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ORIGINAL ARTICLE

# Molecular Tools Applied to the Advancement of Fruit Growing in South Tyrol: a Review

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**Abstract** Molecular biology techniques have become indispensable tools in various areas of research and routine applications. In South Tyrol (northern Italy), molecular methods have been applied to the field of fruit growing over the last decade, and the main applications have been related to plant pathology and identification of apple cultivars. This review article illustrates how the implementation of existing methods and the development of new assays have contributed to gain more insights about the spread and epidemiology of phytoplasma diseases affecting apple trees and grapevine in South Tyrol, to identify and characterise the causal agents of newly emerging disorders, or to describe the local genetic diversity of the apple.

**Keywords** Apple proliferation · Bois noir · *Cacopsylla melanoneura* · *Cacopsylla picta* · '*Candidatus* Phytoplasma mali' · '*Candidatus* Phytoplasma prunorum' · Cultivar identification · *Erwinia amylovora* · European stone fruit yellows · Fire blight · Flavescence dorée · Grapevine yellows · *Hyalesthes obsoletus* · Latent infection · *Malus domestica* · Microsatellite DNA · Molecular diagnostics · Molecular typing · Pathogen quantification · Real-time PCR · Root grafts · *Scaphoideus titanus* · *Tilletiopsis* spp. · Vector · *Vitis vinifera* · White haze

**Anwendung molekularbiologischer Methoden im Obstbau Südtirols – ein Überblick**

**Zusammenfassung** Molekularbiologische Methoden haben sich zu einem unverzichtbaren Hilfsmittel in verschiedenen Bereichen der Forschung und in Routineanwendungen entwickelt. In Südtirol (Norditalien) werden molekularbiologische Methoden seit einem Jahrzehnt im Bereich des Obstbaus angewandt, mit Schwerpunkt auf Pflanzenkrankheiten und die Identifizierung von Apfelsorten. Dieser Review zeigt den Beitrag der Implementierung bestehender Verfahren und der Entwicklung neuer Methoden zur Gewinnung profunder Erkenntnisse über die Ausbreitung und Epidemiologie von Phytoplasmen-Krankheiten des Apfels und der Rebe in Südtirol, zur Identifizierung und Charakterisierung neuer Schaderreger sowie zur Beschreibung der lokalen genetischen Vielfalt des Apfels.

**Schlüsselwörter** Apfeltriebsucht · Schwarzholzkrankheit · Weißdornblattsauger · Sommerapfelblattsauger · '*Candidatus* Phytoplasma mali' · '*Candidatus* Phytoplasma prunorum' · Sortenbestimmung · *Erwinia amylovora* · Europäische Steinobstvergilbung · Feuerbrand · Goldgelbe Vergilbung · Vergilbungskrankheiten der Rebe · Winden-Glasflügelzikade · latente Infektion · Apfel · Mikrosatelliten DNA · Molekulare Diagnostik · Molekulare Typisierung · Erreger-Quantifizierung · Real-time PCR · Wurzelverwachsung · Amerikanische Rebzikade · *Tilletiopsis* spp. · Vektor · Rebe · Weißer Hauch

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**Abbreviations**

| | |
|---|---|
| AP | Apple proliferation |
| ESFY | European stone fruit yellows |
| FD | Flavescence dorée |
| BN | Bois noir |
| PCR | Polymerase chain reaction |
| MAS | Marker assisted selection |
| SE | Standard error |
| RFLP | Restriction fragment length polymorphism |
| SSRs | Simple sequence repeats |

## Introduction

Molecular biology techniques have become indispensable tools in various areas of research and routine applications. The wide implementation of these techniques in different types of medical, biological, and also agricultural laboratories since the 1990s was largely accelerated by the invention of the Polymerase Chain Reaction (PCR), which simplified the manipulation and detection of defined nucleic acid fragments (Rabinow 1997). The potential applications of molecular methods in the area of agriculture are endless and include detection and characterisation of plant pathogens, genotyping for identification and variety protection, assessment of genetic diversity, genome mapping and marker assisted breeding or biotechnological crop improvement.

South Tyrol in northern Italy harbours the largest consistent apple producing area in Europe comprising 18,538 ha, while grapevine, which is grown on 5,319 ha, represents the second important perennial crop culture in this region (APBZ 2012). Since 1976, the agricultural industry of South Tyrol has been supported by active public research and experimental activities performed at the Research Centre for Agriculture and Forestry Laimburg (reviewed in Dalla Via and Mantinger 2012). In late 1999, the idea for the establishment of a Molecular Biology Laboratory was born, which was intended to act as a platform for collaborative research requiring the application of molecular tools in order to address a wide range of issues within agriculture of South Tyrol (Dalla Via and Mantinger 2012).

After being equipped in 2001, the Molecular Biology Laboratory started its research activity in early 2002 (Mantinger and Dalla Via 2002) and has since been involved in numerous projects, some of which even address biodiversity conservation of freshwater organisms (e.g. Baric et al. 2005a, b, 2010d; Meraner et al. 2010). The research activities in the field of fruit growing have thus far concentrated on two major tasks: the molecular diagnosis of plant pathogens and the application of molecular markers for apple cultivar identification, both of which shall be presented in greater detail in the following review.

## Molecular Diagnostics of Plant Pathogens

PCR-based methods have quickly been recognised as powerful and versatile tools for diagnosis of plant diseases, in particular when large sensitivity and specificity are required (e.g. detection of pathogens that occur at low frequencies or are difficult to culture or identify) as is the case for certification and quarantine applications (Henson and French 1993; Ward et al. 2004). Since PCR is based on the amplification of defined nucleic acid regions, only small amounts of starting material are required for the assay and the detection time can be considerably reduced compared to traditional culture approaches (Ward et al. 2004). Moreover, the direct analysis of DNA allows a number of questions to be addressed regarding pathogen populations, genetic variability, ecology, epidemiology and interaction with host organisms as well as to track and control the spread of pathogens (Ward et al. 2004).

Molecular genetic methods have, for instance, developed into a key instrument in diagnostics as well as in taxonomic and epidemiological studies of phytoplasmas (Mollicutes) (Lee et al. 2000). Phytoplasmas are obligate intracellular parasites that colonise the phloem tissue of several hundreds of plant species and despite extensive attempts, to date it has not been possible to cultivate any of these bacteria *in vitro* and thus to apply classical microbiological procedures for their investigation (Lee et al. 2000). Due to the emergence or outbreaks of phytoplasma diseases affecting fruit trees and grapevine in South Tyrol in the last decade, this group of plant pathogens has become a research focus of the Molecular Biology Laboratory.

### Apple Proliferation (‘*Candidatus* Phytoplasma mali’)

The spread of apple proliferation (AP) in South Tyrol, caused by ‘*Candidatus* Phytoplasma mali’, was one of the major challenges for the apple growing industry in the 2000s. Although the disease sporadically occurred in orchards on seedling rootstocks since the 1960s, it was in 1998 that AP was noticed for the first time in an intensive apple orchard in the surroundings of Bozen/Bolzano. In the following years the disease spread throughout the territory of South Tyrol, reaching a peak in 2006 with more than half a million diseased and uprooted trees (reviewed in Baric et al. 2010a). Proliferation of auxiliary shoots (witches’ brooms) and enlarged stipules are regarded as specific disease symptoms, while unspecific symptoms include stunting, chlorosis, yellowing or early leaf reddening (Kartte and Seemüller 1988). Other unspecific symptoms, such as inferior fruit size and quality as well as overall yield reduction, can all lead to serious economic losses.

*Detection of 'Ca. P. mali'*

Considering the sudden spread of AP, one of the first duties of the Molecular Biology Laboratory was the implementation of a PCR-based detection protocol to support experimental work on this task in South Tyrol and provide a sensitive testing tool for the control of propagation material. However, it quickly became evident that up until then commonly applied a number of PCR primers, thought to specifically target '*Ca*. P. mali', have a tendency to bind to the DNA of environmental bacteria, producing non-specific amplicons and false-positive test results (Baric and Dalla Via 2005). In addition, certain primer combinations used in conventional PCR were found to be susceptible to inhibition and thus false-negative results could occur in samples evidently infected by the pathogen (Baric et al. 2006b). For this reason, a new approach for detection of '*Ca.* P. mali' relying on real-time PCR was developed. More specifically, a duplex TaqMan real-time PCR assay was established, which is based on the simultaneous amplification of gene fragments coding for the pathogen 16S rRNA and the host plant chloroplast tRNA leucine (Baric and Dalla Via 2004). This was the first diagnostic method for a phytoplasma that employed an internal analytical control and allowed distinction between uninfected plant material and false-negative results.

Compared to conventional PCR methods, the new real-time PCR procedure combined highest test sensitivity with highest test specificity and due to the minimal number of handling steps displayed a high potential for automation and the application in large-scale testing procedures (Baric and Dalla Via 2008). The major disadvantages of the real-time PCR assay were the comparably higher expenses of instrumentation, consumables and reagents, which however could be compensated for by reduced costs of labour and hazardous waste disposal (Baric et al. 2006b).

The availability of a rapid and powerful testing procedure finally made it possible to address questions that require the analysis of larger sample numbers such as the infection rates of natural vector populations with '*Ca*. P. mali' (Wolf et al. 2003; Baric et al. 2005c, 2010c), alternative transmission routes (Baric et al. 2008a), the incidence of latent infections in apple orchards (Baric et al. 2003, 2006a, 2007; Unterthurner and Baric 2011) or the testing of propagation material.

*Transmission of 'Ca. P. mali' by Insect Vectors*

Apple proliferation was proven to be transmitted by two psyllid species, *Cacopsylla picta* and *C. melanoneura* (Frisinghelli et al. 2000; Tedeschi and Alma 2004), both of which at present occur in South Tyrol (Walch 2006). An adapted real-time PCR protocol was applied in order to assess the infection rates of natural psyllid populations and to evaluate the role of the different species in the transmission of AP in this region (Wolf et al. 2003; Baric et al. 2010c). A total of 801 specimens comprising the species *C. picta*, *C. melanoneura*, *C. mali* and *Trioza urticae*, which were collected in 2006 by beating trays from 18 orchards covering the major part of the apple growing area of South Tyrol, were each individually tested. The analyses evidenced mean infection rates with '*Ca*. P. mali' of 0, 0.6, 0.9 and 11.1 % for *T. urticae*, *C. melanoneura*, *C. mali* and *C. picta*, respectively (Baric et al. 2010c).

*Cacopsylla mali* is a common species of abandoned and untreated apple orchards, which has the ability to acquire '*Ca*. P. mali', but despite extensive transmission experiments was not demonstrated as acting as a vector (Seemüller et al. 2004). *Cacopsylla melanoneura* is considered the main vector of '*Ca*. P. mali' in north-western Italy (Tedeschi and Alma 2004), while in other European regions, including South Tyrol, transmission trials involving this species did not succeed in infecting healthy test plants (Mayer et al. 2009; Wolf et al. 2003) or the transmission rate was very low (0.36 %) (Mattedi et al. 2008). In contrast, *C. picta* was proven to be a highly efficient vector of '*Ca*. P. mali' (Frisinghelli et al. 2000; Mattedi et al. 2008; Seemüller et al. 2004) and due to its frequent occurrence in the commercial orchards of South Tyrol (Walch 2006) and the high infection rates with '*Ca*. P. mali' amounting locally up to 30 % (Baric et al. 2010c), this species was considered to represent the main risk for the spread of AP in South Tyrol on which control strategies should focus.

*Testing of Propagation Material*

While sap-sucking psyllids are considered crucial for propagation of AP within orchards (Mayer et al. 2009), the pathogen can also be transmitted anthropogenically by grafting infected propagation material (Kartte and Seemüller 1988). For this reason, the Molecular Biology Laboratory was involved in testing samples taken from propagation and/or nursery material by the Phytosanitary Service of the Autonomous Province of Bozen/Bolzano. Between 2004 and 2010 more than 500 samples (root material) were tested and in none of the cases was a positive sample found, indicating that propagation material has so far played a negligible role for the dissemination of AP in South Tyrol (Baric et al. 2006a).

*Transmission of 'Ca. P. mali' through Root Grafts*

Another transmission route of AP, the relevance of which is however not yet fully understood, are root anastomoses or root bridges that were shown to form between apple trees under both experimental and natural conditions (Ciccotti

et al. 2008). A case study in South Tyrol addressed this question in a 24-year old commercial apple orchard with vigorous rootstock, where the presence of root grafts was discovered after treatment of the stubs of AP-diseased trees with high dosages of glyphosate and the herbicide damage spread to a large number of neighbouring trees (Baric et al. 2008a). Glyphosate is translocated through the phloem and could only have propagated from the treated stubs to neighbouring trees through naturally formed root bridges. Real-time PCR analyses performed in order to detect '*Ca.* P. mali' in phloem tissue of root samples (Baric and Dalla Via 2004) offered strong evidence of the spread of the pathogen across natural root grafts and emphasised that this transmission route could be relevant in older orchards with vigorous rootstocks (Baric et al. 2008a). Nevertheless, further research is needed in this area to better understand the mechanism of formation of root anastomoses in regard of different rootstocks, planting densities and age of trees (Guerriero et al. 2012).

*Latent Apple Proliferation Infections*

Apple trees infected with '*Ca.* P. mali' were shown to persistently carry the pathogen in their rootstocks although symptom expression varied extensively, and occasionally symptoms remitted or never became evident (Carraro et al. 2004; Seemüller et al. 1984). Because of this, assessments based merely on the observation of symptoms do not necessarily infer the true infection rates of orchards but may rather provide underestimated values. With the aim to reveal the extent of latent AP infections, two case studies were performed in South Tyrol by collecting and testing root samples using the previously described real-time PCR procedure (Baric and Dalla Via 2004).

The first case study involved a 7-year-old commercial apple orchard that displayed a cumulative AP infection rate of 5 % since its establishment based on the assessment of visual symptoms (Baric et al. 2003). A total of 345 trees from eight consecutive rows, representing approximately a third of the entire orchard, were sampled in 2003 and the testing revealed an actual infection rate of 3.8 % compared to 1.5 %, which was discovered by visual inspection alone. Accordingly, the latent infection rate was determined to amount to 2.3 % and the true infection rate was found to be more than twice as high as assessed by the monitoring of symptoms (Baric et al. 2007).

The second case study involved a young orchard which was monitored in collaboration with the South Tyrolean Extension Service for Fruit Growing over a period of 6 years (Unterthurner and Baric 2011). This particular orchard was selected because the young trees were infested by *C. picta* immediately after planting (2005), and therefore in the autumn of the same year, ten percent of the apple trees (N = 105) were randomly selected and sampled for testing. The application of the real-time PCR assay revealed an infection rate of 10.5 % although no symptoms of AP were evident at that time, but first occurred 1.5–2 years after the assumed infection (Baric et al. 2007).

The trees initially tested negative for AP were re-sampled and re-tested for 5 consecutive years in order to monitor the efficiency of the recommended disease control measures of uprooting and vector control, which were strictly followed by the owner of the orchard in the following years. The laboratory testing confirmed the efficacy of the measures as in the second year after planting, the new infections were reduced to 1.1 % (which corresponds to a single tree) and in the subsequent third, fourth, fifth and sixth year, were reduced to zero (Unterthurner and Baric 2011). An interesting observation was made for the aforementioned tree infected in the second year, which first displayed specific symptoms of AP 4 years after infection. In conclusion, the study demonstrated that young field-infected apple trees remained latently infected by AP for at least 1.5 and up to 4 years after infection (Unterthurner and Baric 2011).

*Quantitative Detection of 'Ca. P. mali'*

The colonisation of apple trees by '*Ca*. P. mali' is erratic and follows a seasonal pattern (Seemüller et al. 1984). The pathogen persists in the rootstock throughout the year, from where it can colonise the canopy, and from which it is in turn eliminated during winter time due to the complete degeneration of the phloem tissue in the above-ground parts of the tree (Schaper and Seemüller 1982). It was reported that the presence of '*Ca*. P. mali' in the canopy is associated with the expression of symptoms, while in latent and recovered trees the pathogen remains restricted to the rootstock (Carraro et al. 2004).

With the purpose to measure the phytoplasma concentration in roots and branches of infected apple trees and to correlate the quantities to symptom expression, the previously developed diagnostic real-time PCR assay was refined (Baric and Dalla Via 2004). By contemporaneous amplification of a single-copy gene of the host plant and the 16S rRNA gene fragment of the pathogen in the same tube, and the application of external standard curves, it was possible to obtain normalised estimates of phytoplasma concentration that were expressed as the number of phytoplasma cells per host plant cell (Baric et al. 2011b). The analysis of samples from symptomatic and asymptomatic trees collected at 18 time points over two growing seasons in an orchard in South Tyrol evidenced higher phytoplasma quantities in shoots of symptomatic trees (9.4 ± 1.04 SE) compared to asymptomatic trees (0.7 ± 0.13 SE), while no significant difference between the two groups was found in the pathogen load of the roots (59.8 ± 5.68 SE and 55.1 ± 9.83 SE, respectively).

Moreover, the study for the first time revealed pronounced seasonality of phytoplasma titer in the roots of both symptomatic and asymptomatic apple trees, with highest levels detected between December and May (Baric et al. 2011b).

It needs to be emphasised, that the application of the standard curve-based quantitative real-time PCR procedure to a large number of samples with replicates can be rather impractical because the analysis of serially diluted standards considerably decreases the number of free sample wells on a microtiter plate, and leads to increased analysis time and reagent costs (Baric 2012). A recent study thus proposed a simpler quantification procedure for '*Ca*. P. mali', which does not require the analysis of external standard curves but relies on the comparative threshold cycle method (Baric 2012). This simple and robust quantitative real-time PCR procedure could become an interesting means for studies that require the analysis of high sample numbers.

*Molecular Typing of 'Ca. P. mali'*

Molecular genetic methods have been widely applied to assess the genetic diversity of '*Ca*. P. mali' over large parts of its distribution range and to reveal the pathological importance of distinct strains (Cainelli et al. 2004; Casati et al. 2010; Danet et al. 2011; Jarausch et al. 2000; Martini et al. 2008; Schneider and Seemüller 2009; Seemüller and Schneider 2007; Seemüller et al. 2010). In order to analyse the epidemiology and spread of AP in South Tyrol, a method for simultaneous typing of '*Ca.* P. mali' at two genetic loci on an automated sequencing system was established (Baric et al. 2011a). The two loci comprised a putative rhodanese-like protein gene that displays two point mutations which allow differentiation of three different subtypes (Jarausch et al. 2000) and the ribosomal protein L22 gene which carries a variable copy number of a 12-nucleotide repeat sequence, and based on the number of copies, four different subtypes could previously be distinguished (Martini et al. 2008).

The combined molecular typing method was applied to analyse 310 DNA isolates from apple trees sampled in South Tyrol between 2002 and 2010, and tested positive for '*Ca.* P. mali'. Additionally, 15 '*Ca.* P. mali'-positive samples of *C. melanoneura* and 19 of *C. picta* were typed (Baric et al. 2011a). The distribution of subtype frequencies in apple trees over time indicated that AP may have spread in two separate waves in South Tyrol and that the explosive outbreak of the disease in 2006 was likely related to the spread of the efficient vector species *C. picta* (Baric et al. 2010a), which occurred for the first time in South Tyrol in 2004 (Wolf and Zelger 2006). The data furthermore suggest that there may be a co-adaptation of specific '*Ca.* P. mali' subtypes with distinct insect vector species, a hypothesis that needs to be tested in future experiments (Baric et al. 2011a).

European Stone Fruit Yellows ('*Candidatus* Phytoplasma prunorum')

Another significant phytoplasma disease of fruit trees, European Stone Fruit Yellows (ESFY), is caused by '*Candidatus* Phytoplasma prunorum' that affects plants of the genus *Prunus* and is vectored by *Cacopsylla pruni*. The disease is widespread in central and southern Europe and has developed into a serious impediment for the production of apricots (reviewed in Marcone et al. 2010). The disease is also common in South Tyrol and its control is regulated by the Resolution of the Provincial Government No. 3160 of 24 Sept. 2007, which directs reporting obligation and mandatory uprooting of ESFY-diseased trees (APBZ 2012). With the aim of supporting the disease control programme in South Tyrol, PCR-based methods for the detection of '*Ca*. P. prunorum' have been implemented in the Molecular Biology Laboratory and applied for testing of propagation material and/or orchard trees with ambiguous symptom expression.

Grapevine Yellows (Bois noir and Flavescence dorée)

Two grapevine yellows diseases, Flavescence dorée (FD) and Bois noir (BN) (or Vergilbungskrankheit), that are caused by phytoplasma species of the 16SrV or Elm yellows group and the 16SrXII-A or Stolbur group, respectively, are currently common in Europe (Lee et al. 2000). The symptoms of the two diseases (chlorosis and downward rolling of leaves, stunted shoots, lack of cane lignification, shrivelling of berries) are very similar and do not allow to distinguish the two pathogens. However, due to the transmission by different vector species, considerable differences in the epidemiology of the two diseases exist, requiring distinct phytosanitary measures. While FD is transmitted from grapevine to grapevine by the monophagous planthopper *Scaphoideus titanus* (Caudwell 1990), the polyphagous planthopper *Hyalesthes obsoletus* accidentally transmits BN to grapevine, which represents a dead-end host for this phytoplasma species (Maixner 1994).

Both pathogens, BN and FD, are present in Italy and in some regions even co-occur (Belli et al. 2010). In the 1990s, some of the most important winegrowing areas of northern Italy were affected by considerable economic damage due to an extensive outbreak of FD, resulting in the implementation of the Ministerial Decree No. 32442 of 31 May 2000 which stipulates mandatory control of FD on the entire territory of Italy by uprooting infected vines, planting disease free material and insecticide spraying against *S. titanus* (Decreto Ministeriale 32442/2000 2000).

*Detection and Differentiation of Two Grapevine Yellows Phytoplasmas*

Since reliable detection and discrimination of the two grapevine yellows phytoplasmas is only possible by the application of molecular methods, collaboration between the Phytosanitary Service of the Autonomous Province of Bozen/Bolzano and the Molecular Biology Laboratory was initiated, and a continuous monitoring programme for grapevine yellows has been followed since summer 2002 (Baric et al. 2012a). In the course of the monitoring programme, leaf samples from grapevines with yellows symptoms were collected and the nucleic acid isolates obtained were tested by a nested PCR-RFLP procedure (Pasquini et al. 2001; EPPO 2007) and/or a triplex real-time PCR (Pelletier et al. 2009) with the aim to detect and determine the phytoplasma species involved. Over a period of 10 years, more than 700 samples were tested and BN was identified as the prevalent grapevine yellows species in South Tyrol, while FD was detected in only two isolated cases (Baric et al. 2012a).

In order to assess the genetic diversity of the BN phytoplasma, Langer and Maixner (2004) analysed the elongation factor Tu gene (*tuf*) from different isolates from Germany by RFLP and revealed the existence of three BN-subtypes that could be specifically associated with different herbaceous host plants of *H. obsoletus*: *tuf*-type I with *Urtica dioica*, *tuf*-type II with *Convolvulus arvensis* (but also *Solanum nigrum* and *Prunus spinosa*) and *tuf*-type III (restricted to the Mosel valley) with *Calystegia sepium*. All three subtypes were present in both grapevine and *H. obsoletus*. The application of the typing method to BN-positive grapevine samples collected in South Tyrol over a period of 10 years demonstrated the occurrence of *tuf*-types I and II (Baric and Dalla Via 2007; Baric et al. 2012a). However, in contrast to Germany where *tuf* II was the prevalent BN phytoplasma subtype (Langer and Maixner 2004), in South Tyrol *tuf* I was predominant and was even the only subtype until the first appearance of *tuf*-type II in 2004 (Baric and Dalla Via 2007). These data indicate that two different epidemiological cycles of the BN phytoplasma, though to a varying degree, play a role in the propagation of the disease in South Tyrol.

The increasing incidence of BN in South Tyrol in the 2000s required extension of the monitoring programme to the vector *H. obsoletus* and its herbaceous host plants in order to define disease management strategies. A total of 659 H. *obsoletus* and 516 herbaceous plants of 41 potential host plant species from BN-affected vineyards in South Tyrol were tested by nested PCR for the presence of BN-phytoplasma over a period of up to 4 years (Berger et al. 2009b). The mean infection rate of *H. obsoletus* amounted to 24.1 %, while the two most important herbaceous host plants, *C. arvensis* and *U. dioica*, revealed infection rates of 25.1 % and 4.5 %, respectively (Berger et al. 2009b).

The application of a high-throughput real-time PCR allelic discrimination assay for the distinction of the two BN-phytoplasma subtypes *tuf* I and *tuf* II, that was developed in the Molecular Biology Laboratory (Berger et al. 2009a), demonstrated that both subtypes were indeed present in BN-positive *H. obsoletus* samples from South Tyrol, with similar frequencies (dominancy of *tuf*-type I) as previously reported for grapevines (Baric and Dalla Via 2007; Berger et al. 2009b). The typing of BN-positive herbaceous vineyard plants confirmed that they were infected with their associated BN-subtypes, although *tuf*-type II was found not only in *C. arvensis* but also in four other plant species (Berger et al. 2009b), confirming the lower degree of specificity of this subtype with regard to its host plant (Langer and Maixner 2004). The high percentage of BN-infected samples of *H. obsoletus* and its herbaceous host plants, as well as the correspondence of the frequency of BN-subtype distribution, emphasised the importance of *H. obsoletus* as the vector and *U. dioica* and *C. arvensis* as the inoculum sources for BN-phytoplasma in the vineyards of South Tyrol. Specific measures for elimination of the weed plants from vineyards were thus recommended so as to minimise the further risk of BN infection spread.

Fire Blight (*Erwinia amylovora*)

Fire blight is caused by the Gram-negative bacterium *Erwinia amylovora* (Enterobacteriaceae) which primarily affects plants of the subfamily Maloideae of the Rosaceae familiy (Vanneste 2000). The pathogen is considered an A2 quarantine pest in Europe as it can cause devastating damage to apple and pear production (EPPO 1997). Transmission occurs via insects, birds, wind, rain or pruning tools, and the plant is invaded through natural openings (including flowers and stomata) or wounding. The pathogen can colonise the tree rapidly and provoke symptoms such as withering and death of blossoms, shoots and limbs or even the death of the entire tree. If recognised in time, the infected parts of the plant can be removed by pruning using sterilised equipment, while eradication is performed in the case of advanced infection to prevent the spread of the disease to neighbouring trees or orchards (streptomycin use in agriculture is not permitted in Italy)(Waldner et al. 2012; Zelger 2008).

*Erwinia amylovora* is native to North America and was introduced to Western Europe in the 1950s and to southern Italy in 1990, and first emerged in South Tyrol in 1999 (Lindner 2004b). Since its first appearance, three severe disease outbreaks were reported in South Tyrol: in 2003, 2007 and 2011 with 189, 163 and 945 fire blight cases, respectively (Waldner et al. 2012). The diagnostic protocol

for *E. amylovora* in Italy is regulated by law and involves a series of microbiological tests and an immunofluorescence assay (Decreto Ministeriale 356/1999 1999), which were optimised by Lindner (2004a) such as to obtain reliable test results within 3–5 days. In addition to the official testing method applied in the Microbiology and Virology Laboratory, a PCR-based procedure for the detection of *Erwinia amylovora* as recommended by EPPO (2004) has been implemented in the Molecular Biology Laboratory and applied since 2005 to test propagation and nursery material for potential latent and/or inapparent infections.

### White Haze (*Tilletiopsis* spp.)

Since 1999, a cosmetic disorder on the surface of apple fruit, characterised by whitish to greyish extensive mycelial growth of smut fungi, has been observed in the orchards of South Tyrol (Lindner and Baric 2006). Pronounced occurrence of the symptoms can have a negative impact on the marketability of the produce resulting in economic losses to the farmers (Lindner 2009). A thorough examination of the causal agent of this disorder based on morphological and molecular genetic traits allowed its determination as the genus *Tilletiopsis* spp. (Basidiomycota, Ustilaginomycotina, Exobasidiomycetidae) (Lindner and Baric 2006). While a contemporaneous study in the Netherlands described white haze mainly as a new postharvest disorder of the apple cultivar 'Elstar' (Boekhout et al. 2006), in South Tyrol the syndrome was evident on apple fruit of various cultivars already prior to harvest (Lindner and Baric 2006; Baric et al. 2010b). Recently, white haze has been confirmed as a new late-season disorder in Germany (Weber and Zabel 2011).

In order to assess the genetic diversity of *Tilletiopsis* spp. inducing white haze in South Tyrol, the nuclear ribosomal internal transcribed spacer region DNA of 48 isolates derived from different orchards in northern Italy was sequenced. The study revealed six haplotypes that clustered into three distinct phylogenetic groups (Baric et al. 2010b), one of which (*T. washingtonensis*) was associated with white haze for the first time. The sudden appearance of this new cosmetic fruit disorder in the last decade could be attributed to moister weather conditions and modified cultivation practices, such as the increased installation of hail nets or more intensive use of foliar fertilisers (Rizzoli and Acler 2009; Baric et al. 2010b).

## Molecular Markers for Identification of Apple Cultivars

Apple (*Malus*×*domestica*) is one of the most important fruit trees grown in temperate zones all over the world (Dalla Via and Baric 2012), and although several thousands of different apple varieties have been described, commercial production relies on only a small fraction of this vast genetic diversity (Janick et al. 1996). In South Tyrol, for example, approximately 1.1 million metric tons of apples are produced annually, but 95 % of the total harvest derives from only eight globally-grown cultivars (Dalla Via and Mantinger 2012). Accordingly, driven by the increasing intensification of orchards, negligence of old trees and/or their replacement by new commercial cultivars, many locally-grown apple varieties have disappeared from this region or are at risk of extinction.

With the aim of identifying, describing and protecting the remaining local genetic diversity of the apple, two projects funded within the INTERREG IIIA programme between Italy and Austria were accomplished and an ERDF-funded project is currently carried out (Baric and Dalla Via 2009). The function of the Molecular Biology Laboratory within these projects has been to provide reliable molecular tools for the identification and characterisation of apple varieties sampled throughout the investigation area.

### Implementation of the Genotyping Procedure

Phenotypic traits (i.e. pomological characters of fruit) have long been the only means for description and identification of apple genotypes. The sole dependence on this approach, however, carries difficulties due to the potential impact of environmental factors on the expression of phenotypic characters and/or the long juvenile stage of apple trees. For this reason, markers relying on the direct analysis of DNA have been developed and applied for fingerprinting of fruit tree species since the 1990s (Wünsch and Hormaza 2002). Among the different molecular markers, microsatellites (or SSRs) have been the most widely applied for cultivar identification and characterization of germplasm resources, because of their high variability and reproducibility, their random occurrence in the genome, co-dominant inheritance and selective neutrality as well as the potential for analysis on automated systems (e.g. Garkava-Gustavsson et al. 2008; Guarino et al. 2006; Hokanson et al. 1998; Pereira-Lorenzo et al. 2007).

A collaborative study involving the Molecular Biology Laboratory and the Institute of Plant Sciences of the University of Graz addressed the potential for the exchange of genotyping data produced in different laboratories, since the integration of different datasets would be useful for direct comparisons of the genetic diversity of germplasm resources within and among regions or for identification of synonym or homonym cultivars (Baric et al. 2008b). Each group analysed the same set of 61 DNA samples at five microsatellite loci by applying their own laboratory protocols. Comparative analysis of the two datasets revealed that 99.3 % of the single-locus genotypes corresponded with at least one

allele, but also evidenced the occurrence of inconsistencies due to dropout of longer alleles, mis-scoring of stutter peaks or complete allele mismatch, that could impede the applicability of merged databases (Baric et al. 2008b). It was thus concluded, that an interchange of genotyping data needs to be preceded by an *a priori* standardisation of analysis protocols across different laboratories. In addition, the application of a conversion procedure for the presentation of allele sizes as absolute values, based on the exact allele sizes in standard cultivars, was proposed.

Genotypes of domesticated apple are highly heterozygous and the combined analysis of a set of variable microsatellite markers should contribute to elevated degrees of discrimination power. By the analysis of a set of eight microsatellite loci, Hokanson et al. (1998) calculated a probability of approximately 1 in 1 billion that two randomly selected genotypes matched by chance. In the Molecular Biology Laboratory a protocol for genotyping at 14 microsatellite loci was adopted and applied to the analysis of apple varieties collected from various European germplasm collections (Baric et al. 2009). Among other results, ten genetic profiles were revealed, each of which was shared by at least two accessions differing in their names (Baric et al. 2011c). In order to evaluate whether the number of markers was insufficient to distinguish potentially closely related cultivars, the number of microsatellite loci was extended to a total of 48. The analysis of a higher number of loci, however, did not result in an increased discrimination power, but confirmed the previous assumptions of synonym pairs, spur mutants or incorrect name assignments (Baric et al. 2011c).

### Establishment of a Reference Database and its Application

Accurate cultivar identification and verification of cultivar authenticity are major requirements not only for efficient management of germplasm resources but also for their exploitation in breeding programmes (Evans et al. 2011), as well as for the entire trade process from propagation and planting material to fruit retail. In order to provide a reliable instrument for cultivar identification, a database with molecular genetic profiles of reference cultivars based on genotyping at 14 microsatellite loci was established (Baric et al. 2009).

The database has been applied to the identification of local apple varieties collected in South Tyrol and northern Tyrol (Austria), and allowed identification of approximately 70 % of the trees sampled, resolving several cases of pomological mis-determination (manuscript in preparation). For one of the most common genotypes sampled in the Austrian part of the investigation area, for instance, the combined pomological and molecular approach revealed the spurious denomination of ‘Zigeunerapfel’ for the actual cultivar ‘Roter von Simonffi’ (Holler et al. 2012). The analysis of molecular data furthermore demonstrated a strong impact of intensive apple production on the remaining local genetic diversity of the apple, as the number of different genotypes in northern Tyrol, where extensive meadow orchards predominate, was twice as high as in South Tyrol, where intensive orchard production prevails (manuscript in preparation). In addition to pure cultivar identification, the database provides a much wider array of applications and even proved useful for parentage assessment of apple cultivars (Baric et al. 2012b; Storti et al. 2012).

## Conclusion

Molecular biology methods have been applied over the last decade in research, experimental work and routine practice in the field of fruit growing (and viticulture) in South Tyrol, and have contributed to a number of important findings. The major application so far has been in the area of plant pathology, which will continue to rely on sensitive and specific tools for pathogen diagnosis because of recurring disease outbreaks or the emergence of new plant diseases. It will certainly be necessary to expand the array of molecular methods to new areas such as plant virology or soil heath (Berger and Oberhuber 2011). Apart from the application of molecular markers as cultivar identification tools, there will be a reinforcement for their use in marker assisted selection (MAS) in breeding for quality and resistance of new apple cultivars (Berger and Oberhuber 2011), a research line that has been followed at the Research Centre Laimburg in collaboration with other institutions since the early 2000s (Costa et al. 2005; Dalla Via and Baric 2007; Kouassi et al. 2009).

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