Clavicipitaceous Fungi Associated with Ergoline Alkaloid-Containing Convolvulaceae

Mahalia A. Ahimsa-Müller,[†] Anne Markert,[†] Sabine Hellwig,[†] Volker Knoop,[‡] Ulrike Steiner,[§] Christel Drewke,[†] and Eckhard Leistner*[,†]

Institut für Pharmazeutische Biologie der Rheinischen Friedrich-Wilhelms-Universität Bonn, Nussallee 6, D-53115 Bonn, Germany, Institut für Zelluläre and Molekulare Botanik der Rheinischen Friedrich-Wilhelms-Universität Bonn, Kirschallee 1, D-53115 Bonn, Germany, and Institut für Nutzpflanzenwissenschaften and Ressourcenschutz (INRES) der Rheinischen Friedrich-Wilhelms-Universität Bonn, Nussallee 9, D-53115 Bonn, Germany

Received June 28, 2007

Ergoline alkaloids are a group of physiologically active natural products occurring in taxonomically unrelated fungal and plant taxa Clavicipitaceae (Hypocreales) and Convolvulaceae (Solanales). We show in the present paper that clavicipitaceous fungi are associated with four different ergoline alkaloid-containing plant taxa of the family Convolvulaceae. These fungi are macroscopically visible on the adaxial surface when young leaf buds are opened or are detectable by molecular biological techniques in seeds. Detectability of the fungus correlates with the absence or presence of ergoline alkaloids within the respective plant organ. The fungi contain the gene (dmaW) responsible for the committed step in ergoline alkaloid biosynthesis. Sequencing of ribosomal DNA (18S rDNA and internal transcribed spacer) as well as the dmaW gene (partial) and construction of phylogenetic trees show that the fungi are clavicipitaceous, not identical but very closely related.

Ergoline alkaloids (syn. ergot alkaloids) (Figure 1) are L-tryptophan-derived natural products of high physiological activity. They are used in therapy as nootropics,[25] were responsible for food poisoning during the Middle Ages,[11,12] and cause severe toxicosis in cattle feeding on grass infected with ergoline alkaloid-producing clavicipitaceous fungi.[4,12,21] The fungi defend the plants against pests and confer drought resistance and fitness to their hosts.[5]

Ergoline alkaloids are also present in different genera of a dicotyledonous plant family, the Convolvulaceae.[14] Certain representatives of this family are used in the uplands of southern Oaxaca in Mexico for divinatory and hallucinatory purposes.[7,14] The physiological potential of ergoline alkaloids is therefore of interest in medicine and agronomy as well as ecological and anthropological studies.

It was one of the unresolved questions why these alkaloids occur in such diverse taxa as the fungal Clavicipitaceae and Eurotiaceae on one hand and in Convolvulaceae, a higher plant family, on the other. An explanation for this enigma has recently been given. There are strong indications that *Ipomoea asarifolia* Roem. et Schult plants (Convolvulaceae) are colonized by an ergoline alkaloid-producing clavicipitaceous fungus (provisionally named IasaF13) that is responsible for the accumulation of ergoline alkaloids within the plant.[18,28] Treatment of *I. asarifolia* with fungicides resulted in the elimination of a leaf-associated fungus and concomitant loss of alkaloids from the plant.[18] This fungus is equipped with genetic material essential for the synthesis of ergoline alkaloids (Markert and Leistner, unpublished) and is seed-transmitted.[28] The fungus is specialized for growth on *I. asarifolia* plants, as indicated by its inability to grow on different known synthetic media designed for fungal growth.[18,28]

In order to look for further support of our observations, we investigated additional ergoline alkaloid-containing Convolvulaceae plants for the presence of fungal colonization. We here demonstrate that highly similar fungi are also present on Convolvulaceae plants other than the white-blooming *I. asarifolia* species. Molecular biological analyses of the fungal isolates show that they are clavicipitaceous but not identical, albeit very closely related.

* Author for correspondence. Tel: +49-228-733199. Fax: +49-228-733250. E-mail: eleistner@uni-bonn.de.
[†] Institut für Pharmazeutische Biologie.
[‡] Institut für Zelluläre and Molekulare Botanik.
[§] Institut für Nutzpflanzenwissenschaften und Ressourcenschutz (INRES).

Results and Discussion

Plant Material and Alkaloid Content. For the experiments reported here four different plant taxa belonging to the family Convolvulaceae were chosen. The first two are *I. asarifolia* occurring as white- and red-blooming individuals (Figure 2A). Both may represent different varieties but have not yet been described as such. It is for this reason that we use the term "white-blooming" or "red-blooming" individuals or *I. asarifolia* types in this publication. The third plant chosen was *Ipomoea violacea* L., and as a fourth experimental system *Turbina corymbosa* (L.) Raf. (syn. *Rivea corymbosa* (L.) Hall. f.) was selected (Figure 2A).Voucher specimens of all four plant taxa were deposited in our herbarium.

The four plant taxa were investigated for the quantity of ergoline alkaloids by an optical test based on the Van Urk reaction.[18] The seeds are the main site of accumulation of ergoline alkaloids (Table 1), an observation that is not unexpected.[10] Although alkaloids were reported to be present in aerial parts of *I. violacea*,[10,22] alkaloids were detected only in seeds but not in shoots of *I. violacea* plants investigated in the present work. This will be discussed later.

Ergoline alkaloids occurring in Convolvulaceae plants were first isolated and identified by spectroscopic methods from seeds of *R. corymbosa* (L.) Hall.f. (i.e., *T. corymbosa* (L.) Raf.) and *I. violacea* L.[14] We investigated the ergoline alkaloids present in shoots (both types of *I. asarifolia* and *T. corymbosa*) or seeds (*I. violacea*) because there are conflicting reports on the presence in Convolvulaceae of these natural products,[1,26] which were often identified by paper- or thin-layer chromatography alone.[3,8,29] The alkaloids present in the red *I. asarifolia* plants had not been investigated previously. Identification was carried out by HPLC-MS and comparison with authentic material. The results are listed in Table 2. The spectrum of major alkaloids present in both types of *I. asarifolia* turned out to be qualitatively but not quantitatively identical. As opposed to previous publications we were unable to detect agroclavine and elymoclavine in *I. violacea* (Table 2).

Co-occurrence of Alkaloids and Fungus. It is our experience that ergoline alkaloids and the clavicipitaceous fungus IasaF13 always co-occur in the intact *I. asarifolia* (white-blooming) plant.[18,28] Indeed, visual inspection of young leaf buds that were opened by manipulation (Figure 2B) showed that the fungus was macroscopically visible as white mycelial layers on the adaxial leaf surfaces of both *I. asarifolia* plants and *T. corymbosa* but not on *I. violacea* (Figure 2B). The typical distribution of the fungal colonies

10.1021/np070315t CCC: $37.00
Published on Web 11/22/2007

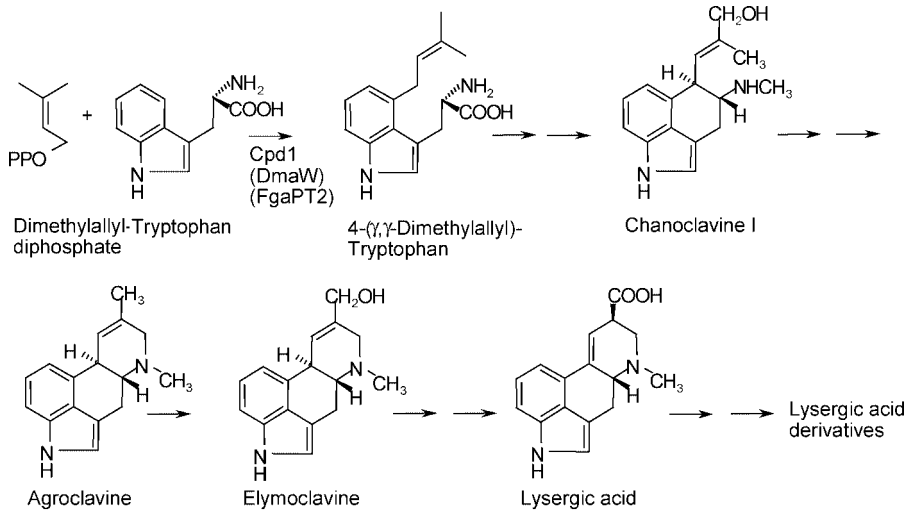

Figure 1. Biosynthetic pathway leading to lysergic acid and its derivatives.[9,12,13,31]

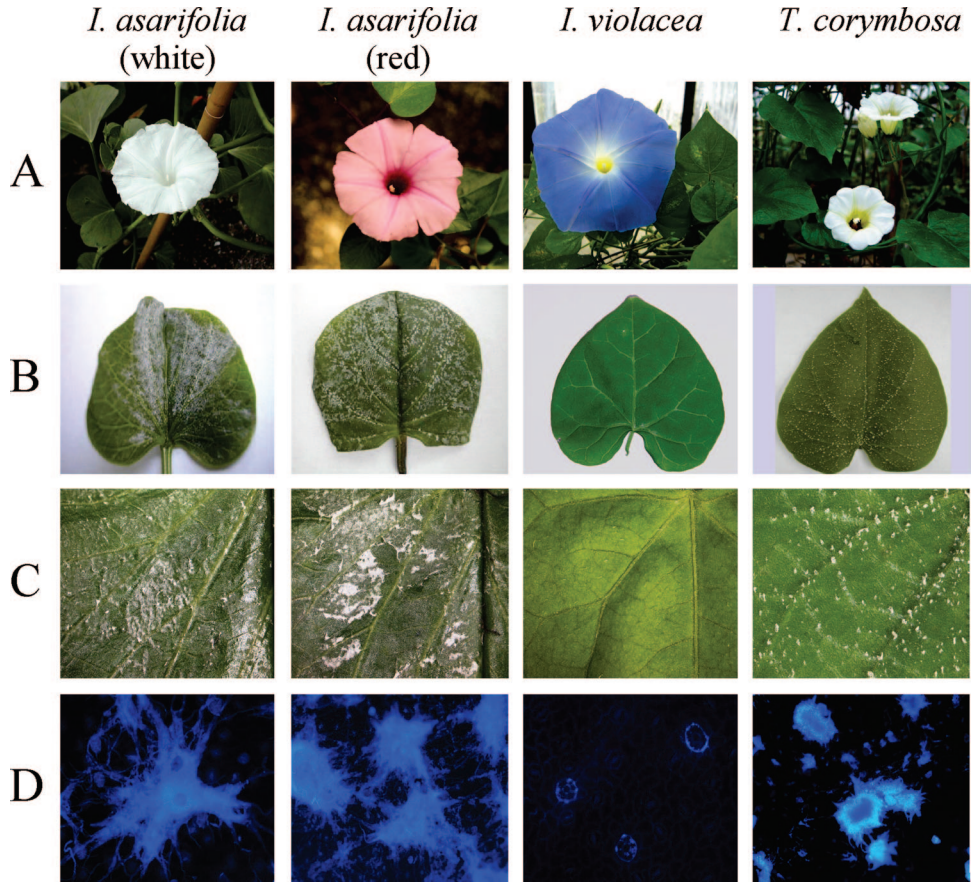

Figure 2. Plants investigated: Phenotype (A) and macroscopic (B) and microscopic (C, D) views of the adaxial leaf surface of leaf buds opened by manipulation. Magnification is 10-fold (C) or 100-fold (D). Fungal cell walls were stained with Uvitex 2B (D). The leaf surface of *I. violacea* (D) shows three secretory glands but no fungal colonies.

on the leaf surface is shown in Figure 2C, after staining with Uvitex 2B (Figure 2D). Staining is directed toward the carbohydrate cell walls of the fungal hyphae but leaves the underlying plant cuticle unaffected; the mycelium forming the fungal colonies became very well visible (Figure 2D).

Since the *I. violacea* seeds do contain alkaloids (Table 1), a clavicipitaceous fungus should be present in the seeds, whereas the leaves of this plant should be devoid of both fungus and alkaloids. This is actually observed as documented in Figures 2B, C, and D and Tables 1 and 2.

Construction of Phylogenetic Trees. DNA was extracted from

Table 1. Quantitative Estimation of Ergoline Alkaloids [μg alkaloids $\times$ (g fw)$^{-1}$] in Different Organs of Plants Belonging to the Family Convolvulaceae (data are given as ergonovine maleate equivalents)

	leaves and stems	seeds
I. asarifolia (white)	7.0	370[a]
I. asarifolia (red)	11.3	
I. violacea	0.0	240
T. corymbosa	14.6	220

[a] Amount taken from ref 17. This reference does not distinguish between the white- and the red-blooming plants of *I. asarifolia*.

Table 2. Ergoline Alkaloids from Shoots (*I. asarifolia,* both types; *T.corymbosa*) and Seeds (*I. violacea*) as Identified by HPLC-MS and Comparison with Authentic Reference Compounds[a]

	I. asarifolia (white)		*I. asarifolia* (red)		*I. violacea*		*T. corymbosa*	
alkaloids	this work	refs	this work	refs	this work	refs	this work	refs
chanoclavine	++	16, 18, 29	+	–	++	3, 8, 10, 15, 27	+	3, 8, 15, 26, 28, 29
lysergic acid-α-hydroxy ethylamide and isolysergic acid-α-hydroxy ethylamide	+	16, 18, 29	+++	–	++	3, 8, 10, 15, 27	+++	3, 8, 15, 26, 28
lysergic acid amide and isolysergic acid amide	++	16, 18, 29	+	–	+++	3, 8, 10, 15, 27	++	3, 8, 15, 26, 28, 29
ergonovine	++	18, 29	+	–	+++	3, 8, 27	+++	3, 8, 15, 26, 28, 29
agroclavine	nd	–	nd	–	nd	3, 8, 27	+++	3, 8, 15, 26, 28
elymoclavine	nd	–	nd	–	nd	3, 8, 27	+++	3, 8, 15, 26, 28, 29

[a] The alkaloids were estimated to be minor (+), medium (++), or major (+++) components of the alkaloid fraction. n.d. = not detected. Ergobalansine, which is a constituent of *I. asarifolia* (*I. piurense*), remained unidentified due to a lack of authentic material.[16]

seeds of *I. violacea*, and the mycelia were collected from leaf buds of the three other plants shown in Figure 2 in order to characterize the fungi. The nuclear small subunit ribosomal DNA (18S rDNA), the internal transcribed spacers (ITS1 and ITS4), and the 4-(γ,γ-dimethylallyl)tryptophan synthase gene (dmaW, i.e., cpd1 or FgaPT2, compare Figure 1) were targeted for PCR amplification, and the products obtained were sequenced. The dmaW gene was chosen because it catalyzes the determinant step in ergoline alkaloid biosynthesis, introducing a dimethylallyl residue into the 4-position of tryptophan (Figure 1).[9,12,13,30,31] Sequencing was carried out on both strands of three (dmaW gene) or at least five (18S rDNA, ITS) selected clones.

The 18S rDNA sequences of all four fungal DNA isolates turned out to be identical, indicating that the fungi are closely related and belong to the order Hypocreales, as shown previously[28] for the fungus IasaF13 present on *I. asarifolia* (white blooming). The provisionally named fungal isolates and the accession numbers of their 18S rDNA sequences are as follows: *I. asarifolia* (white blooming), IasaF13-DQ119128; *I. asarifolia* (red blooming), IasaredF01-DQ641918; *I. violacea,* IviolF01-DQ641919; *T. corymbosa,* TcorF01-DQ127128.

In order to possibly obtain a better fine resolution for the closely related fungal taxa, we analyzed the ITS region. The amplified ITS sequences of the fungi associated with all four plant taxa were first compared by single-strand conformation polymorphism (SSCP). The amplified ITS DNA of the fungi IasaF13, IasaredF01, and TcorF01 were indistinguishable by the SSCP method, but all three showed a clear difference from those of IviolF01 (data not shown).

Subsequently the ITS DNA of the fungi on *I. asarifolia* (both types) and on *T. corymbosa* were sequenced and found to be identical, whereas the ITS sequence of the fungus from *I. violacea* exhibits 84.3% similarity to the sequences of the fungi on the other three plant taxa. The ITS sequences were deposited in GenBank: *I. asarifolia* (white blooming), IasaF13-AY937227; *I. asarifolia* (red blooming), IasaredF01-DQ641920; *T. corymbosa,* TcorF01-AY995219; *I. violacea*, IviolF01-DQ641921.

Construction of phylogenetic trees based on 18S rDNA and ITS sequences had previously shown that IasaF13 is a clavicipitaceous fungus.[28] Since IasaredF01 and TcorF01 have sequences (18S rDNA and ITS) identical to those of IasaF13 (white blooming), it is concluded that they also belong to the family Clavicipitaceae. Integration of the ITS sequence of IviolF01 into a phylogenetic tree (Figure 3) demonstrates that this fungus is also clavicipitaceous and has a root in common with the fungi on the other three plant taxa.

Finally, internal sequences from the dmaW genes encoding the 4-(γ,γ-dimethylallyl)tryptophan synthase in IasaF13, IasaredF01, and TcorF01 were analyzed. It turned out that the sequences in IasaF13 (DQ121453) and IasaredF01 (DQ 647955) were again identical, whereas the sequence in TcorF01 (DQ121454) showed 93% similarity on DNA level and 93.6% similarity on protein level, respectively. Construction of the phylogenetic tree shows the close relationship among the dmaW genes of the isolates, clearly nested within a clade of homologues in other Clavicipitaceae (Figure 3).

During these experiments we were unable to amplify the dmaW gene from a total DNA plant extract (leaves or seeds), although the plant was clearly colonized by the fungus. This was experienced in spite of the fact that DNA purified by electrophoresis or PCR and nested PCR were employed sequentially. Only DNA from an isolated fungal mycelium gave a dmaW product. Thus, the sequence of the dmaW gene can be given only for *I. asarifolia* (both types) and *T. corymbosa* but not for *I. violacea*.

We have previously shown that a clavicipitaceous fungus (IasaF13) occurring on the white-blooming *I. asarifolia* plant very likely is responsible for the accumulation of ergoline alkaloids within the plant. The fungus was provisionally named IasaF13, is seed-transmitted, hitherto noncultivable on synthetic media, occurs on the adaxial leaf surface, and contains genetic material required for the synthesis of ergoline alkaloids (Markert and Leistner, unpublished).[18,28] The presence of alkaloids within the plant shoots can be predicted from the presence of mycelium on the adaxial leaf surface of leaf buds (Figure 2).

These observations challenge the hypothesis that the occurrence of ergoline alkaloids in such diverse taxa as clavicipitaceous fungi and convolvulaceous plants may be explained by a horizontal gene transfer that occurred during evolution between these taxa or that the biosynthetic pathway leading to ergoline alkaloids was repeatedly "invented" during evolution. It seems more appropriate to attribute the occurrence of ergoline alkaloids in dicotyledonous plants to the presence of a plant-associated clavicipitaceous fungus.

Data presented in this publication extend these observations and show that epibiotic (shoots of *I. asarifolia*, both types; *T. corymbosa*) and endophytic (seeds of *I. violacea*) fungi occur in association with different taxa of the family Convolvulaceae and are closely related: The 18S rDNA is identical among the fungi from each plant source investigated, whereas the ITS sequences were identical among the fungi from both *I. asarifolia* types and *T. corymbosa*, with the fungus on *I. violacea* exhibiting 84.3% similarity when compared to the ITS sequences of the fungi present on the other three plant taxa. Sequencing of the dmaW gene differentiates between the fungi present on both *I. asarifolia* types on one hand and the gene present in the fungus present in *T. corymbosa* on the other (93% similarity when compared to the fungi on both types of *I. asarifolia*). The view that these fungi are responsible for alkaloid accumulation in the four different plant taxa investigated here is supported by the observation that alkaloids and fungi always co-occur, as has been experienced for the intact *I. asarifolia* plant (white blooming).[28]

Co-occurrence of alkaloids and fungi is also evident from the investigation of *I. violacea* alone: Alkaloids (Tables 1 and 2) and fungus (Figure 3) are present in seeds, but shoots of the plant are devoid of both fungus (Figure 2B, C, D) and alkaloids (Table 1).

The nondetectability of alkaloids within shoots of *I. violacea* may be unexpected because alkaloids were previously reported to

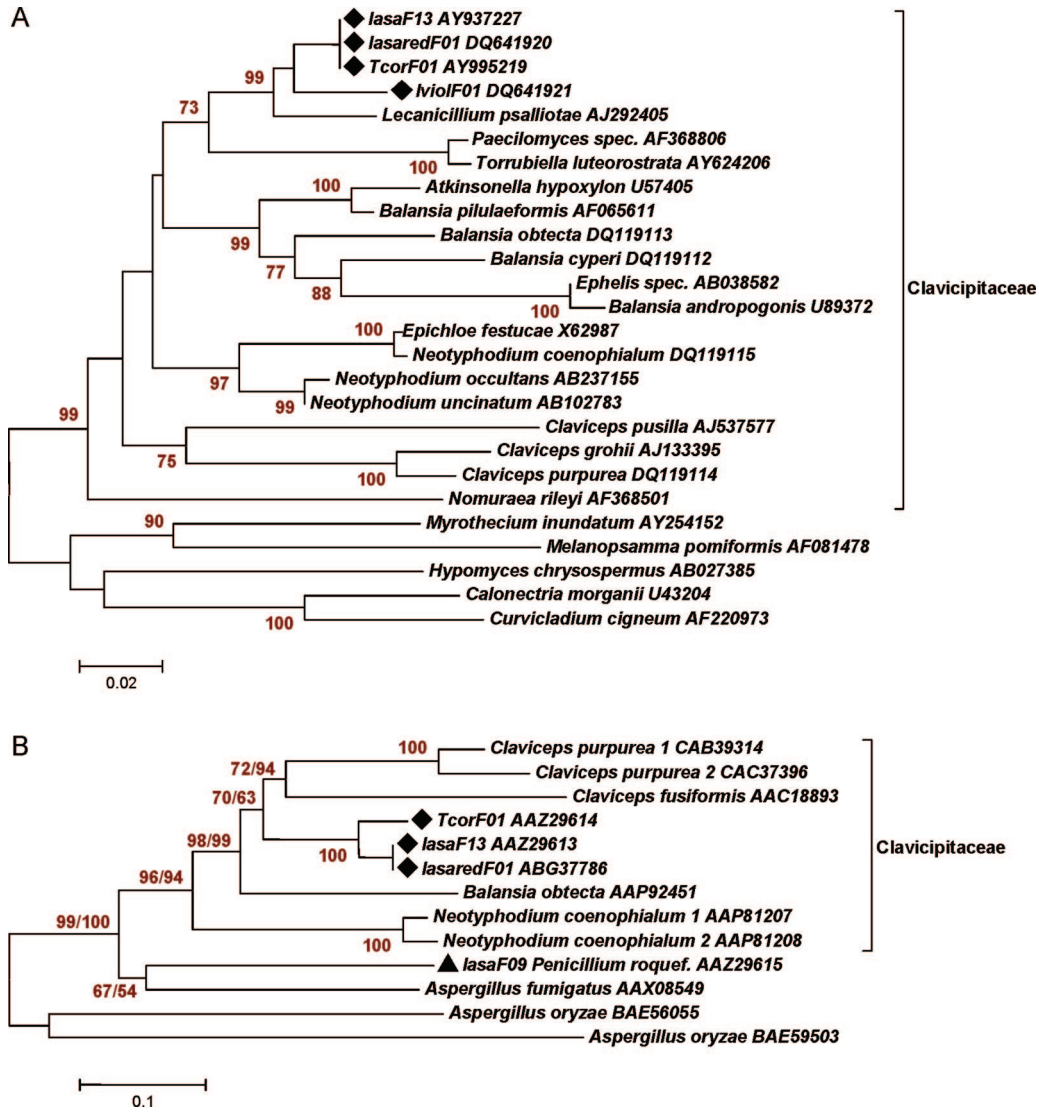

Figure 3. Phylogenies of the ITS sequences (A, 695 alignment positions) and the dmaW, cpd1, and FgaPT2 genes (B, 395 alignment positions) of the fungal isolates under investigation with additional homologous sequences taken from the database. Accession numbers are indicated. Only most similar sequences outside the Clavicipitaceae were included in each case for rooting the clade. Vertical lines indicate identity of sequences in the new fungal isolates (diamonds). Phylogenetic trees shown are neigbor joining trees based on Kimura-2-parameter (A) and Poisson-corrected amino acid distances (B), respectively. Node support based on 10 000 bootstrap replicates is shown only where at least 70. Additionally, support from 1000 bootstrap replicates using Dayhoff (PAM) distances is given after the slash in B where deviating.

be also present in shoots of this plant.[10,22] There are, however, conflicting reports about the presence and absence of ergoline alkaloids within various species of the family Convolvulaceae.[1]

Similar observations were made with maytansinoid ansa antibiotics, which are constituents of the *Actinosynnema pretiosum* (Actinomycetes) but also of higher plant species within the families Celastraceae, Rhamnaceae, and Euphorbiaceae. Investigation of *Putterlickia verrucosa*, being a member of the Celastraceae, showed that maytansinoid antibiotics are present in some individual plants but are not detectable in others. This was taken as an indication that the plants are associated with a microorganism responsible for the accumulation of ansa antibiotics in the plant. The microorganism may be present or absent,[2,24] viable or nonviable,[32] or whose natural product synthesis was induced or noninduced possibly depending on the presence of another challenging microorganism triggering natural product synthesis as part of a defense reaction.

It is possible that the viability of the clavicipitaceous fungus in *I. violacea* seeds is limited, resulting in a plant devoid of alkaloids after germination of the seed. A similar observation has been reported for seeds of Poaceae infected by a clavicipitaceous fungus. Endophyte survival was influenced by temperature, length of storage, and seed moisture content.[32]

Nevertheless, the fungus present in *I. violacea* seeds was very well detectable by its 18S rDNA and ITS sequences and adds another example to the observation that the absence or presence of ergoline alkaloids in Convolvulaceae plants may be predicted by viewing the adaxial leaf surface of young leaf buds: no mycelium was seen in leaf buds of *I. violacea* and no alkaloids were detected in shoots of the plant as opposed to representatives of the other three taxa, which contain both alkaloids and show the mycelium responsible for alkaloid production (compare Tables 1 and 2 and Figure 2).

Another point deserves attention: The fungal 18S rDNA and ITS sequences from mycelia located in leaf buds of the two *I. asarifolia* plant types and *T. corymbosa* are identical, but a difference was observed when compared to the ITS sequence obtained from the fungus in *I. violacea* seeds (84.3% similarity). On first sight one may expect that the fungi living on plants of the genus *Ipomoea* are closest relatives and that the fungus on *T. corymbosa* belongs to a separate taxonomically defined group. It is intriguing, however,

that such an observation was also made with clavicipitaceous fungi on Poaceae, demonstrating that phylogeny of an associated fungus does not mirror the phylogeny of the host plant.[23] This may indicate that during evolution the jump of a clavicipitaceous fungus to a plant species belonging to the family Convolvulaceae occurred on different occasions. Alternatively it may have happened that fungi switched host plants during evolution.

The sequence data presented here show that the fungi on all four plant taxa investigated are close relatives. Since the fungi show no differences in their 18S rDNA and only moderate differences in their ITS and dmaW sequences, we hypothesized that this may also reflect the composition of alkaloids isolated from the plants. In this respect it is essential to note that the alkaloids accumulate in the plant tissue (Hellwig, Steiner, and Leistner, unpublished), whereas the genetic material for the synthesis of alkaloids is present in the plant-associated fungus (vide supra), indicating that the biosynthesis of ergoline alkaloids takes place in the symbiotic microorganism. Thus, both symbiotic organisms (plant and fungus) are in contact with the alkaloids and may contribute to the spectrum of alkaloids qualitatively and quantitatively.

Following early experiments with clavicipitaceous fungi present on grasses, it has been noted that the composition of alkaloids is hereditary but is also influenced by the nutrition or physiological state of the host plant.[19] This is in agreement with studies of others and our own experience with the composition of ergoline alkaloids in Convolvulaceae plants.[20,27,28]

The alkaloids present in the plants under investigation (Figure 1) can be divided into biosynthetic precursors (chanoclavine I, agroclavine, elymoclavine) and derivatives (lysergic acid-α-hydroxy ethyl amide including its isomer, lysergic acid amide including its isomer and ergonovine) of lysergic acid.[10,12,13,30] The derivatives of lysergic acid are detectable with our HPLC-MS system in each plant investigated. Agroclavine and elymoclavine, however, were only detected in *T. corymbosa* but must also be present in the other taxa because they are precursors of lysergic acid including their derivatives (Figure 1). We have to assume that these two precursors went undetected due to their scarcity in *I. asarifolia* (both types) and *I. violacea* (Table 2). The difference in the ergoline alkaloid composition of all four taxa investigated therefore must be primarily a quantitave rather than qualitative difference.

This is also evident from the following observation: Both types of *I. asarifolia* have identical 18S rDNA, ITS, and dmaW sequences, but the composition of alkaloids in both plant types is not identical; data obtained by HPLC-MS analysis (data not shown) and determination of alkaloid content of both types by the Van Urk technique (Table 1) show that the red *I. asarifolia* contains twice the amount of alkaloids present in the white variety, and while lysergic acid-α-hydroxy ethylamide and its isomer are the main components in the red variety, it is only a minor component in the white variety, in which chanoclavine, lysergic acid amide, and its isomer as well as ergonovine are dominating constituents. The assumption that both symbiotic organisms contribute to the composition of alkaloids has also been postulated in the case of maytansinoids[2] and is in agreement with the observation[10] that ergoline alkaloids can be metabolized by a plant belonging to the Convolvulaceae. This is in line with our own observation, which showed that ergoline alkaloids disappear from white *I. asarifolia* as well as *T. corymbosa* plants when the fungus is removed by fungicides.[18,28] The alkaloids present in the plants apparently are subject to a turnover independent of the fungal symbiotic partner.

Experimental Section

Plant Material. Sources and culture conditions of *I. asarifolia* and *T. corymbosa* were previously described.[28] Both the red- and the white-blooming *I. asarifolia* plants were obtained from Dr. E. Eich, Berlin, Germany, and were cultured as described for the white-blooming variety. Seeds of *I. violacea* were obtained from "Samen Mauser" in Quedlinburg, Germany (harvested in 1999, experiments were carried out in 2005).

Single-Strand Conformation Polymorphism (SSCP). The SSCP procedure and the oligomers employed were described.[28] SSCP of DNA from seeds of *I. violacea* was carried out after extraction of DNA from seeds as described.[6] The DNA was submitted to PCR using oligomers ITS1F and ITS4. The products (ca. 600 bp) were cloned and the ITS inserts sequenced. Clones of clavicipitaceous origin were submitted to PCR using oligomers ITS1F and ITS2P and analyzed by electrophoresis following the procedures for SSCP.[28] For the SSCP analysis of ITS sequences of the fungal mycelium collected from leaf buds the two oligomers ITSF1 and ITS2P were directly incubated with DNA.

Microscopy. The fungi on the leaf surfaces were visualized with a Leica MZ 16 F stereomicroscope (Leica, Bensheim, Germany) with 10-fold magnification. Fluorescence microscopy was carried out with a Leitz DMR photomicroscope (Leica, Benheim, Germany) with 100-fold magnification. Fungal structures on the leaf surface were stained in the dark with 0.05% Uvitex 2B (w/v) (Syngenta, Basel, Switzerland) in 0.1 M Tris/HCL buffer, pH 8.0. The specimen was covered with a coverslip and observed under fluorescence light with filter combination BP340-380/FT400/LP430. Both microscopes were equipped with a KY-F75 digital camera and Diskus software (TB Hilgers, Königswinter, Germany) for image processing.

Experimental Techniques. The following experimental techniques were carried out as described:[28] Amplification of 18S rDNA, ITS, 4-(γ,γ-dimethylallyl)tryptophan synthase gene (dmaW, cpd1, FgaPT1); construction of phylogenetic trees; microscopic techniques; identification of alkaloids by HPLC-MS.

Acknowledgment. This research was supported by a grant from the Deutsche Forschungsgemeinschaft to E.L.

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NP070315T