The *Trichoderma harzianum* demon: complex speciation history resulting in coexistence of hypothetical biological species, recent agamospecies and numerous relict lineages

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**Abstract**

**Background:** The mitosporic fungus *Trichoderma harzianum* (*Hypocrea*, Ascomycota, Hypocreales, Hypocreaceae) is an ubiquitous species in the environment with some strains commercially exploited for the biological control of plant pathogenic fungi. Although *T. harzianum* is asexual (or anamorphic), its sexual stage (or teleomorph) has been described as *Hypocrea lixii*. Since recombination would be an important issue for the efficacy of an agent of the biological control in the field, we investigated the phylogenetic structure of the species.

**Results:** Using DNA sequence data from three unlinked loci for each of 93 strains collected worldwide, we detected a complex speciation process revealing overlapping reproductively isolated biological species, recent agamospecies and numerous relict lineages with unresolved phylogenetic positions. Genealogical concordance and recombination analyses confirm the existence of two genetically isolated agamospecies including *T. harzianum* sensu stricto and two hypothetical holomorphic species related to but different from *H. lixii*. The exact phylogenetic position of the majority of strains was not resolved and therefore attributed to a diverse network of recombining strains conventionally called ‘pseudoharzianum matrix’. Since *H. lixii* and *T. harzianum* are evidently genetically isolated, the anamorph - teleomorph combination comprising *H. lixii/T. harzianum* in one holomorph must be rejected in favor of two separate species.

**Conclusions:** Our data illustrate a complex speciation within *H. lixii* - *T. harzianum* species group, which is based on coexistence and interaction of organisms with different evolutionary histories and on the absence of strict genetic borders between them.

**Background**

The unique nature of fungi, when the closely related organisms exploit incomparably different strategies for reproduction (mostly sexual vs. exclusively asexual vs. sexual and asexual), leads to existence of a variety of overlapping species concepts. Some fungal species can be differentiated based on sexual compatibility of their members, which are in turn reproductively isolated from the other species (i.e. the biological species concept). The situation is more complicated with those fungi which either do not mate in vitro or have lost the ability to reproduce sexually in nature. In addition, the taxonomy of almost every large group of fungi suffers from certain historical biases which usually come from the applied value of the organisms. The introduction of molecular methods in evolutionary mycology has already resulted in the dramatic taxonomic changes [1] though the effort is still needed to rich the clarity for individual genera. The mitosporic genus *Trichoderma* (*Hypocrea*, Ascomycota, Hypocreales, Hypocreaceae), a fungus beneficially used in agriculture, is a striking example for this: the number of its morphologically recognized species (i.e. the morphological species concept) is still around 30 [2-4], while the application of genealogical concordance between unlinked DNA loci (i.e. the phylogenetic species concept) resulted in 100 phylogenetic
species recognized by 2006 [5] and this number is growing quickly.

The special scientific interest to this genus is largely connected with the modern pace towards the development of the market for organic farm products, which already covers approximately 2% of total world farmland and more than 10% in some European countries such as Austria, Switzerland and Sweden [6]. The management of organic farms requires the integration of biological pest control (biocontrol) in agricultural practices. The control or prevention of some plant diseases by such mycoparasitic fungi or ‘biofungicides’ such as Trichoderma is an attractive alternative to the use of chemical fungicides [7] and therefore is an important component of modern organic farming. These fungi not only antagonize plant pathogens [8,9], but are also rhizosphere competent and can enhance plant growth in endophyte-like associations [10]. However, as the prerequisite to applying biofungicides to farm fields, the biology of every active agent should be understood. Some molecular aspects of Trichoderma mycoparasitism and endophytism - such as the role and regulation of formation of cell wall hydrolytic enzymes and antagonistic secondary metabolites - have been intensively investigated [8]. On the other hand, the genetic stability of the fungus in the environment, its population structure, reproduction strategies and geographic distribution, have received little attention and remain poorly studied.

The asexual (or anamorphic) T. harzianum is the most familiar Trichoderma species as it is the most frequent Trichoderma sp. in the majority of samples worldwide [11-13]. It has often even been synonymized with Trichoderma biocontrol agents in general [14], as it is the principal component in several commercial biofungicide formulations. Although the biology of T. harzianum has not been studied in detail, it was studied taxonomically. It was originally defined as a “species aggregate” [15], but Chaverri et al. [16] reported that it consists of seven genetic lineages which would fulfil the basic criteria of cryptic phylogenetic species within a large morphological species. The latter authors also stated that H. lixii is a teleomorph (sexual stage) of T. harzianum thus raising the probability of genetic recombination in nature. Despite the detected genetic polymorphism, the authors proposed the existence of a single although complex H. lixii/T. harzianum species. However, no more definitive data have been published on the reproduction, global geographic distribution and speciation within the H. lixii/T. harzianum.

In this paper we reveal the complex speciation history within H. lixii/T. harzianum sensu Chaverri et al. [16] and show that it consists of several hypothetical biological species, some recent agamospecies and numerous relict lineages with a monophyletic origin altogether.

We also show that T. harzianum sensu stricto and H. lixii s.s. are genetically isolated and therefore are two separate species.

Results
Sample set
As a first prerequisite for this study we established a representative sample set, originally comprising > 300 strains of worldwide origin which were identified as H. lixii/T. harzianum by the ITS1 and 2 barcode [11] and diagnostic morphophysiological characteristics [15,16]. The sample was condensed to 93 strains which were representative for the total genetic and geographic variation observed. The final sample set included 10 strains which were collected as Hypocrea telemorphs from decaying wood (Table 1), whereas the strains isolated as Trichoderma anamorphs were predominantly from various soil ecosystems. Representative strains of all neighbour species (the Harzianum-Catoptron Clade; [17,18]) were included in order to determine the speciation history within the clade, to set up genetic borders for morphological H. lixii and T. harzianum species, and to be used as a negative control for recombination analyses. The spans of the morphological species (separately considered for telemorphs and anamorphs) are shown on the right inset in Figure 1. Species names abbreviations presented in Figure 1 correspond to atrog - H. atro-gelatinosa CBS 237.63, tawa - H. tawa CBS 246.63, alni - H. alni CBS 120633, agr - T. aggressivum CBS 433.95, epi - H. epymiyes CBS 120534, pleuroti - T. pleuroticola Z.D. 56, pleurotu - T. pleurotum C.P.K. 2113, brunn - H. bruneo-viridis CBS 120928, velu - T. velutinum DAOM 320014, stram - H. straminea G.J.S. 02-84, ceri - T. ceri-num DAOM 230012, tom - T. tomentosum Z.D. 28, cato - H. catoptron G.J.S. 02-76, C.P.K. 410 - T. sp. C.P.K. 410.

Detection of phylogenetic species
We screened among five potential phylogenetic markers for their ability to differentiate within our sample. Besides the finally analyzed loci the initial set included exon fragments of RNA polymerase (rpb2) and translation elongation factor 1-alpha (tefl1) genes. However, only three unlinked loci were sufficiently variable - intron containing regions of the calmodulin (call) and tef1 genes and the coding fragment of the GH18 chitinase gene (chi18-5). Their nucleotide characteristics are given in Additional file 1. We analyzed them as a combined dataset as well as individually.

As seen in Figures 1 and 2, all phylograms confirmed a monophyletic origin for selected 93 strains. Despite the monophyletic origin, the level of intraspecies genetic polymorphism (deduced from the branch lengths) within H. lixii/T. harzianum sensu Chaverri et al. [16] was comparable with that of interspecific variation within the
| Strain no. | Other collections | Origin |
|-----------|-------------------|--------|
| **T. harzianum** |                  |        |
| hz.01**   | C.P.K. 1052       | DAOM231646 | Kirstenbosch, South Africa |
| hz.02     | C.P.K. 1070       | DAOM 222343 | Ireland |
| hz.03     | J.B.T 1244        | J.B.T 2181 | Almonte, ON, Canada |
| hz.04     | C.P.K. 1093       | DAOM 222183 | Leamington, ON, Canada |
| hz.05     | C.P.K. 1104       | DAOM 176235B | Vancouver Island, Canada |
| hz.06     | C.P.K. 204 ex-type | CBS 226.95 | Sheffield, UK |
| hz.08     | C.P.K. 206        | TUB F-477 | Ural, Russia |
| hz.09     | C.P.K. 2111       | SzMC 3203 | Hungary |
| hz.10     | C.P.K. 217        | TUB F-690 | Moscow, Russia |
| hz.11     | C.P.K. 261        | TUB F-743 | Krasnoyarsk, Siberia |
| hz.12     | C.P.K. 265        | TUB F-790 | Vladimir, Russia |
| hz.13     | C.P.K. 1116       | DAOM 167088 | Kananaskis, AB, Canada |
| hz.14     | C.P.K. 360        | IMI 359823 | North Ireland, UK |
| hz.15     | J.B. RO11-1       | C.P.K. 2654 | Brasov, Romania |
| hz.16     | C.P.K. 1818       | PPRC J12 | Jimma, Ethiopia |
| **Lixii subclade** |                  |        |
| li.01     | C.P.K. 1068       | DAOM 229959 | Wisconsin, USA |
| li.02     | C.P.K. 1081       | DAOM 229903 | Wisconsin, USA |
| li.03     | C.P.K. 1102       | DAOM 222136 | Campbellville, ON, Canada |
| li.04     | C.P.K. 1107       | DAOM 222137 | Leamington, ON, Canada |
| li.05     | C.P.K. 1108       | DAOM 22151 | Temple, PA, USA |
| li.06     | C.P.K. 1110       | DAOM 190830 | Kingston, ON, Canada |
| li.07     | C.P.K. 1720       | G.J.S. 05-82 | Cameroon |
| li.08     | C.P.K. 1722       | G.J.S. 05-22 | Cameroon |
| li.09     | C.P.K. 1724       | G.J.S. 05-32 | Cameroon |
| li.10     | C.P.K. 588        | CBS 115334 | El-Fayum, Egypt |
| li.11     | C.P.K. 1069       | DAOM 229907 | Konza Prairie, KS, USA |
| li.13     | C.P.K. 2784 ex-type | G.J.S. 97-96 | Saraburi Prov., Thailand |
| li.14     | C.P.K. 1596       | W.M.J. 2317 | Styria, Austria |
| li.15     | C.P.K. 334        | G.J.S. 98-65 | Unknown |
| li.16     | C.P.K. 335        | G.J.S. 98-64 | Unknown |

| Subclade II T. sp. | 'afroharzianum' |
|-------------------|----------------|
| Il.01              | C.P.K. 238 |
| Il.02              | C.P.K. 807 |
| Il.03              | C.P.K. 588 |
| Il.04              | C.P.K. 246 |
| Il.05              | C.P.K. 845 |
| Il.06              | C.P.K. 2618 |
| Il.07              | C.P.K. 51 |
| Il.08              | C.P.K. 1061 |

Table 1 Strains of *H. lixii/T. harzianum* species complex used in this study.
Table 1: Strains of *H. lixii/T. harzianum* species complex used in this study. (Continued)

| Subclade IIa | Strain Code | Country | Location | GenBank Accession Numbers |
|--------------|-------------|---------|----------|--------------------------|
| IIa.09 | C.P.K. 2624 PPRC RW20 | Harerge, Ethiopia | coffee rhizosphere | FJ716621 FJ577759 FJ623104 |
| IIa.09 | C.P.K. 2624 PPRC RW20 | Harerge, Ethiopia | chernozem soil, corn field | EF191339 FJ577769 EF191295 |
| IIa.09 | C.P.K. 2624 PPRC RW20 | Harerge, Ethiopia | park soil | AY605788 FJ577735 FJ623097 |
| IIa.09 | C.P.K. 274 TUB F-771 | Ghaze, Nepal | forest soil | AY605834 FJ577695 AY605880 |
| IIa.09 | C.P.K. 245 CBS 115343 | Costa Rica | maize field | EF191318 FJ577689 EF191252 |
| IIa.09 | C.P.K. 2710 PPRC S33 | Dilla, Ethiopia | soil | FJ577790 FJ577771 FJ623106 |

| Subclade III | Strain Code | Country | Location | GenBank Accession Numbers |
|--------------|-------------|---------|----------|--------------------------|
| III.01 | C.P.K. 1075 DAOM 229908 | Wisconsin, USA | A1 horizon, forest soil | EF191322 FJ577730 EF191266 |
| III.02 | C.P.K. 276 TUB F-773 | Nepal | bark | AY605850 FJ577696 AY605881 |
| III.03 | C.P.K. 272 TUB F-769 | Nepal | bark | AY605849 FJ577694 AY605879 |
| III.04 | C.P.K. 2301 UNISS 13b-11 | Cuglieri, Sardinia | forest land | EF488114 FJ577755 EF392736 |
| III.05 | C.P.K. 2646 | Costa Rica | maize field | EF191329 FJ577760 EF191285 |
| III.06 | C.P.K. 271 TUB F-768 | Geirigan, Nepal | on Quercus | AY605847 FJ577693 AF399267 |
| III.07 | C.P.K. 2673 PPRC R12 | Bako, Ethiopia | coffee plantation | AY605856 FJ577690 EF191238 |
| III.08 | C.P.K. 1084 DAOM 229978 | Western Australia | on *Eucalyptus* | AY605870 FJ577691 EF191268 |
| III.09 | J.B. RSA122 DAOM 231651 | Kirstenbosch, South Africa | soil under *Erica* | EF191338 FJ577768 EF191294 |

| Subclade IV | Strain Code | Country | Location | GenBank Accession Numbers |
|--------------|-------------|---------|----------|--------------------------|
| IV.01 | C.P.K. 590 | Atherton, Australia | rhizosphere | EF191320 FJ577740 EF191254 |
| IV.02 | C.P.K. 693 TUB F-961*** | Beijing, China | park soil | AY605848 FJ577697 AF399265 |
| IV.03 | J.B. NZ1-2 DAOM 233825 | Urupakapaka, New Zealand | under *Leptospermum* | EF191330 FJ577761 EF191286 |
| IV.04 | J.B. NZ7-2 DAOM 233281 | Mt. Pureora, New Zealand | soil | EF191332 FJ577763 EF191288 |
| IV.05 | C.P.K. 2610 PPRC RM6 | Bako, Ethiopia | coffee rhizosphere | FJ577787 FJ577765 FJ623102 |
| IV.06 | C.P.K. 53 PPRC 3909 | South Africa | unknown | EF113551 FJ577682 EF191249 |
| IV.07 | C.P.K. 1044 DAOM 231412 | Kigali, Rwanda | sandy clay cultivated soil | AY605764 FJ577719 EF191255 |
| IV.08 | C.P.K. 1058 DAOM 231435 | Kigali, Rwanda | parkland soil | EF191321 FJ577721 EF191258 |

| Subclade V | Strain Code | Country | Location | GenBank Accession Numbers |
|--------------|-------------|---------|----------|--------------------------|
| V.01 | C.P.K. 1065 DAOM 231405 | Isla Mujeres, Q.R., Mexico | sandy soil | AY605774 FJ577725 EF191261 |
| V.02 | C.P.K. 646 TUB F-613*** | Hookena, Hawai | decaying grass | FJ577780 FJ577760 FJ623086 |
| V.03 | C.P.K. 727 TUB F-1082*** | Trinandrum, India | plant debris | FJ577781 FJ577709 FJ623088 |
| V.04 | C.P.K. 743 TUB F-1236*** | Embudu, Maldives | dead bark | FJ577782 FJ577710 FJ623089 |
| V.05 | C.P.K. 1059 DAOM 231425 | Cancun, Q.R., Mexico | plant soil | EF605768 FJ577722 EF191259 |

| Subclade X’/no clade | Strain Code | Country | Location | GenBank Accession Numbers |
|-----------------------|-------------|---------|----------|--------------------------|
| X.01 | C.P.K. 836 | El-Fayum, Egypt | cotton field | AY605838 FJ577713 FJ623090 |
| X.02 | C.P.K. 837 CBS 115333 | El-Fayum, Egypt | maize field | AY605839 FJ577714 FJ623091 |
| X.03 | C.P.K. 1505 UNISS10.5M | Aoujet, Mauritania | soil | FJ577784 FJ577743 FJ623098 |
| X.04 | C.P.K. 3408**** PPRC R12 | Papua New Guinea | deep sea sediment | FJ577791 FJ577773 FJ623108 |
| X.05 | C.P.K. 3409**** | Papua New Guinea | deep sea sediment | FJ57792 FJ577774 FJ623109 |
| X.06 | J.B. NZ11-1 DAOM 233829 | Kichappes, New Zealand | soil | EF191333 FJ577764 EF191289 |
whole Harzianum-Catoptron Clade (Figure 1 left inset).
The application of the genealogical concordance phylogenetic species recognition concept [19], using the cri-
ted in any of the others) identified two distinct
independent evolutionary lineage [phylogenetic species]
H. lixi/Trichoderma harzianum has been recognized [19],

Table 1: Strains of H. lixi/Trichoderma harzianum species complex used in this study. (Continued)

| Strain | Collection | Location | Type | GenBank Accession Numbers |
|--------|------------|----------|------|----------------------------|
| X.07   | J.B. NZ2-4 | DAOM 233823 | Urupakapaka, New Zealand | Soil under tree fern | EF191331, FJ577762, EF191287 |
| X.08   | C.P.K. 596 | TUB F-1035 | Victoria, Brazil | Soil | FJ577779, FJ577705, FJ623085 |
| X.09   | C.P.K. 709 | TUB F-1035 | Iguazu Falls, Brazil | Tropical rain forest | AY605851, FJ577708, AY605884 |
| X.10   | C.P.K. 878 | Z.D. 57 | Iran | Soil | AY602977, FJ577717, FJ623094 |
| X.11   | C.P.K. 1066 | DAOM 231402 | Kaia village, Q.R., Mexico | Garden soil | AY650775, FJ577726, EF191262 |
| X.12   | C.P.K. 1717 | G.J.S. 05-62 | Vietnam | Unknown | EF191325, FJ577746, EF191281 |
| X.13   | C.P.K. 1064 | DAOM 231408 | Chichen Itza, Q.R., Mexico | Forest soil | AY605773, FJ577724, EF191260 |
| X.14   | J.B. NZ2-4 | DAOM 233966 | Iquitos, Peru | Soil | EF191334, FJ577765, EF191290 |
| X.16   | C.P.K. 239 | CBS 115342 | Costa Rica | Maize field | EF191317, FJ576888, EF191251 |
| X.17   | C.P.K. 333 | G.J.S. 91-159 | Unknown | Unknown | FJ577775, FJ57698, FJ623080 |

Strains used in this work were either obtained by one of the authors, or taken from previous published work [cf. refs. [11,13,16-19] and citations therein]. "Other collection" names are names that have been used for these isolates in earlier papers. * corresponds to species or clades revealed in this study; ** strain code used in Figures 1 and 2; *** strains obtained in the course of a bilateral research project between TU Vienna to C.P.K. and the Technical University of Budapest (Hungary) to George Szakacs; **** only DNA material available, supplied by Katja Fisch (University of Bonn, Germany); bold font highlights strains isolated from teleomorphs;
Figure 1 Multilocus phylogenetic map of *H. lixii* - *T. harzianum* species complex. The main body of the figure shows the radial Bayesian tree resulting from the analysis of the concatenated sequences of *tef1*, *cal1* and *chi18-5*. Nodes supported by posterior probabilities (PP) > 0.94 and 0.89 < PP < 0.95 are indicated by black and grey dots respectively. Names of subclades, as used in the text, are written along the most internal branches leading to them. Full squares at OTUs indicate teleomorph isolates. The color code indicates the geographic region from which the isolates were obtained, as explained in the right top inset. Isolates with yellow color indicate uncertain geographic origin. The ex-type strains are underlined. The four putative phylogenetic species are indicated by rectangular and round backgrounds for agamospecies and holomorphic species, respectively. Isolates for which a recombination history was detected are grouped by irregular shape frames with grey background. Isolates used and results from the PHT test are shown by arrows and the respective P values, ‘rec +’ specifying positive recombination result and ‘rec -’ specifying no recombination detected. The left bottom inset shows the results from the same analysis when representative strains of the all studied *H. lixii*/*T. harzianum* and the representative strains of the Harzianum-Catoptron Clade were also included. The yellow background separates strains of the ‘pseudoharzianum matrix’. Other symbols are used as above, but no geographic color code is applied. The right bottom inset shows the recombination network, determined by SplitsTree (NJ mode) from the combined dataset. Taxa with "harzianum"-like morphology are shown by a light yellow background and dotted lines; other anamorph morphologies are shown by different colours and dotted lines; individual teleomorph morphologies are indicated by different end symbols. Representative sequences for species from Harzianum-Catoptron Clade may be retrieved from NCBI Entrez search engine using [species strain locus] keywords.
strain had an unresolved position in the ‘pseudoharzia-
num matrix’.

The detected phylogenetic species, hypothetical phylo-
genetic species and newly introduced terms with and
without taxonomic significance are listed in Table 2.

Recombination between and within T. harzianum, T. sp.
‘afroharzianum’ and strains of the “pseudoharzianum
matrix”

The uneven distribution of teleomorph isolates in the H.
lixii - T. harzianum species complex, particularly their
absence in T. harzianum s.s. and T. sp. ‘afroharzianum’
but their presence in the Lixii subclade, may suggest
that either the tested strains are virtually genetically
identical and likely clonal (≡ clonal sterility) or that all
teleomorph stains within the sample were completely
reproductively isolated (≡ interspecific sterility). As these
fungi can not be crossed in vitro, we applied three alter-
native computational methods for detection of genetic
recombination from sequence data. To this end we used
the T. harzianum strains as a control sample for clonal
sterility and seven representative strains of species from
the Harzianum-Catoptron Clade which were genetically
most distant to H. lixii and T. harzianum as a control
for interspecific sterility.

First, the partition homogeneity test (PHT; [21]) was
used to examine the congruence between gene trees.
This test produces artificial datasets by multiple
(10 000) re-sampling and random swapping of observed
datasets and subsequent construction of maximum-par-
simony trees for every newly sampled ‘gene’ sequence.
For clonally reproducing populations (= no sexual
recombination), the sums of the lengths of the gene
trees for the observed and re-sampled data should be
similar. However, under recombination the sums of the
tree lengths should be longer than that for the actual
data because of introduction of homoplasy into unlinked
sites. This test confirmed our analysis of topologies of
single locus trees - the clades containing T. harzianum
and T. sp. ‘afroharzianum’ showed congruence of data.
suggesting their clonality (Figure 1). Other clades, which were incongruent in different trees, provided evidence for recombination. However, recombination was also not detected with strains occurring at unresolved positions on the combined tree (X.01 - X.16) including the ex-type strain of *T. inhamatum* (X.15 = CBS 273.78) and two strains isolated as teleomorphs (Figure 1). As none of these strains fulfilled the criteria of a phylogenetic species, we assume that they represent multiple relict lineages which are incompatible of recombination either due to the loss of sexuality or due to mating incompatibility evolved in a course of habitat isolation. The relatively long genetic distances between them support this view. The controls (= representatives of Harzianum-Catoptron Clade) produced essentially negative results (Figure 1, right inset).

As a second means, we used the index of association (IA) test on a subset of ‘clone corrected’ data (i.e. individuals with identical alleles of the three loci were excluded so that each haplotype was represented only once; cf. [22]). In this test, complete panmixia (sexual compatibility resulting in recombination) would be indicated by a value of 0 (= the null hypothesis). This value was neither obtained with the complete dataset nor with any of the individual clades (data not shown). Yet the Lixii subclade, and Subclades III and IV gave values lower than 1, thus supporting the null hypothesis of sexual compatibility between strains within them. In contrast *T. harzianum* and *T. sp. ‘afroharzianum’ yielded values above 1, rejecting the recombination. Subclade V was not analyzed as it consisted of only two concatenated haplotypes. In accordance with results from PHT, IA values for strains at unresolved positions (X.01 - X.16) also showed no evidence for recombination.

Finally we applied the Phi-test, which uses the pairwise homoplasy index (PHI, Ö) to detect refined incompatibility [23]. This method assumes the infinite sites model of evolution [24] in which the detection of incompatibility for a pair of sites indicates recombination. Application of this test to the same subsamples based on phylogenetic species and clades of the combined tree confirmed the results of the previous analyses, and also detected no recombination in *T. harzianum*, *T. sp. ‘afroharzianum’ and between representative strains of the Harzianum-Catoptron Clade (*P* = 0.1897, *P* = 0.2773 and *P* = 0.3406, respectively). All other subclades showed positive recombination signals (*P* < 0.05). The corresponding network, produced by Splitstree, is shown on the inset on the right bottom of Figure 1.

Since the Phi-test is a very robust means which can detect recombination even in the presence of recurrent mutation, we decided to use this method to define the borders of recombining populations. To this end, we first set up a non-recombining sample consisting of the most terminal strains of a clade to be investigated, and the most distant strains from other phylogenetic species. Then we gradually added phylogenetically closer strains until evidence for recombination was detected (*P* < 0.05). To determine the outer border of the recombining population, we excluded the first strain which indicated recombination and started to add phylogenetically more distant isolates from the same subclade or clade. Such approach has the advantage that it also allows the analysis of small subclades which can not be analysed alone due to insufficient data. This technique was applied to all meaningful combinations of species/clades/subclades which were present on the phylograms shown (around

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### Table 2 Conventional nomenclature derived from phylogenetic analyses

| Phylogenetic species: | Taxonomic value |
|-----------------------|-----------------|
| *T. harzianum*         | yes             |
| *T. sp. ‘afroharzianum nom. prov.’ | yes, awaiting description |

Hypothetical phylogenetic species:

| Species and taxonomic units: | Taxonomic value |
|-----------------------------|-----------------|
| *H. lixii*                  | yes             |
| *T. inhamatum*              | yes             |

Lone lineages with unresolved phylogenetic position but belonging to established taxonomic species:

| Species and taxonomic units: | Taxonomic value |
|-----------------------------|-----------------|
| *paralixii nom. prov.*      | no, awaiting verification |
| *cameroonense nom. prov.*   | no, awaiting verification |

Above species taxonomic units:

| Species and taxonomic units: | Taxonomic value |
|-----------------------------|-----------------|
| *H. lixii - T. harzianum species complex or aggregate = H. lixii/T. harzianum sensu Chaveri et al., [16] | no |

Conventional terms for groups of strains with unresolved relations:

| Species and taxonomic units: | Taxonomic value |
|-----------------------------|-----------------|
| ‘pseudoharzianum matrix’ = *H. sp. ‘pseudoharzianum nom. prov. dub.’ | no |
| Lixii Subclade               | no |
| Subclades Ila, III, IV, V and X | no |
90 individual tests). The positive results (recombination, \( P < 0.05 \)) obtained are shown by shadowed areas on the main part of Figure 1, and provided interesting insights into the reproduction history of the clades found. For example, although the isolates of *T. harzianum* were proven to be clonal by all three methods used, they still revealed a recombination history with strains X.01 - X.03 from Northern Africa which occupied an otherwise unresolved phylogenetic position in the *tefl, cal1* and *chi18*-*S* trees. Another presumably recent agamospecies, *T. sp. ‘afroharzianum’*, exhibits a history of recombination with five phylogenetically plesiotypic strains of different geographic origins (Figure 1). Not all strains of the Lixii subclade showed a recombination history in this test. Nevertheless two groups with positive recombination signal were detected - one includes strains of *H. sp. ‘paralixii’* and two temperate strains from North America and Europe; the second covers the latter two strains, *H. sp. ‘cameroonenense’* and the *ex*-type strain of *H. lixii*. A limited recombination was also detected for strains from Subclade III occupying terminal positions - three Austrian telemorphs and two anamorphic strains isolated from Sardinian soils were recombining with each other but not with strains occupying basal branches of the subclade. Similarly, the four terminal strains from a Subclade IV (all from Africa) display a history of recombination. In addition, all strains of the panmictic subclade V showed evidence for recombination. This result corresponds to the fact that they occupy conflicting positions in the single gene trees but belong to the same subclade of *ech18*-S phylogram indicating their common evolutionary path. The most genetically polymorphic isolates of the ‘pseudoharzianum matrix’, which formed almost no phylogenetic structure (Subclade X), showed no evidence for recombination signal when they were confronted either with each other or with other subclades. However, when some of them were analysed together with representatives of the Harzianum - Catoptron Clade, a significant recombination signal was detected indicating traces of interspecific sexual compatibility (open shadow area on Figure 1).

**Population divergence and stability**

To estimate the degree of differentiation within the ‘pseudoharzianum matrix’, we applied methods for analysis of populations and computed the \( F_{ST} \) values [25] for the main subclades of the combined phylogram. Qualitatively, an \( F_{ST} \) value in the range close to 0 indicates low differentiation (= fixation of characters) between populations (compared populations are composed of equally different individuals) and close to 1 indicates great differentiation when populations are composed of similar individuals but there is a big difference between populations [26]. \( F_{ST} \) values between *T. harzianum* and other subclades were in the range of 0.67 - 0.83 indicating essential genetic separation of this species. The \( F_{ST} \) value between Subclades III and IV of the ‘pseudoharzianum matrix’ was low (0.17) which may indicate that some genetic exchange still occurs between their strains.

The population parameters \( 0 \) (for haploids 2 Nm, where N is effective population size and m is the mutation rate per site and generation) and population growth rates were calculated using LAMARC package (see Methods). Consistent with the occurrence of *H. lixii - T. harzinum* species complex as one of the most frequent taxon of the genus, the growth rate of most subclades is large, which is indicative of a large effective population size and population expansion (data not shown). The recombining subclades had the highest growth rate. Only *T. harzinum* exhibited a significantly smaller value for population growth (5.90) and also a 5-20-fold smaller \( 0 \)-value than the other subclades. This suggests that this species apparently occupies a niche with a limited potential for expansion.

**Discussion**

The perception of fungal species as dynamic entities which arise, persist for a longer or shorter period, modify, decline and then become extinct and replaced by other species leads to a variety of existing species concepts in mycology. In addition, the taxonomy of almost every large fungal genus is biased either because of its importance to mankind (e.g. for convenient differentiation of pathogenic or industrial organisms) or by its history of taxonomic description. Here we have analysed *H. lixii - T. harzinum* species complex, one of the most commonly sampled groups of fungi because of its dominant presence in the majority of soil ecosystems worldwide and its occupation of a broad diversity of ecological niches. From the results of this paper, the evolutionary success of the *H. lixii - T. harzinum* species aggregate may be attributed to the very complex structures of the contemporary populations of these fungi, which can be differentiated into nearly all possible ‘types’ of fungal species: reproductively isolated biological species, sympatric and allopatric phylogenetic species, recent agamospecies and numerous relict lineages with unresolved phylogenetic positions. This complexity comes from the fact that many of these species ‘types’ are overlapping and therefore it frequently happens that two closely related organisms become attributed to different species recognized based on incomparable criteria. Our data illustrate a speciation history within *H. lixii - T. harzinum* species complex, which is based on coexistence and interaction of organisms with different evolutionary strategies and on the absence of strict genetic borders between them.
The genealogical concordance phylogenetic species recognition [GCPSR, [19]] concept is the most advanced approach to defining species in modern fungal taxonomy. Yet our results demonstrate that even this method has its limits within a population of isolates with different recombination histories. Basically, the whole of the *H. lixii* - *T. harzianum* species complex might be considered as a single species as its monophyletic origin is supported by all three loci tested in this paper. Chaveri et al. [16] used the indistinguishable morphology of these fungi in favour of such a conclusion. However, this approach appears to be invalid when *T. harzianum* sensu Chaveri et al. [16] is compared with its genetically closest neighbouring species. The genetic distances calculated within *H. lixii* - *T. harzianum* species complex are comparable to the distances between well diverged species of the Harzianum-Catoptron Clade, which are characterized by different phylogenies, morphologies, physiologies and ecological characteristics [17]. Within the *H. lixii* - *T. harzianum* complex, a conservative application of the GCPSR concept justifies two anamorphic phylogenetic species (*T. harzianum* and *T. sp. ‘afroharzianum’), and does not contradict postulating two further holomorphic phylogenetic species (*H. sp. ‘paralixii’* and *H. sp. ‘cameroonensis’*). No consistent phylogenetic structure could be detected, however, in the remaining strains.

The results from this work also shed new light on the concept of the *H. lixii/T. harzianum* holomorph and the synonymization of *T. inhamatum* with *T. harzianum*. All methods used clearly differentiated *T. harzianum* sensu stricto from the remaining isolates as an agamospecies with global distribution in temperate ecosystems. Since *T. harzianum* and *H. lixii* are genetically isolated and evidently do not appear in the same life cycle, the holomorph *H. lixii/T. harzianum* must therefore be rejected. Also, the unclear phylogenetic position of the ex-type strain of *H. lixii*, and the fact that telemorphs with ‘*H. lixii*’ morphology are also present in subclade III which is reproductively isolated from *H. lixii* sensu stricto, render the naming of most of the isolates investigated here as ‘*H. lixii*’ doubtful. Finally, we show that *T. inhamatum* is a separate phylogenetic lineage only distantly related to *H. lixii* or to *T. harzianum*, and its name should therefore be maintained.

Recombination is a powerful evolutionary force that merges historically distinct genotypes. Yet the extent of recombination within many organisms is unknown, and even determining its presence within a set of homologous sequences is a difficult question. Molecular traces of sexual recombination were detected for the majority of tested strains and phylogenetic species, and were correlated with the occurrence of most of the teleomorph isolates within recombining clades. This finding is the opposite of what has been seen with other apparently asexual fungi such as the human pathogens *Coccidioides posadasii* [27], *C. immitis* [28] and *Paracoccidioides brasiliensis* [29], the facultative pathogen *Aspergillus fumigatus* [22], and the mycotoxin producer *A. flavus* [30]. In all these cases, telemorphs were not found, whereas phylogenetic evidence for recombination was obtained. In our sample, such a situation was seen only for Subclade V which is composed of evidently recombining strains exclusively isolated as anamorphs.

In this study, we introduced a trial-and-error approach to detect borders of recombination within a sample. The limited distribution of sexual recombination within the phylogenetic clades underlines that these fungi pass through periods of sexual and asexual recombination. Based on the unresolved structure of the “pseudoharzianum matrix” in the individual and combined phylogenetic trees, we originally expected sexual compatibility between all of its strains. Yet our data showed that distantly related strains from the same subclades had already lost their ability for genetic exchange. The most striking example was the absence of recombination between the *H. lixii* ex-type strain and two other teleomorphic strains from the same subclade, which further supports the postulation of *H. sp. ‘paralixii’* as a separate albeit closely related taxon.

With the exception of *H. sp. ‘paralixii’* and Subclade IV (whose isolates were exclusively derived from Africa, South-East Asia, Australia and New Zealand), all other clades exhibited a global geographic distribution. While a worldwide distribution of fungi was at one time believed to be the rule, the application of molecular genetic methods has recently shown that most of these globally distributed taxa actually consist of several alloparratic cryptic species. To the best of our knowledge, the only other similar finding of panmixis for a mitosporic fungus has recently been presented for *A. fumigatus* [22]. The fact that most of the clades detected in this study could maintain (in part) a panmictic population is interesting, because it is known (e.g. for insects) that dispersal and subsequent alloparratic speciation can occur in very short times, even in the absence of severe population bottlenecks [31,32]. This lack of alloparratic speciation - which otherwise seems to occur in several other species of *Hypocreae/Trichoderma* [cf. [33,34]] - must be due to a continuous, unrestricted gene flow, whose mechanism warrants being identified.

*T. harzianum* sensu Chaveri et al. [16] is one of several *Trichoderma* species which are successfully used in biological control of plant pathogenic fungi. The results from this study show that the respective strains must be members of one of several phylogenetic species with different recombination frequencies. In a preliminary test of a few commercially used “*T. harzianum*” biocontrol
agents, none of them showed the gene sequences characteristic for the strains from the clonal *T. harzianum* and *T. sp.* ‘afroharzianum’ clades, and therefore may be members of the recombining populations and phylogenetic species (C.P. Kubicek, unpublished data). Recombination of a biocontrol strain clearly could have a significant impact on its stability in the field. A study aiming at clarifying this situation is currently in progress. Fungi belonging to ‘pseudoharzianum matrix’ are abundantly isolated from various environments and are frequently selected as biocontrol strains. Therefore their correct identification is of great importance. This study shows that because some of them are able to recombine while others are probably sexually incompatible, their attribution to a single taxon would be very doubtful. We suggest the introduction of the provisional temporary name *H. sp.* ‘pseudoharzianum nom. prov. dub.’ in order to separate strains belonging to ‘pseudoharzianum matrix’ from phylogenetic species such as *T. harzianum s.s.*, *T. sp.* ‘afroharzianum’, *H. lixii s.s.* and *T. inhamatum*, which can be correctly identified by the analysis of *tef1* 4th large intron sequence using either the similarity search tools or phylogeny. The development of an integrated bioinformatic tool for the haplotype-based identification of fungi within *H. lixii - T. harzianum* species complex and for the potential prediction of their mycoparasitic abilities has been started in the laboratory of the corresponding author.

**Conclusions**

In this work we did not defeat the ‘harzianum demon’. However, we demonstrated its power, span and perhaps its limits, and believe that the current study provides some understanding of the forces driving speciation in these fungi. A major challenge of future work will be the elaboration of standardized methods by which the phylogenetic species and the ‘pseudoharzianum matrix’ detected here can be conveniently differentiated with predictable biological activities. Preliminary data in our laboratory showed that the use of 95-carbon source phenotype microarrays may be helpful in this regards (J. Bissett and I.S. Druzhinina, unpublished data).

**Methods**

**Strains and gene sequences**

The strains, sequences and NCBI GenBank accession numbers are listed in Table 1, and are maintained in long-term storage facilities at Vienna University of Technology (Austria) and ECORC (Canada) laboratories.

**DNA extraction, PCR amplification and sequencing**

Mycelia were harvested after 2 - 4 days of growth on malt extract agar at 25°C and genomic DNA was isolated using QIAGEN DNeasy’ Plant Maxi Kit following the manufacturer’s protocol. Amplification of DNA fragments comprising ITS1 and 2 and the 5.8S rRNA gene, the endochitinase chi18-5 (= ech42; [35]) and the large 4th intron of *tef1*, amplicon purification and sequencing was performed as described in detail previously [36]. Previously published sequences used for phylogenetic analyses in this study were retrieved from GeneBank and are identified by their respective accession numbers.

**Phylogenetic analyses**

For the phylogenetic analysis DNA sequences were aligned with Clustal X 1.81 [37]. As the 4th large intron of *tef1* is highly polymorphic, the alignment contained several ambiguous areas which could contain homoplasious characters resulted from multiple substitutions and/or saturation. In order to detect such areas we have processed the concatenated alignment using the Gblocks server [[38], URL: http://molevol.cnmima.csic.es/castresana/Gblocks_server.html]. When the default stringent parameters of Gblocks were applied, nearly complete locus of the *tef1* intron has been removed (not shown); when the less stringent options were used, only 10% of the alignment was removed leaving the considerable part of *tef1* intron in the analysis (Additional file 2). The poorly aligned areas of *tef1* selected by Gblocks have been carefully edited manually by inserting extra gap columns in order to reduce the difference between sequences originating from hypothetically homoplasious characters. The original and edited alignments are available from Additional file 3. The possibility of intragenic recombination, which would prohibit the use of the respective loci for phylogenetic analysis, was tested by linkage disequilibrium based statistics as implemented in DnaSP 4.50.3 [39]. The neutral evolution of coding fragments (*call* and *chi18-5*) was tested by Tajima test implemented in the same software. The interleaved NEXUS files were formatted using PAUP*4.0b10 [40]. The best nucleotide substitution model for the each locus was determined using jMODELTEST [41]. As Akaike and Bayesian Information criteria (AIC [42] and BIC [43], respectively) selected different nucleotide substitution models for every locus and due to the relatively small size of individual datasets (1379 characters per 107 sequences for the biggest) the unconstrained GTR + I + G substitution model was applied to all sequence fragments (Additional file 1). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed using MrBayes v. 3.0B4 with two simultaneous runs of four incrementally heated chains that performed 5 million generations. The length of run (number of generations) for each dataset was determined using AWTY graphical system [44] to check the convergence
Population divergence and stability

Population growth parameters and theta (θ) values were inferred using the program LAMARC 2.0 [50]. All polymorphic sites were used to assess the population parameters. To estimate the population growth parameters, we used 10 initial chains with 2,000 genealogies sampled and two final chains with 20,000 genealogies sampled. Population parameters were inferred using both Bayesian and Maximum Likelihood criteria.

Detection of Recombination

The congruence or incongruence of the three genealogies was used to infer recombination between isolates of the *sensu stricto* group (see Results). To this end, three different tests were employed: the incongruence length difference/partition homogeneity test (ILD/PHT) [21,46] using a score of $P < 0.05$ to reject the null hypothesis of congruence between loci; the Index of Association [IA; [47]] test, in which the data were compared to the IAs of artificially recombined datasets [27,48]; and the Phi-test implemented in SplitsTree, which uses the pairwise homoplasy index, PHI (= Φ) statistic, to detect refined incompatibility indicating recombination [23].

In addition we applied split decomposition implemented in SplitsTree program, version 4.0 [49], using pairwise distances under the Kimura 3-ST model [24]. This method visualizes recombination events by depicting the shortest pathway linking sequences, rather than forcing them into a bifurcating tree [49].

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Authors’ contributions

ISD wrote the final version of the paper, and performed the phylogenetic and recombination analyses and interpretations; CPK designed the study and the sample; performed the population analyses and participated in writing a draft version of the paper; MKZ and TBM performed the molecular laboratory work; JB provided additional strains and their sequences and contributed to the text of the final version of the paper. All authors read and approved the final manuscript.

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