Chapter 1
Introduction

Symbiotic association, a perfect example of division of labor between two organisms, the outcome of which may lead to endeavors like biological nitrogen fixation (BNF). Reduction of atmospheric N_2 to ammonia and its further assimilation into amino acids and other bio-molecules enables gaseous nitrogen to incorporate into life processes. As all organisms need Nitrogen to survive, nitrogen fixation is probably the second most important biochemical pathway after carbon fixation. In nature, there are two major ways of fixing nitrogen. Natural abiotic nitrogen fixation, which can be mediated by lightning or fires, oxidizes N2 to nitrate (NO3-). The NO3- produced in this way can be washed out from the atmosphere with precipitation and is thus deposited in terrestrial ecosystems. The other way of nitrogen fixation involves activity of certain soil bacteria that absorb atmospheric N2 gas and convert it into ammonium. This process is known as BNF. However, the ability to fix nitrogen is found only in one biological kingdom, the Prokaryota (Sprent and Sprent, 1990). Thus other organisms have exploited the ability of prokaryotes to fix nitrogen by establishing various types of interactions (Werner, 1992). Cyanobacteria and plant microbe symbiosis are considered to be among the major milestones in evolution of life on Earth, bringing together the two most essential biochemical pathways, carbon fixation and nitrogen fixation.

These occur two main types of symbiosis between nitrogen fixing bacteria and vascular plants. One between *Rhizobium* and leguminous plants, and other between *Frankia* and actinorhizal plants (Wall, 2000). A large number of woody dictyledonous plants making symbiotic association with actinomycetes, belonging to the genus *Frankia* are called actinorhizal plants (viz. *Alnus nepalensis*, *Eleagnus pyriformis*, *Myrica negi*, *Casuarina eqisetifolia*, *Coriaria nepalensis* and *Hippophae* sp. etc).

The rhizobia-legume symbiosis involves more than 1700 plant species of the family Fabaceae (Leguminosae) distributed in three sub-families: Mimosoideae, eaesalpionoideae and Papilionoideae (Wall, 2000) with bacterial partners belonging to the family Rhizobiaceae (*Rhizobium*, *Azorhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Mesorhizobium*) (Mousavi *et al*., 2014). Unlike *Rhizobium*, *Frankia* form symbiosis with 24 different genera of dictyledonous mostly woody plants belonging to 8 families. These plants called actinorhizal plants comprise 260 species associated with the filamentous actinomycetes *Frankia*. Actinorhizal plants rival legumes in the amount of nitrogen they fix in global basis (Schwintzer, 2012; Mirza *et al*., 2007), yet knowledge of their biology and uses is, for the most part, very recent. They have in common a predilection to grow in marginally fertile soils and often serve as pioneer species early in successional plant community development (Kennedy *et al*., 2010). Because they often thrive on marginal soils, actinorhizal plants have current and potential applications in reclaiming and conditioning soils, producing timber and pulp and acting as wind break ornamental and fuel wood plants. Besides, globally they have potential for integrating into schemes for addressing issues of reforestation (Benson and Silvester, 1993; Bose and Sen, 2006).

However in all cases of *Rhizobia*-legume or *Frankia*-actinorhizal symbiosis a new plant organ, the nodule, is developed in which the bacteria proliferate, express the most vital enzyme, the nitrogenase and fix nitrogen into ammonia. These compounds are then assimilated and transported to the rest of the plant parts (Hirsch, 1992; Pawlowski and Bisseling 1996; Franche *et al,*. 1998). Several genes of both host and bacteria are involved in the process of

nodulation and nitrogen fixation. Actinorhizal nodules are characterized by a central vascular bundle and peripheral infected tissue surrounded by cortical nodule parenchyma. Actinorhizal nodules are ontologically related to roots. The nodule also provides an environment with a low O2 content, inside the host system. This environmrnt in the root nodules are essential, as the enzyme nitrogenase has to get protection from free oxygen that produce into the nodules. Nitrogenase, the enzyme that catalyzes nitrogen fixation, is oxygen labile. Some oxygen however must be provided so that the bacteria can respire and produce energy required both for survival and to drive N2 fixation. A special O2-transporting protein, called haemoglobin (Hb) thus eventually plays a crucial role in shielding the nitrogenase from oxygen and supplies controlled amount of O2 carefully.

Hb is a ubiquitous protein, present in all life forms, which functions to provide reversible binding of gaseous ligand such as oxygen and nitric oxide (Dordas *et al*., 2003). Usually, Hb reminds us of mammalian and circulatory system of blood but significance of Hb are to exist ubiquitously almost in all life forms and has been reported to be critically involved in oxygen transport and circulation mechanisms. Plant haemoglobins (pHbs) are different from mammalian globins and were first identified in the leguminous plant (*Glycine max*) which is nodulated by rhizobia (Kubo, 1939). The presence of Hbs in the plant nodules accounted for its red pigmentation and these proteins were termed as leghaemoglobin (LHb) (Dordas *et al*., 2003). PHb proteins were initially thought to be restricted to plant species carrying out symbiotic nitrogen fixation, but were later found in non-nodulated plants (Bogusz *et al*., 1988) as well.

There are three distinct types of pHbs: symbiotic haemoglobins (sHbs), nonsymbiotic haemoglobins (nsHbs) and truncated haemoglobins (ptHbs) (Dordas *et al*., 2003). Previously it was thought that sHb is mainly found in the root nodules of plants which go to symbiotic association with Rhizobia (Kubo, 1939; Guldner *et al*., 2004), but further research revealed the presence of Hbs in the actinorhizal root nodules also (Benson and Silvester, 1993).

Parasponia Hb have originated from sHbs, and are classically thought to have non-symbiotic roles (Appleby *et*

al., 1983). These Hbs are also called as LHb.

The sHbs are exclusively named when found in actinorhizal root nodules (Kubo, 1939; Appleby, 1984) and termed as LHb when present in Rhizobial symbiosis (Appleby, 1984; Perazzolli *et al*., 2004).

SHbs are required for symbiotic nitrogen fixation, but not for cellular functions of plants (Vinogradov *et al*., 2006). The ratio of oxygen and nitric oxide concentration in symbiotic nodules is maintained by the nsHbs. Initially, pHbs were assumed to exist only in symbiotic plant systems due to their abundance in symbiotic root nodules (Bogusz *et al*., 1990). However, investigation of various plant species like *Parasponia andersonii*, *Trema tomentosa*, *Celtis australis* and others showed many of these Hbs are nonsymbiotic in nature (Reeder and Hough, 2014).NsHbs are found dwelling mainly in leaf and stem. Nevertheless, it is present in roots of the seedlings, roots tissues exposed to flooding stress and oxygen-stressed aleurone tissues (Taylor *et al*., 1994). sHbs have a greater affinity to oxygen than sHbs (Hill, 2012). Nonsymbiotic haemoglobins are of two types: Class 1 and Class 2 haemoglobins. However, the definite functions of these two types of haemoglobins are not fully determined yet. The presences of different types of Hb in various plant tissues indicate it has other functions besides oxygen transportation (Hebelstrup *et al*., 2013).

A third type of pHb, i.e. ptHB, is found to be distributed in wide range of life forms, starting from bacteria to unicellular eukaryotic to higher plant (Sasakura *et al*., 2006; Vinogradov *et al*., 2006). PtHb have a 2-on-2 sandwich primary sequence (Igamberdiev *et al*., 2004) instead of the 3-on-3 classical Hb fold (Vinogradov *et al*., 2006; Bogusz *et al*., 1990; Ciaccio *et al*., 2015), and are also 20-40 amino acids shorter than the classical form of haemoglobins (Vinogradov *et al*., 2006; Jokipii-Lukkari *et al*., 2009). Conversion of hexa to penta coordination arrangement in ptHb, takes around 20 minutes which is extremely slow compared to other classes of Hbs (Jokipii-Lukkari *et al*., 2009; Ascenzi *et al., 2017*). The above evidence revealed that the ptHbs are structurally different from the two other classes of pHb (sHbs and nsHbs). Some plant species contain two ptHbs, but the functions of these Hbs still remain elusive (Jokipii *et al*., 2008).

Although ptHbs are longer than bacterial haemoglobins (Vinogradov *et al*., 2006; Jokipii *et al*., 2008), a phylogenetic tree based on sequence alignment placed ptHbs closer to the haemoglobins of gram-positive bacteria (Wittenberg *et al*., 2002; Hoy & Hargrove, 2008).

Some work has been done on the expression of pHb in response to symbiotic and pathogenic bacteria (Nagata *et al*., 2008; Sasakura *et al*., 2006) but, virtually no work has been done in respect to actinorhizal pHb particularly the alder (*A. nepalensis*), found in Sub-Himalayan West Bengal and Sikkim region. No report has been found on three dimensional structure of actinorhizal pHb proteins, their expression pattern in different plant parts specially in Alder of sub-himalayan West Bengal and Sikkim till date. The evolution of ptHbs in actinorhizal plant system, their functions in plant system. The general trend of actinorhizal pHb in respect to other pHbs in gene as well as protein level.

Alder being a major constituent of forest flora, particularly in the high altitude Himalayan region play a pivotal role in early succession of forest ecosystem and maintaining the fertility of forest soil (Benson and Silvester, 1993). Although no work has been done on the ecological aspect, genetic diversity, population genetics study in host plant i.e *A. nepalensis* in Sub-Himalayan West Bengal and Sikkim region.

The above information prompted us to an in-depth study regarding the characters, phylogeny and the expression of different Hb genes and proteins found in actinorhizal plants with special reference to the actinobacterial Hbs (bHbs), specially the symbiotic counterpart of *Alnus* i.e *Frankia*. In the present study, both experimental methods as well as *in-silico* analysis were done to understand the divergent of the functionality of different pHbs as well as bHbs.

Keeping in mind, the above information the present study entitled "Characterization and diversity of selected actinorhizal haemoglobin genes and proteins with reference to *Alnus-Frankia* symbiosis" has been designed with the following objectives:

- Determination of the ecology of *A. nepalensis* in sub Himalayan West Bengal and Sikkim.

- Determination of the population genetics and genetic diversity of sub Himalayan *A. nepalensis.*
- Characterization of actinorhizal pHb genes with reference to all kind of pHbs available in public domain (*in-sillico*).
- Determination of functional region as well as specific residues responsible for actinoHbs (actinorhizal pHbs and bHbs).
- Comparative codon usage analysis of selected 100 bHbs (*in-sillico*).
- Determination of functional diversity of bHbs.
- Determination of 3D model of actinorhizal pHb proteins using homology modeling technique and their intrinsic dynamics study.
- Comparative phylogenetic analysis amongst action-Hbs based on their structures, sequences and presence of functional motifs among them.
- Structural resemblance determination among actinoHbs.
- Determination of functional divergent of action-Hbs to describe the evolution of ptHbs into plant system.
- To learn the expression of *A. nepalensis* Hb genes in response to *Frankia*.