.) Fries (Kemp, 1970). However, degenerate hybrids of *Typhula* Fries have been forced in the laboratory, but are not found in nature (Christen & Bruehl, 1979). The evidence given above leaves no doubt that mating compatibility studies are helpful in developing species concepts in the Agaricales. This is the approach which we have adopted in our study of the *A. praecox* species.

Table 1. Sources of stock material used for mating experiments

Isolate code	Collector's number	Location	Date	Substrate/habitat	Mating types recovered
Species I					
QB1	CG84/27	Quebec, Canada	v.84	Mulch/campus	3
NJ1	GB750	New Jersey	v.84	Mulch/suburb	4
NJ2	GB754	New Jersey	v.84	Mulch/suburb	4
NJ3	GB751	New Jersey	v.84	Mulch/suburb	3
NY1	TF546	New York	v.84	Mulch/suburb	3
NJ5	GB747	New York	v.84	Mulch/suburb	nd
NJ4	GB749	New Jersey	v.84	Mulch/suburb	nd
NY3	GB755	New York	v.84	Mulch/suburb	3
NY4	CHAM1795	New York	v.84	Mulch/campus	3
VA1	TF350	Virginia	v.84	Mulch/garden	3
VA2	TF532	Virginia	v.84	Mulch/campus	nd
VA5	TF780	Virginia	v.85	Mulch/suburb	nd
VA6	TF795	Virginia	v.85	Mulch/surburb	nd
D1	TF877	BDR	vi.85	Spruce; larch/forest trial	nd
DK1	RV84/161	Denmark	vi.84	Deciduous/forest trail	3
DK2	RV84/163	Denmark	vi.84	Deciduous/forest trail	3
DK3	RV84/164	Denmark	vi.84	Deciduous/forest trail	4
F1	RV84/66	France	vi.84	Sequoia/trail	3
GB1	TF554	Scotland	vi.84	Grass	4
NL1	RV84/112	Netherlands	vi.84	Deciduous litter/meadow	nd
NL2	RV84/121	Netherlands	vi.84	Deciduous litter/meadow	nd
NL3	RV84/110	Netherlands	vi.84	Deciduous litter/meadow	nd
NL4	RV84/113	Netherlands	vi.84	Deciduous litter/meadow	nd
NL6	PIP1476	Netherlands	vi.85	Litter/replanted forest	nd
NL5	PIP1475	Netherlands	vi.85	Litter/replanted forest	nd
NL7	JVD6/5	Netherlands	vi.85	Litter/replanted forest	nd
MT1	TF80	NW Montana	vii.83	Conifer/forest trail	4
MT2	TF114	NW Montana	vii.83	Conifer/forest trail	3
MT3	TF817	NW Montana	vi.85	Conifer/forest trail	nd
MT4	TF818	NW Montana	vi.85	*Betula*/forest	nd
WA1	SAR84/117	Seattle, WA	v.84	Mulch/campus	4
ID11	OKM20133	NC Idaho	vi.84	Conifer/forest trail	4
Species II					
CA1	TF488	N California	iii.84	Soil/shrubs	4
CA2	OKM20987	N California	vi.84	Manured conifer/corral	nd
CO1	TF674	C Colorado	viii.84	Aspen/forest	3
CO2	TF697	C Colorado	viii.84	Aspen, spruce/forest	4
AL2	TF837	SW Alberta	vii.85	Conifer/forest	nd
AL3	TF839	SW Alberta	vii.85	*Salix*/alpine bog	nd
AL4	TF847	SW Alberta	vii.85	Conifer/picnic area	nd
ID1	OKM20119	NC Idaho	vi.84	Conifer/forest	4
ID2	OKM20120	NC Idaho	vi.84	Conifer/logging road	4
ID3	OKM20123	NC Idaho	vi.84	Conifer/forest	3
ID4	TF799	NC Idaho	vi.85	Conifer/logging road	nd
ID5	TF800	NC Idaho	vi.85	Conifer/logging road	nd
ID6	TF802	NC Idaho	vi.85	Conifer/logging road	nd
ID7	TF813	NC Idaho	vi.85	Conifer/logging load	nd
ID8	TF814	NC Idaho	vi.85	Conifer/logging road	nd
ID9	TF815	NC Idaho	vi.85	Conifer/road	nd
ID10	TF816	NC Idaho	vi.85	Conifer/forest	nd
MT5	TF819	NW Montana	vi.85	Conifer/forest	nd
MT6	JLG43	NW Montana	vi.85	Conifer/forest	nd
OR1	OKM20113	Oregon	v.84	Manured conifer/corral	3
CH1	OKM21272	Switzerland	viii.84	Conifer/forest stream	3
Species III					
VA3	TF576	SW Virginia	vii.84	Maple/campus	3
VA4	TF596	SW Virginia	vii.84	Maple, oak/forest	3
VA7	TF798	SW Virginia	v.85	Maple, sycamore/forest	nd
VA8	TF894	SW Virginia	vi.86	Maple, elm, ash/forest	nd
MA1	TF905	Massachusetts	viii.86	Maple, ash/forest	nd
MA2	TF913	Massachusetts	viii.86	Maple, ash/forest	nd
Species IV					
DK4	RV84/165	Denmark	vi.84	Deciduous-conifer/forest	3
DK5	RV84/166	Denmark	vi.84	Deciduous-conifer/forest	4

Table 1. (*cont.*)

Isolate code	Collector's number	Location	Date	Substrate/habitat	Mating types recovered
Agrocybe molesta					
	TF405	Virginia	ix.83	Grass/lawn	nd
	TF528	Virginia	v.84	Grass/disturbed lawn	nd
	TF529	Virginia	v.84	Grass/disturbed lawn	nd
	TF536	Virginia	v.84	Grass/lawn	3
	TF538	Virginia	v.84	Grass/lawn	nd
	CG84/28	Quebec	vi.84	Grass/campus	4
	TF559	Virginia	vi.84	Grass/disturbed lawn	nd
	TF565	Virginia	vii.84	Grass/campus	nd
	TF566	Virginia	vi.84	Grass/lawn	nd
	TF567	Virginia	vii.84	Grass/lawn	nd
	TF569	Virginia	vii.84	Grass/lawn	nd
	TF573	Virginia	vii.84	Grass/lawn	nd
	TF573	Virginia	vii.84	Grass/lawn	nd
	TF583	Virginia	vii.84	Grass/lawn	nd
	TF640	Virginia	vii.84	Grass/campus	3
	TF683	Colorado	viii.84	Grass/riparian	nd
	OKM21914	Hawaii	x.84	Grass/trail	nd
	TF791	Virginia	v.85	Grass/lawn	nd
	TF885	Virginia	v.85	Grass/campus	nd
	TF886	Virginia	v.85	Grass/fairy ring	nd
	TF915	Massachusetts	vii.85	Grass/lawn	nd

nd = not determined; NW = north west; SW = South West; C = Central; NC = north central.

MATERIALS AND METHODS

Holotypic and authentic specimens were obtained and studied. Living specimens were obtained from North America and Europe during four collecting seasons (spring–summer 1983–6). The source of each isolate, habitat, year collected, and mating type recovered are given in Table 1. Spore prints were made on 25% cotton rag paper, placed in manila coin envelopes and stored at 2 °C for later use. Fungi were routinely isolated and maintained on modified Melin–Norkrans (MMN) agar (Molina & Palmer, 1982) amended with 1 g/l yeast extract and substituting 10 mg/l ferric di-(*o*-hydroxy phenylacetate) for the $FeCl_3$. For all mating experiments, the medium was 0·5% (w/v) malt extract, 1·0% (w/v) agar medium, which reduced hyphal density. This facilitated easier examination of hyphae stained with 1% (w/v) Safranin-O (Sigma S-2255) and 1% (w/v) Na-acetate dissolved in 1:1 H_2O, 95% EtOH (v/v). Two home-made tools, 'hockey sticks' and 'transfer spatulas', were helpful in making single-spore isolations. The hockey sticks, used to spread spores, were made by bending glass rods to an obtuse angle. Transfer spatulas are made by flattening the tips of steel straight pins with 'vice-grip' pliers and melting them into glass tubing handle.

Several described methods for the isolation of single-spore colonies (Korhonen & Hintika, 1980; Watling, 1981), were attempted, but the 'hockey stick' method proved best because spore clusters were mechanically broken and well separated. A minute amount of spores, obtained either from a spore print or piece of lamella, were transferred on to the isolation medium using a sterile bacterial transfer loop. The transferred spores were then smeared and separated with a sterile hockey stick, often aided with a Petri dish turntable. Spore dilutions were incubated at 20° and checked for signs of germination; most spores germinated within 2–10 days. Young colonies were magnified with a dissecting microscope using transmitted light and were transferred on to MMN agar using a transfer spatula. Five single-spore isolates, four peripheral and one central, were placed on each Petri dish to give maximum colonial diameter and incubated at 20°. They were checked for clamp-connexions when they reached 15–20 mm diam. These original single-spore isolates were stored at 2° during the selfing experiments, and representatives of each mating type were saved.

Selfing experiments and non-sib matings

Selfing matrices of ten single-spore isolates per collection were combined on large 14 × 2 cm Petri dishes containing 0·5% malt extract agar. Small cubes of agar (2–4 mm^3) containing mycelium were cut with a flamed scalpel. The cubes were placed side by side almost in contact. The selfing matrices were incubated at 20° for 7 d, and subcultures taken from the junction line were transferred to a daughter matrix and incubated for an additional 7 d at 20° (Jurand & Kemp, 1973; Chase & Ullrich, 1983); the parent matrix was stored at 2°. After 14 total days of incubation, the subcultured daughter matrix was examined for clamp connexions and placed at 2° to slow growth. Blocks of agar taken from the colonial margin were stained by placing a drop of Safranin-O solution on the block followed by a drop of 3% KOH. The stained mycelium was examined for the presence of clamp-connexions, pseudo-clamps, or no reaction. Representatives of each identified mating type were placed in the Virginia Tech culture collection.

The non-sib matings used to identify biological species were composed to two compatible or randomly chosen single-

Fig. 1. Non-sib cross matrix among monokaryons obtained from morphologically distinct taxa. See Table 1 for origins of OR1, ID3, MT1, VA2. *A. molesta* = MOL, *A. smithii* = SMT, *A. pediades* = PED. +, Clamp-connexions; a blank indicates no reaction.

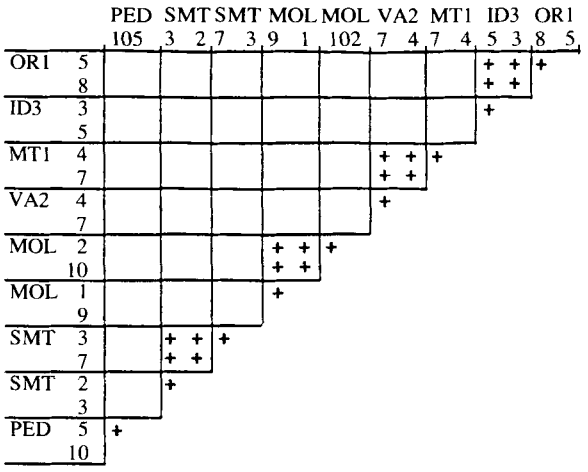


Fig. 2. Non-sib cross matrix confronting monokaryons from morphologically similar stock collections. See Table 1 for isolate origins. +, Clamp-connexions; −, barrage zone formation; ○, indicates neither reaction.

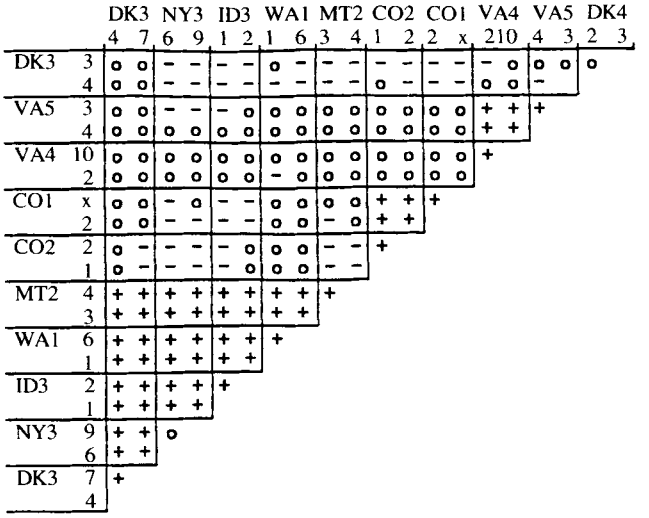
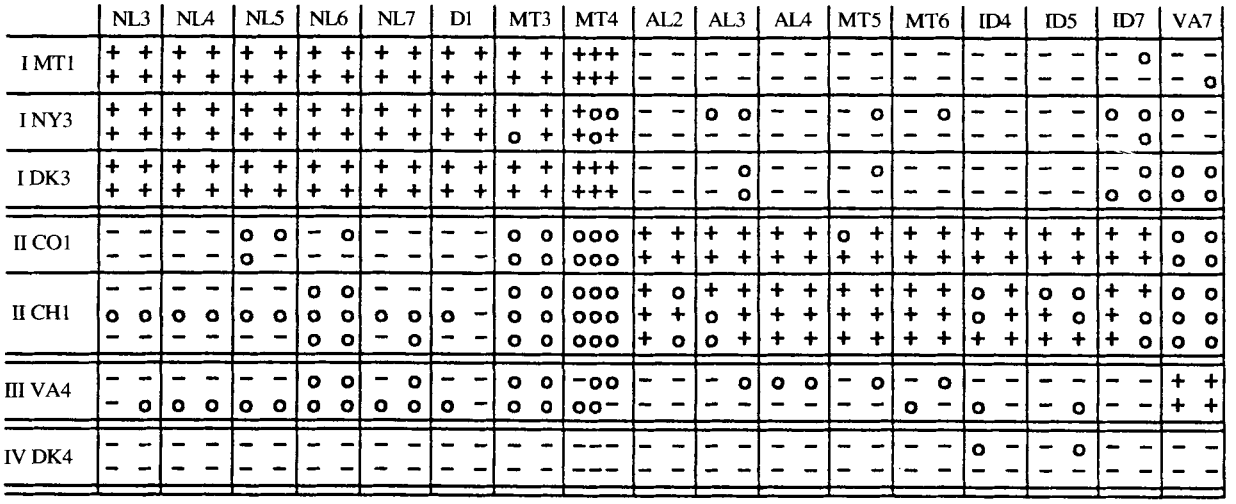


Fig. 3. Non-sib cross matrix confronting intercompatibility group tester strains with monokaryon strains obtained from stocks collected in later years and new locations. See Table 1 for isolate origins. +, Clamp-connexions; −, barrage zone formation; ○, no reaction.



spore isolates representing different collections. Matrices of 18 × 18 combinations were inoculated on to the culture medium on 38 × 24 × 2·5 cm stainless steel trays fitted with a galvanized sheet metal lid. A paper template had been placed under the agar. The incubation regime was the same as the selfing experiments. Each subcultured confrontation in the matrix was evaluated for the presence of barrage zones and clamp-connexions using the Safranin-O solution. These were stored at 2° to avoid excessive growth.

RESULTS

Selfing experiments revealed that all strains possess a bifactorial, homogenic incompatibility system. Pseudoclamp-connexions were routinely observed, indicating common B-factor heterokaryons. Nuclear migration and the Buller phenomenon (Raper, 1966) were not observed in experiments using Korhonen's method (1983).

The validity of interspecific sterility among distantly related taxa (Fig. 1) was tested by confronting monokaryons of *A. molesta*, *A. smithii* Watl. & Bigel., *A. pediades* (Fr.) Fayod, and *A. praecox sensu lato*. These taxa, along with the two isolates of *A. praecox sensu lato*, were to be intersterile.

The following mating compatibility results are based on 14 matrices (3034 confrontations). Four biological species representing populations from North America and Europe are shown in Fig. 2. Some confrontations produced brown-pigmented 'barrage zones' indicating strong antagonistic interactions. The mycelium in these barrage zones was brown, highly vacuolated and thick-walled. The pattern of barrage

Fig. 4. Size distribution of spores for the biological species and halotypes. Solid lines connect one standard deviation of peripheral mean values (10 spores per collection) labelled by biological species number; dotted lines connect one standard deviation of mean length and width of the single holotypic and authetic collections. A, *A. acericola*, H, *A. howeana*, L, *A. alachuana*, P, *A praecox*, T, *A. temnophyla*, V, *A. vermiflua*.

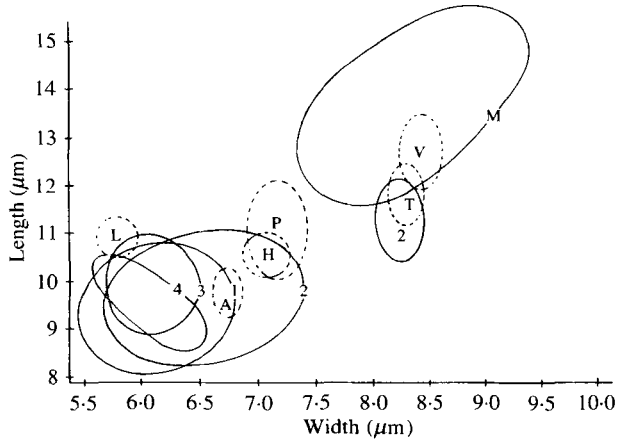
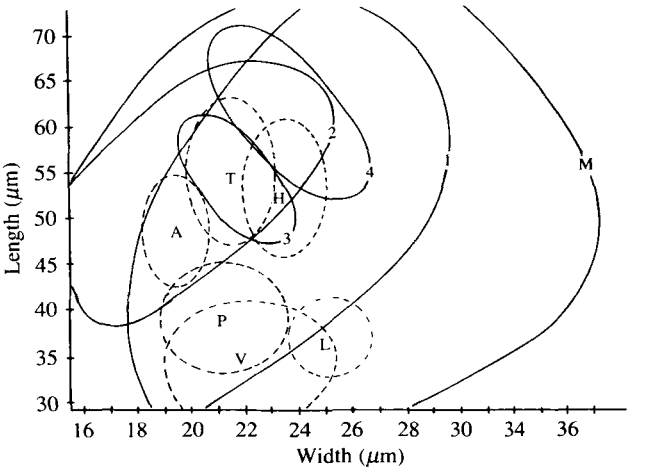


Fig. 5. Size distribution of pleurocystidial dimensions. Methods and labelling are the same as in Fig. 4.



formation could not be attributed to either inheritance or geographic origin.

Homokaryons representing populations from Idaho, Montana, Virginia, and the Netherlands sampled for two collecting seasons were confronted with tester strains (Fig. 3). This experiment shows that the biological species exist through time, and rarely collected populations such as the Montana populations of species I and the Virginia populations of species III can be recovered. The range and number of species III isolates was increased again in 1986, when two new collections were obtained from Massachusetts and another in Virginia. Additional population samples from Denmark and Switzerland could not be obtained. When 19 collections of *A. molesta* representing Virginia, eastern Canada, Colorado and Hawaii were confronted among themselves, they were found to belong to a single biological species.

Figure 4 shows the size distribution of spores for the biological species and holotypic specimens. Ten spores from each collection were measured, averaged and plotted. The lines connect one standard deviation of the peripheral means. Two groups, *A. molesta* and *A. praecox*, can be distinguished by differences in spore dimensions. Species within the *A. praecox* group, however, cannot be distinguished by spore size. The holotypes, based on single individuals, routinely yield smaller distributions. It is noteworthy that the variation in pleurocystidia (Fig. 5) yielded similar results. Field observations listed in Table 1 indicate that isolates in each biological species are capable of degrading a variety of woody substrates. The habitats of *A. molesta* and the biological species related to *A. praecox* overlap in many instances, but substrates utilized by these species do not. The *A. praecox* complex group degrade fragmented wood, branches, twigs and forest litter, whereas *A. molesta* is restricted to graminicolous substrates.

Species I usually occurs in urban habitats where fragmented wood chips are used to mulch ornamental plantings. It is distributed across North America and Europe, and is regionally sympatric with the other biological species. Species I fruits during May and June in lower latitudes, but fruits later in N.W. Montana. Although this species lives primarily in urban habitats some collections from Montana, Idaho, and Europe are found in forested areas along heavily used foot trails. This was particularly evident in Glacier National Park, and the University of Montana Biological Station in the northwestern United States.

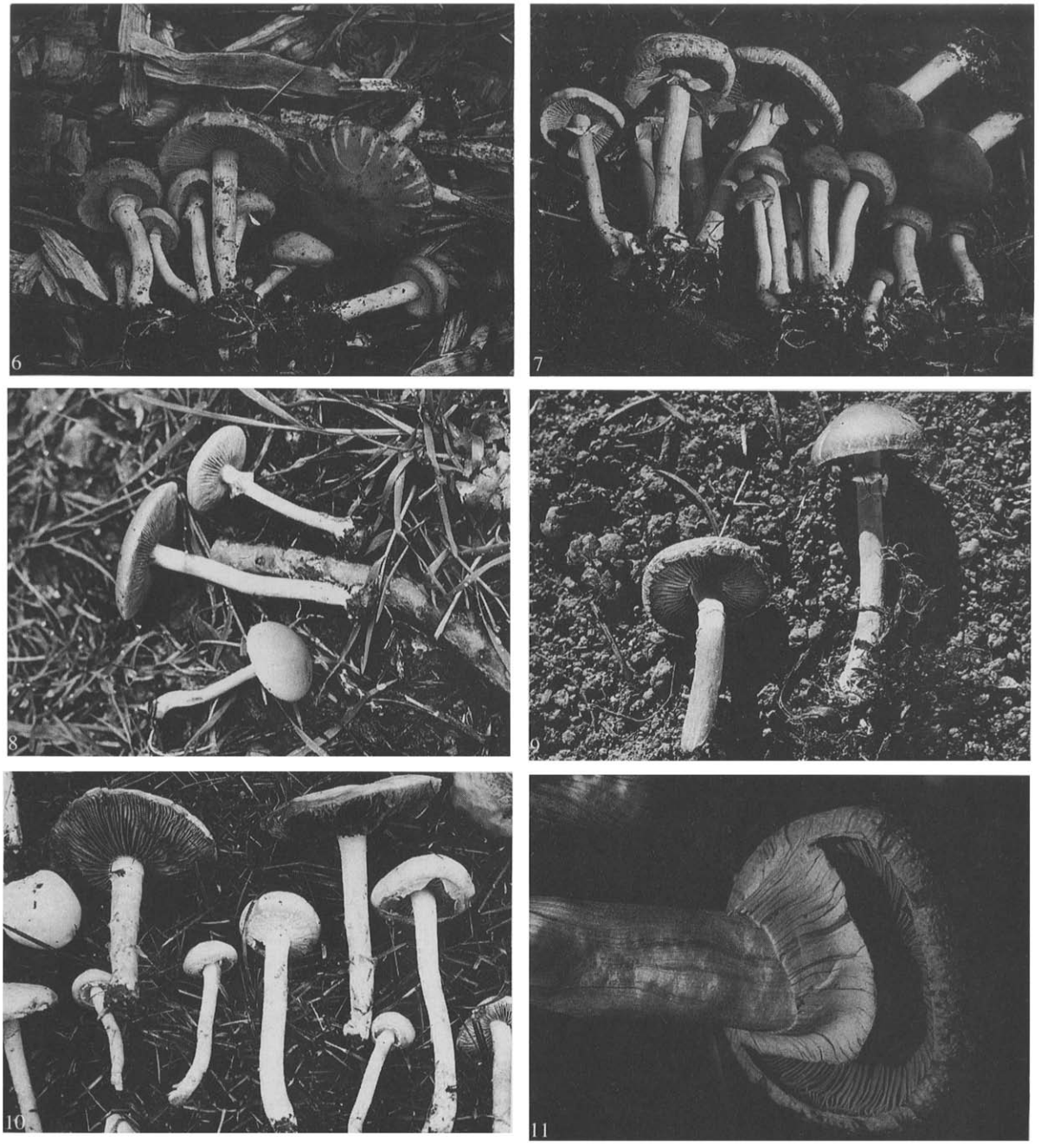
Species II degrades fragmented wood in forests in western North America. It is distributed along the Rocky Mountains from Colorado to Alberta, Canada, and the coastal range of northern California. A single collection was also made in Switzerland, where it may be more widespread. Species II usually occupies areas rarely frequented by man, but some collections were found along foot trails or logging roads where soil had recently covered organic matter. Species II lives in both aspen- or conifer-dominated forests, and fruits when spring comes to the higher elevations and latitudes, usually from May to August.

Species III is restricted to eastern North America, living in mesic northern-hardwood communities dominated by maple, beech, sycamore and oaks. Collections have been made in the mountains of Virginia and western Massachusetts and may extend further south. It fruits on fragmented wood in litter, and sometimes on large well-decayed logs.

Species IV was collected only once in a deciduous forest park in Denmark. It is not compatible with any of the other isolates used in this study (Figs 2–3). More data are needed to ascertain the distribution of this taxon.

Agrocybe molesta is a distinct member of the section Agrocybe, because it degrades graminicolous substrates in grassy fields or bare soil, but it is not found on wood substrates. It is found throughout North America, most often in urban settings such as lawns, golf courses and city parks. *Agrocybe molesta* usually fruits in May, one to two weeks before the other species, and continues until August. It will fruit again in late September and October often flushing concurrently with *Agaricus campestris* (L.) Fries.

Figs 6–11. Comparative macroscopic variation within select biological species.
Fig. 6. Species I in New Jersey suburb. **Fig. 7.** Species II in Idaho mountains (near McCall). **Fig. 8.** Species I in France along tree-lined foot trail. **Fig. 9.** Species II near McCall, Idaho. **Fig. 10.** *A. molesta* at VPI campus. **Fig. 11.** Species II near McCall, Idaho.



DISCUSSION

Five biological species have been identified in this study. Four of the species fit the concept of *A. praecox* and are morphologically indistinguishable from each other. These biological species, as a group, conform to the concept of sibling species (Dobzhansky, 1970; Mayr, 1965). Unfortunately, many of the taxa allied to *A. praecox* are not supported by type specimens. Recognition of these taxa relies only on macroscopic characteristics such as the areolate pileipellis of *A. cutifracta*, the unusually large and robust basidiocarps of *A. britzelmayrii*, the delicate stature of *A. paludosa*, and the appendiculate partial veil fragments of *A. gibberosa*. Each biological species, however, is capable of expressing these phenotypic characteristics (Figs 6–11). For example, the individuals in Figs 6–7 are different species (species I and II, respectively), but they are more similar than conspecific individuals shown in Figs 6 and 8 (species I), and Figs 7, 9 and 11 (species II). Furthermore, Fig. 9 (species II) looks identical to Bulliard's icone of *A. sphaleromorpha*, and the distinctive coloration shown in Fig. 11 (species II) could merit species status if the mating relationships were not known.

Agrocybe molesta (Fig. 10) has similar stature to Fig. 6 (species I), but its cottony, loosely interwoven partial veil and graminicolous nutrition separate it from the *A. praecox* species complex. These figures show that macroscopic variation within a species is equivalent to the variation between species, and the previous attempts at species delineations do not work. Microscopic characteristics also vary continuously (Figs 4–5), but some qualitative modality can be seen within each biological species.

The field observations, linked with the compatibility results, indicate that many described taxa (*A. cutifracta, A. britzelmayrii, A. gibberosa, A. sphaleromorpha*) represent variation most likely caused by abiotic factors such as moisture, temperature, humidity, light and substrate quality. Since these factors do affect basidiocarp morphogenesis and hence the phenotype as shown in the Bolbitiaceae (Watling, 1975), *Rhodotus palmatus* (Bull.: Fr.) Maire (Miller *et al.*, 1980), and other species (Taber, 1966), a re-evaluation of species concepts in the *A. praecox* complex is needed.

Substrate and habitat are useful for clustering biological species of the *A. praecox* complex. Ecological patterns are also diagnostic for biological species of *Auricularia* Bull. (Duncan & MacDonald, 1967), *Hirschioporus* Donk (Macrae, 1967), *Heterobasidion* Bref. in Europe (Korhonen, 1978*b*) and *Paxillus* E.M. Fr. (Fries, 1985). Many animals, such as the Lace-wing insect, follow a similar pattern (Tauber & Tauber, 1977*a*, *b*). Mating studies have elucidated diagnostic morphological characteristics for *Exidiopsis* (Bref.) Möller (Wells & Wong, 1985) and *Tremella* Pers. (Wong *et al.*, 1985). However, neither ecological differentiation nor morphological characteristics can be used to distinguish species of *Sistotrema* Pers. and some species of *Coprinus* Pers. Furthermore, reproductive isolation among morphologically similar biological species of *Collybia* (Fr.) Staude has low DNA similarity (Vilgalys & Johnson, 1987), but remains high within phenotypically variable sympatric interfertile populations. These examples suggest that little change in basidiocarp morphological accompanies genetic isolation and ecological differentiation. It is apparent from this study of *Agrocybe*, and the literature cited above, that the biological species concept is both valid and useful for basidiomycete systematics. The knowledge of mating relationships allows one to cut through confounding variables, and evaluate the relative diagnostic values of morphology, habitat and substrate in the establishment of species concepts.

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