

Signs of resistance to *Melampsora larici-tremulae* on species of *Pinus* hosts of *Melampsora pinitorqua*: implications regarding the taxonomic relationship between the two rust fungi

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Summary. Signs of resistance such as cortical necroses which occurred after artificial inoculations with *Melampsora larici-tremulae* on some species of the genus *Pinus* in the host range of *M. pinitorqua* were evaluated. Similar observations were carried out on *P. sylvestris* inoculated with two provenances of *M. larici-tremulae* from areas with different environmental conditions. In both experiments, the degree of resistance to *M. larici-tremulae* depended on how suitable environmental conditions were for both the host and the fungus. The greatest resistance to *M. larici-tremulae* was shown by *P. sylvestris*. These observations could indicate that *P. sylvestris* is becoming a nonhost of *M. larici-tremulae*. The type of host-parasite interaction between some species of *Pinus* and *M. larici-tremulae*, when compared to analogous interactions between the same species of *Pinus* and *M. pinitorqua*, can shed light on the taxonomic relationship between the two rust fungi.

Key words: cortical necroses, infection on *Pinus*, larch needle rust, pine twist rust, rust systematics.

Introduction

The taxonomic relationship between *Melampsora larici-tremulae* Kleb. (the larch needle rust fungus) and *Melampsora pinitorqua* Rostr. (the pine shoot twist rust fungus) has long been debated (Longo *et al.*, 1975). At present, it is not clear if these two fungi should be considered separate species, or *formae speciales* of *Melampsora populnea* (Pers.) Karst.

Evidence from the literature indicates that the two rust fungi have very similar morphological features and both have their uredial-telial stage on *Populus tremula* L. However, they differ in their natural pycno-aecial hosts: *M. pinitorqua* infects the growing shoots of many species of *Pinus*, while *M. larici-tremulae* infects the needles of *Larix*.

Longo *et al.* (1970, 1975) and Naldini Longo *et al.* (1985), during inoculation trials on seedlings of species of both *Pinus* and *Larix* (in their first growth cycle with primary needles), observed that some species of *Pinus* were susceptible to *M. larici-tremulae*, while conversely some species of *Larix* were susceptible to *M. pinitorqua*. They concluded that the pycno-aecial host ranges of the two rust

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fungi partially overlapped. However, they highlighted some morphological and pathogenic differences between the two taxa, which suggested the need for further study as regards their identity at a taxonomic level. The two rust fungi differed significantly in teliospore and urediniospore size. They also differed in their pathogenic behaviour with regard to the range of susceptible species (*P. sylvestris* L. was not susceptible to *M. larici-tremulae*), in the degree of pathogenicity (the number of infected seedlings and the speed of rust development were greater on their specific hosts), and in the location of the infection caused (*M. larici-tremulae* infected only the host needles).

Highlighting these differences between the two rust fungi on seedlings in their first growth cycle, Naldini Longo *et al.* (1985) held that the two taxa were more different *inter se* than mere formae speciales and hence should be considered distinct species.

Since these experiments indicated that *P. sylvestris* was a discriminating species for these rust fungi, artificial inoculations with many Italian provenances of rusted *Populus tremula* were carried out both on *P. sylvestris* and on *Larix decidua* Mill. in epidemiological experiments carried out over a period of several years (Naldini Longo *et al.*, 1988, 1991; Naldini *et al.*, 1992) to explore the distribution of *M. pinitorqua* and *M. larici-tremulae* in such provenances. In the case of *P. sylvestris*, seedlings in their second growth cycle with developing shoots were used; these shoots were usually infected under natural conditions with *M. pinitorqua*. In the course of these experiments, cortical necroses with rust mycelium were unexpectedly observed on shoots of *P. sylvestris* that had been inoculated with *M. larici-tremulae* from the central Alps and the northern Apennines. Cortical necroses were also observed on *P. sylvestris* after inoculation with *M. larici-tremulae* from the Maritime Alps (Naldini *et al.*, unpublished data). The necroses had very different macroscopic features, and generally appeared on only a small percentage of inoculated seedlings after a rather variable, but often quite long, incubation period (Longo *et al.*, 1997).

The sudden death of plant cells invaded by a rust fungus has generally been reported as due to a "host resistance" defence mechanism (Heath, 1981; Elmhirst and Heath, 1987). This reaction was

observed in the shoots of *P. sylvestris*, which was considered to be a nonhost of *M. larici-tremulae* (Naldini Longo *et al.*, 1985). A cyto-histological study was therefore carried out to determine the type and level of resistance shown by shoots of one year old seedlings of *P. sylvestris* to this rust fungus and thus to clarify the uncertain nonhost status of this pine species (Longo *et al.*, 1997). This study revealed that: 1) direct and intra-epidermal infection structures of the two rust fungi on their pycno-aecial hosts and of *M. larici-tremulae* on *P. sylvestris* were morphologically similar; 2) both pre- and post-haustorial resistance mechanisms to *M. larici-tremulae* occurred in *P. sylvestris*. These results, together with the morphological, epidemiological and host range data (Naldini Longo *et al.*, 1985, 1991), suggested the need for further study of the taxonomic relationship between the two rust fungi (Longo *et al.*, 1997).

Indeed, according to Heath (1981, 1985) regarding the evaluation of host and nonhost resistance in order to understand the evolution of host-rust interactions, post-haustorial reactions in *P. sylvestris* infected with *M. larici-tremulae* could indicate a "host resistance superimposed on a basic compatibility". The presence of pre-haustorial reactions could be interpreted as a "reintroduced resistance" mechanism of the nonhost type. This could indicate that *P. sylvestris* is a host species that is in the process of becoming a nonhost to *M. larici-tremulae*, and also that *M. larici-tremulae* and *M. pinitorqua* are in the process of diverging.

The aim of this work was to confirm that such a process is taking place, since *P. sylvestris* and *M. larici-tremulae* have a common habitat which ensures an on-going host-parasite interaction. To this end, two experiments were carried out. In the first, Experiment (A), signs of resistance to *M. larici-tremulae* were studied in shoots of some species of *Pinus* other than *P. sylvestris* which, in their first growth cycle, were susceptible to this rust fungus on primary needles (Longo *et al.*, 1975; Naldini Longo *et al.*, 1985). The signs of resistance found were compared with those previously reported on *P. sylvestris* (Longo *et al.*, 1997). The other pine species examined were natural hosts of *M. pinitorqua* like *P. sylvestris*, but their habitat was different. In Experiment (B) the aim was to compare, in all combinations, signs of resistance to two provenances of *M. larici-tremulae* in seedlings of *P. syl-*

vestris from the same two provenances as the rust. The choice of *M. larici-tremulae* provenances was made on the basis of previous knowledge about the distribution of this rust in the Maritime Alps (Naldini *et al.*, unpublished data) and the central Alps (Longo *et al.*, 1975), and having regard to the occurrence and location of *P. sylvestris* in these areas. Seedlings of *Pinus nigra* Arn. subsp. *laricio* Poiret were included to study their resistance to the same two provenances of *M. larici-tremulae*. Seedlings of this species of *Pinus* are known to be resistant only to *M. larici-tremulae* from the central Alps, and only in their first growth cycle (Longo *et al.*, 1975).

Materials and methods

Inoculations were carried out on the following species of *Pinus*: *P. pinea* L., *P. halepensis* Mill. and *P. pinaster* Ait (Experiment A); *P. sylvestris* L., provenance Maritime Alps [L.N.B.S. (Lista Nazionale Boschi da Seme), wood No. 4]; *P. sylvestris* L., prov. central Alps (L.N.B.S., wood No. 28); and *P. nigra* Arn. subsp. *laricio* Poiret (Experiment B). One-year-old seedlings in their second growth cycle were inoculated when needle growth on the apical shoot was just beginning.

The provenances of the rust fungi used for the inoculations were as follows: for Experiment A, *M. larici-tremulae* from Vallombrosa (Firenze), Northern Apennines, 1000 m a.s.l.; for Experiment B, *M. larici-tremulae* from Baiardo (Imperia), Maritime Alps, 800–1000 m a.s.l. and from Luson (Bolzano), central Alps, 1200 m a.s.l.; as a control for both Experiments, *M. pinitorqua* from Monticiano (Siena), Maremma Toscana, 400–500 m a.s.l.¹. Germinating teliospores on leaves of *P. tremula*, collected, over-wintered and incubated as described in previous papers (Longo *et al.*, 1970; Naldini Longo *et al.*, 1985, 1988, 1991; Naldini *et al.*, 1992; Longo *et al.*, 1997), were used as inoculum. Inoculations were carried out in spring at the time of optimal teliospore germinability, which varied with

the fungus and with the over-wintering pedoclimatic conditions encountered (*Tr.*) by the teliospore-bearing leaves (Naldini Longo *et al.*, 1988). Despite these variations in the times of inoculation, the degree of development of the pine shoots at the moment of inoculation was kept unchanged, since the seedlings were made to artificially pre- or post-sprout prior to inoculation as needed. The inoculations were carried out separately for the two rusts as indicated in previous papers (Naldini Longo *et al.*, 1985, 1988, 1991; Naldini *et al.*, 1992; Longo *et al.*, 1997). The inoculated seedlings were left in a controlled environment chamber in the following conditions: temperature, constant 15°C for 3 days and then 12–22°C (night-day); photoperiod, alternating 12 h darkness and 12 h light throughout the entire incubation period and course of infection. Data were collected approx. 45 days from inoculation and included: percentage of seedlings with cortical necroses, percentage of seedlings with aecia on the shoots, presence of twisting on infected growing shoots, presence of aecia on primary needles. Fresh transverse sections were cut from infected shoots using a cryo-cut microtome (American Optical, Buffalo, NY, USA). These sections were stained with lactic acid-cotton blue (Dop and Gautié, 1928) and examined under a Leitz Orthoplan optical microscope (Ernst Leitz, Wetzlar, Germany).

Results

In the comparisons made in Experiments A and B, the necroses evaluated were shoot lesions at an advanced stage of colonization by the rust. They were quite variable in size and colour (Fig. 1a, b). The shoots of some pine species also had one-sided growth and subsequent twisting (Fig. 1c). This twisting, together with the production of aecia, is a characteristic feature of pine shoots infected with *M. pinitorqua* (hence the name, pine twist rust) (Fig. 1d, e). Just as the macroscopic characteristics of the necroses were different, so too there were differences in the tangential and radial extension of the necroses in the cortical parenchyma, in the degree of colonization of this parenchyma by the rust mycelium, and in the continuity of the necrophylactic periderm surrounding the necrotic tissue (Fig. 2a, b). Notably, the type of necrosis was not correlated with a single pine, pine source, pine species, or a source of *M. larici-tremulae*.

¹ The rust provenances were previously determined by the Authors (Naldini Longo *et al.*, 1985, 1988, 1991; Naldini *et al.*, 1992; Naldini *et al.*, unpublished data) to be composed of single species and not mixtures of *M. pinitorqua* with *M. larici-tremulae*.



Fig. 1. a, b, c. Cortical necroses on shoots of *Pinus* inoculated with *Melampsora larici-tremulae* from a, Baiardo; b, Lusón; c, Vallombrosa; a, *Pinus nigra* subsp. *laricio* with small light-yellow necroses; b, *P. sylvestris* with large brownish necroses; c, *P. pinea* with extended necrosis and twisting. d, e. Shoots of *Pinus* inoculated with *M. pini-torqua* from Monticiano; d, *P. pinea* with aecia and characteristic twisting; e, *P. nigra* subsp. *laricio* with aecia.

In Experiment A (Table 1), a high percentage of seedlings of *P. pinea* and *P. halepensis* inoculated with *M. larici-tremulae* had cortical necroses on the shoots; when these same species were inoculated with *M. pinitorqua*, aecia were produced on the shoots of a high percentage of seedlings. In these two pine species, twisting of the shoots was observed after inoculation with either *M. pinitorqua* or *M. larici-tremulae*. In *P. pinaster* inoculated with *M. larici-tremulae*, the percentage of seedlings with cortical necroses was noticeably lower than it was in *P. pinea* and *P. halepensis* inocu-

lated with the same rust fungus, and twisting was completely absent. Also, aecia developed on the primary needles of *P. pinea* and *P. halepensis* inoculated with both *M. larici-tremulae* and *M. pinitorqua*, but not on *P. pinaster* after inoculation with *M. larici-tremulae*.

In Experiment B (Table 2) it can be seen that when seedlings of *P. sylvestris* and *P. nigra* subsp. *laricio* were inoculated with *M. larici-tremulae* from Baiardo, the percentage of seedlings with cortical necroses was clearly higher than when they were inoculated with *M. larici-tremulae* from Lu-

Table 1. Cortical necroses and aecia on shoots of *Pinus pinea*, *P. halepensis* and *P. pinaster* seedlings inoculated with *Melampsora larici-tremulae* from Vallombrosa and *M. pinitorqua* from Monticiano.

Rust fungus	Rust provenance	Pine species	No. of inoculated seedlings	No. of seedlings with necroses (%) ^a	No. of seedlings with aecia (%) ^a
<i>M. larici-tremulae</i>	Vallombrosa	<i>P. pinea</i>	45	100 ^{b, c}	0
		<i>P. halepensis</i>	40	69.7 ^{b, c}	0
		<i>P. pinaster</i>	30	13.2	0
<i>M. pinitorqua</i>	Monticiano	<i>P. pinea</i>	30	0	70.3 ^{b, c}
		<i>P. halepensis</i>	35	0	69.8 ^{b, c}

^a Percentage over total No. of seedlings inoculated.

^b Shoot twisting.

^c Aecia on primary needles.

Table 2. Cortical necroses and aecia on shoots of *Pinus sylvestris* and *P. nigra* subsp. *laricio* seedlings inoculated with *Melampsora larici-tremulae* from Baiardo, *M. larici-tremulae* from Luson and *M. pinitorqua* from Monticiano.

Rust fungus	Rust provenance	Pine species ^a	No. of inoculated seedlings	No. of seedlings with necroses (%) ^b	No. of seedlings with aecia (%) ^b
<i>M. larici-tremulae</i>	Baiardo	<i>P. sylvestris</i> (4)	206	20.8	0
		<i>P. sylvestris</i> (28)	237	23.2	0
		<i>P. nigra</i> subsp. <i>laricio</i>	134	25.4	0
<i>M. larici-tremulae</i>	Luson	<i>P. sylvestris</i> (4)	203	4.7	0
		<i>P. sylvestris</i> (28)	183	3.8	0
		<i>P. nigra</i> subsp. <i>laricio</i>	138	4.3	0
<i>M. pinitorqua</i>	Monticiano	<i>P. sylvestris</i> (4)	91	0	93.4
		<i>P. sylvestris</i> (28)	96	0	98.9
		<i>P. nigra</i> subsp. <i>laricio</i>	48	8.3	87.1

^a The No. in parentheses indicates the source, as L.N.B.S. wood No., of the pine species (see Materials and methods for details).

^b Percentage over total No. of seedlings inoculated.

son. By contrast, when the inoculations of seedlings with each provenance of *M. larici-tremulae* are considered, the percentage of seedlings with cortical necroses did not show appreciable differences, either between provenances of *P. sylvestris*, or between single provenances of *P. sylvestris* and *P. nigra* subsp. *laricio*. The same species and sources of *Pinus* inoculated as controls with *M. pini-torqua* from Monticiano (Table 2) had an extremely high percentage of seedlings with aecia on the shoots. Notably, there were cortical necroses on the shoots of some seedlings of *P. nigra* subsp. *laricio* inoculated with *M. pini-torqua*. These seedlings had only cortical necroses: no seedlings developed both cortical necroses and aecia on the shoots.

Discussion

Cortical necroses on the shoots of *P. sylvestris* inoculated with *M. larici-tremulae* have been studied before, though mainly at their initial stages of development (Longo *et al.*, 1997). Characteristic aspects of those necroses, such as the behaviour of the mycelium in the necrotic parenchyma and the formation of necrophylactic periderm around the necrotic tissue, were similar to the aspects observed in all the species of *Pinus* inoculated in this work. Such characteristics correspond to host post-haustorial reactions, not to nonhost pre-haustorial reactions (Heath, 1981, 1985).

For these reasons, and because a cortical necrosis is a reaction to infection, when such a necrosis occurs in a nonhost, it indicates that an infection has taken place, even though there may have been a subsequent resistance reaction. Hence a high percentage of seedlings with necroses in a nonhost species indicates that many seedlings were infected, i.e., that their resistance was low.

Experiment A explored the resistance of *P. pinea*, *P. pinaster* and *P. halepensis* to *M. larici-tremulae*. These pine species are nonhosts to this rust in natural conditions, even if their primary needles are susceptible to it in artificial conditions. Experiment A revealed that *P. pinea* was less resistant than *P. sylvestris* and *P. nigra* subsp. *laricio*. This is evident if one compares the percentage of seedlings with necroses exhibited by *P. pinea* in Experiment A with that exhibited by *P. sylvestris* and *P. nigra* subsp. *laricio* in Experiment B, and with what was previously observed on *P. sylvestris*

by Longo *et al.* (1997) who reported necroses in only a small percentage of inoculated seedlings. The degree of resistance of *P. pinaster* appeared to be intermediate between *P. pinea* on the one hand and *P. sylvestris* and *P. nigra* subsp. *laricio* on the other in previous experiments on seedlings in their first growth cycle (Longo *et al.*, 1975; Naldini Longo *et al.*, 1985). However, the resistance of *P. pinaster* would be similar to that of *P. sylvestris* and *P. nigra* subsp. *laricio* if percent values from the trials on one-year-old seedlings in Experiments A and B were considered, bearing in mind the absence of shoot twisting in *P. pinaster*.

There was greater resistance to *M. larici-tremulae* in the shoots of *P. sylvestris* and *P. nigra* subsp. *laricio* than in the shoots of other pine species that are hosts of *M. pini-torqua*. This result is in agreement with previous susceptibility trials with *M. larici-tremulae* based on the criterion of aecia production on the primary needles (Longo *et al.*, 1975; Naldini Longo *et al.*, 1985). This greater resistance is why *P. sylvestris* was identified as a 'nonhost' of this fungus.

It must be remembered that in both Experiments the seedlings were inoculated in their second growth cycle, at which time *P. pinea* shoots had only primary needles, *P. pinaster* shoots both primary and secondary needles, while *P. sylvestris* and *P. nigra* subsp. *laricio* shoots already had only secondary needles. Consequently, any differences in resistance based on necroses on the shoots, together with the presence of aecia on the primary needles (see Table 1), could be attributed to the particular type of plant growth and the type of needles (primary or secondary) related to it. However, previous studies (Longo *et al.*, 1975; Naldini Longo *et al.*, 1985) using the seedlings of the same pine species in their first growth cycle (each with only primary needles at the time of inoculation) showed the same differences in resistance to *M. larici-tremulae* as did the seedlings in the second growth cycle. This suggests that such differences are attributable not to phenological but to genetic factors linked to the host-parasite interaction.

Lastly, *P. halepensis* must be considered separately since its resistance to *M. larici-tremulae* was tested for the first time in Experiment A by inoculating seedlings in their second growth cycle, which, like *P. pinea*, had only primary needles. In this tri-

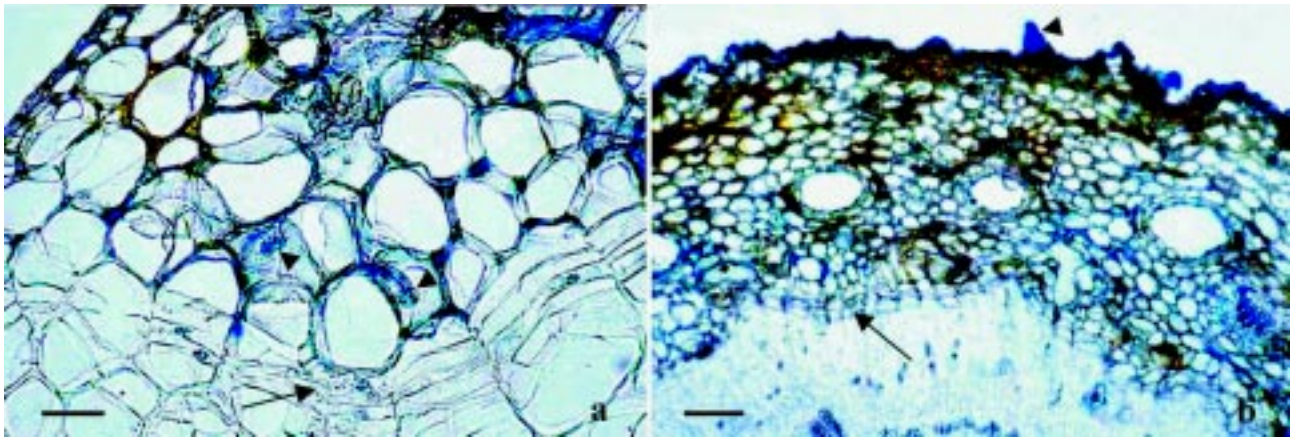


Fig. 2. a, b. Transverse sections of *Pinus* shoots, cryo-cut and stained with cotton blue, with necroses on a, *P. nigra* subsp. *laricio* and b, *P. pinea*, after inoculation with *Melampsora larici-tremulae* from a, Baiardo and b, Vallombrosa; a, small necrosis in the cortical parenchyma and rust haustoria with encasement in the necrotic parenchyma cells (arrow-heads); incomplete necrophylactic periderm bordering the necrotic tissue (arrow); b, necrosis extending tangentially and radially for some distance in the cortical parenchyma colonised by rust mycelium, aborted subcuticular spermogonia (arrow-head) and continuous necrophylactic periderm delimiting the necrotic tissue (arrow). Scale bars: a, 50 µm; b, 100 µm.

al, *P. halepensis* and *P. pinea* had similar levels of resistance to *M. larici-tremulae*.

Experiment B, which compared the resistance of *P. nigra* subsp. *laricio* and *P. sylvestris* from two provenances, to *M. larici-tremulae* from the same two provenances found that *P. sylvestris* of both provenances and *P. nigra* subsp. *laricio* were less resistant to *M. larici-tremulae* from Baiardo (Maritime Alps) than to that same rust from Luson (central Alps). There was no difference in resistance between the two provenances of *P. sylvestris* to each provenance of *M. larici-tremulae*, nor between the two provenances of *P. sylvestris* and *P. nigra* subsp. *laricio*.

The assumption that the cortical necroses on pine species are one of the signs of reaction to infections in both nonhost resistance to *M. larici-tremulae* and host resistance to *M. pinitorqua* was confirmed by Experiment B, in which *M. pinitorqua* caused cortical necroses on 8.3% of *P. nigra* subsp. *laricio* seedlings (Table 2). Indeed, this pine host of *M. pinitorqua* was already known to be less susceptible to *M. pinitorqua* than other hosts of the same rust. This lower susceptibility was initially attributed to phenologic characteristics (Longo *et al.*, 1970, 1980). Since the seedlings of all pine species in Experiment B were inoculated at the same

stage of development, hence with similar phenological characteristics, this lower susceptibility can now be attributed to the true host resistance of *P. nigra* subsp. *laricio*.

If it is borne in mind that the susceptibility/resistance ratio in a pathosystem depends in part on how long a host and a parasite interact in an area characterised by given environmental conditions (Millar and Kinloch, 1991), the following remarks can be made. Firstly, the fact that *P. pinea* has a lower resistance to *M. larici-tremulae* than *P. sylvestris* (both these pine species are hosts of *M. pinitorqua*) (Experiments A, B) can be attributed to the habitat of *P. pinea*, which is ecogeographically further away from that of *M. larici-tremulae* than is the habitat of *P. sylvestris*. Thus *P. pinea* has not been extensively exposed to this pathogen and has not built up resistance to it. Secondly, the lower resistance of *P. sylvestris* to *M. larici-tremulae* from Baiardo (Maritime Alps) than to that from Luson (central Alps) (Experiment B) can be explained by the fact that Baiardo (given its proximity to the sea) presents environmental conditions that are less suitable for the interaction of both *P. sylvestris* and *Larix decidua* with *M. larici-tremulae*. This is all the more likely since *L. decidua* (the natural host of *M. la-*

rici-tremulae) is a microthermic species that prefers altitudes having a continental climate with cold and snowy winters. By contrast, Luson is characterised by environmental conditions suitable both for *P. sylvestris* and *L. decidua* (native to this area) and for *M. larici-tremulae*. Thus *P. sylvestris* in Luson had been extensively exposed to *M. larici-tremulae*, and had built up a greater resistance to it.

In both Experiments, the degree of resistance shown towards *M. larici-tremulae* could be the result of a host-parasite interaction that may or may not trigger a specific genetic arrangement upon which the expression of resistance depends.

In conclusion, the host-parasite interactions between some species of *Pinus* (natural hosts of *M. pinitorqua*) and *M. larici-tremulae*, when compared with the interactions between these same species and *M. pinitorqua*, can shed light on the taxonomic relationship between the two rust fungi. They support the hypothesis stated by Longo *et al.* (1997) that, while the two rust entities are closely related, they follow a diverging route of evolution that may eventually lead to their complete separation as distinct species.

At this point in the research, the molecular characteristics of *M. pinitorqua* and *M. larici-tremulae* at present under study, when compared with morphological and biological data, are important for a clearer differentiation between these two rust fungi.

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